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Cancer Therapy: Preclinical**A Novel Synergistic Combination of Cyclophosphamide and Gene Transfer of Interleukin-12 Eradicates Colorectal Carcinoma in Mice**

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Abstract Purpose: Interleukin-12 (IL-12) is an immunostimulatory cytokine with potent antitumor effects in several animal models. However, serious toxicity has been associated with its systemic application in humans. Gene transfer has emerged as a tool to specifically express therapeutic genes into the tumor/peritumoral milieu, thus avoiding systemic toxicity. The aim of this study was to analyze whether subtherapeutic doses of an adenovirus encoding IL-12 (AdIL-12) might synergize with low immunopotentiating doses of cyclophosphamide in the treatment of colorectal carcinoma.

Experimental Design: The antitumor effect of combining a single low dose of cyclophosphamide with an intratumoral injection of AdIL-12 was evaluated in an *in vivo* murine colorectal carcinoma model. The immune responses achieved with different treatments were monitored, comparing the effect of combining both therapies with individual treatments.

Results: The combined therapy induced a complete tumor regression in >50% of mice in a synergistic fashion, and it significantly prolonged their survival. This strategy was superior to each single treatment in reducing both peripheral and splenic CD4+CD25+Foxp3+ regulatory T cells, increasing the number of activated dendritic cells, and inducing IFN- γ -secreting CD4-positive T lymphocytes. Importantly, the combined treatment generated a powerful tumor-specific CTL response. Consistently, a significant reduction in IL-10 levels was found. Our data suggest that the combination of nontoxic doses of cyclophosphamide with AdIL-12 allows the generation of good antitumoral responses, thus avoiding undesired side effects of both agents.

Conclusions: Our data strongly support the use of a combination of cyclophosphamide and AdIL-12 as a novel therapeutic strategy against colorectal carcinoma. (Clin Cancer Res 2009;15(23):7256–65)

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Colorectal carcinoma is the second most common cause of cancer mortality in western countries (1). Over the last two decades, much attention has been given to immunotherapy as a promising therapeutic strategy for the treatment of certain human tumors, including metastatic colorectal carcinoma (2). One of the most frustrating consequences of the interaction between the immune system and tumors is the ability of cancer cells to escape the immune response through different mechanisms, such as tumor-induced impairment of antigen presentation, activation of negative costimulatory signals, production of immunosuppressive factors, and expansion and/or recruitment of regulatory cells that may contribute to the immunosuppressive network (3).

Systemic administration of cytokines efficiently generates potent immune responses against a number of tumors (4). However, high doses of cytokines are needed to achieve significant antitumor effects that, unfortunately, are associated with severe toxicity (5). Gene therapy has emerged as a strategy to allow required levels of cytokine expression to tumoral/peritumoral milieu (6). Interleukin 12 (IL-12) is one of the most potent

Translational Relevance

We hereby provide preclinical data regarding the anticancer efficacy of a novel immunotherapy combination between cyclophosphamide and adenoviral gene transfer of IL-12 (AdIL-12) for the treatment of colorectal carcinoma. Our approach successfully combines chemotherapy and immunotherapy, which in a synergistic manner was found to result in decreased number of regulatory T cells, increased IFN- γ -secreting CD4-positive T lymphocytes, enhanced activation of splenic dendritic cells, and a potent specific CTL response using suboptimal doses of both agents. As a result, the combined therapy is shown to induce complete tumor eradication in >50% of mice and to prolong their survival. Immunotherapy in different forms, such as cyclophosphamide plus AdIL-12, could be tested for overall clinical benefit on top of conventional treatment regimens that have evidenced improvement of survival. Thus, the combination of cyclophosphamide-based chemotherapy and gene therapy of IL-12 fulfills the criteria to become a useful and strong tool for treatment of colorectal carcinoma patients.

antitumor cytokines. Multiple biological effects can account for its efficacy, including the induction of a Th1 type response and the activation of natural killer (NK) cells/natural killer T cells (NKTs) and CTLs (7). IL-12 also has the ability to increase expression of adhesion molecules on endothelial cells (e.g., VCAM-1), thus facilitating tumor recruitment of activated lymphocytes (8, 9). In addition, tumor angiogenesis is inhibited by downstream mediators of the IL-12/IFN- γ pathway (10, 11). The activity of IL-12 has been investigated in patients with advanced solid and hematologic malignancies. With few exceptions, however, the efficacy was low (12). The efficacy of IL-12 gene transfer for advanced gastrointestinal tumors in animal models has been consistently shown by different groups, including ours (13–17). Intratumoral administration of AdIL-12 in patients with advanced gastrointestinal carcinomas in a phase I trial was a feasible and well-tolerated procedure that exerted only mild antitumoral activity (18). Several factors might account for the lack of clinical success of gene therapy with IL-12 and other immunotherapies, but the immunosuppressive effect induced by cancer cells seems to be the most important (19). It was also shown that IL-12 can synergize its therapeutic effect when it is combined with other procedures involving the simultaneous transfer of other genes or non-gene-based strategies (20).

Cyclophosphamide is a widely known chemotherapeutic agent (21) that has been used by several groups, including ours, as an immunomodulatory agent against murine and human cancers (22–25). We have previously shown that a single low-dose cyclophosphamide can inhibit the metastasis of a B-cell lymphoma (L-TACB) in a rat model, likely due to the induction of potent immunomodulatory mechanisms that result in a significant reduction in IL-10 levels (26). Moreover, cyclophosphamide was shown to induce a Th2/Th1 switch and to increase the proliferative rate in spleen cells (27).

The objective of this study was to investigate the antitumor activity of combined regimens using cyclophosphamide as an immunomodulatory agent and gene transfer of IL-12 as an immunostimulatory tool, aimed at increasing the effectiveness of individual treatments in a murine colorectal carcinoma model. We hereby show that a low dose of cyclophosphamide prior to AdIL-12 intratumoral injection results in a synergistic effect leading to eradication of >50% of colorectal carcinoma. Consistently, a significant decrease in CD4+ CD25+Foxp3+ cells (regulatory T cells, or Tregs) and a significant increase in activated dendritic cells were observed under combined treatment. In addition, the combined strategy induced a significant increase in the number of IFN- γ -secreting CD4-positive T lymphocytes. Thus, intratumor IL-12 adenovirus gene transfer in animals pretreated with an immunomodulatory dose of cyclophosphamide resulted in a significantly increased antitumor effect. Furthermore, a potent enhancement in tumor-specific CTL activity was also found and could account, at least in part, for the improved effects of this therapeutic combination approach.

Materials and Methods

Animals and cell lines. Six- to eight-week-old male BALB/c mice were purchased from Biofucal S.A. The animals were maintained at our Animal Resources Facilities (School of Biomedical Sciences, Austral University) in accordance with the experimental ethical committee and the NIH guidelines on the ethical use of animals. The CT26 tumor cell line, an undifferentiated murine colorectal carcinoma cell line established from a *N*-nitroso-*N*-methylurethan-induced transplantable tumor in BALB/c (H-2d) mice (kindly provided by Prof. Prieto, University of Navarra, Spain) was herein used. Murine BNL methylcholanthrene-transformed liver cell line and HEK 293 cells were obtained from the American Type Culture Collection. YAC-1 cells (Moloney leukemia virus-transformed lymphoma cell line derived from A/Sn mice) were kindly provided by Dr. Mirta Giordano, Academia Nacional de Medicina de Buenos Aires, Argentina. Cells were maintained in DMEM supplemented with 10% heat-inactivated FCS, 2 mmol/L L-glutamine, 100 U/mL streptomycin, and 100 mg/mL penicillin and incubated at 37°C in a 5% CO₂ humidified atmosphere.

Drugs. Cyclophosphamide (Filaxis) was dissolved in sterile water at a concentration of 20 mg/mL and injected i.p. at the doses indicated.

Adenoviral vectors. Construction of a recombinant adenovirus encoding for IL-12 (AdIL-12) and the *lacZ* reporter genes (Ad β -Gal) were previously described (13). Recombinant adenoviruses were isolated from a single plaque, expanded in 293 cells, and purified by double cesium chloride ultracentrifugation (28). Purified viruses were extensively dialyzed against 10 mmol/L Tris-1 mmol/L MgCl₂ and stored in aliquots at -80°C, and were carefully titrated by plaque assay. The concentration of recombinant vectors was expressed as 50% tissue culture infectious doses (TCID₅₀) per milliliter (29).

In vivo experiments. CT26 cells were injected at a dose of 5×10^5 cells s.c. into the right flank of BALB/c mice. Tumors were allowed to reach approximately 85 mm³ in size before treatment was started. Animals were distributed in different groups and then treated: saline i.p.; cyclophosphamide (50 mg/kg i.p., day 8); AdIL-12 (10^9 TCID₅₀ Intratumorally, day 9); cyclophosphamide (50 mg/kg i.p.) plus AdIL-12 (10^9 TCID₅₀ i.t.); Ad β -Gal (10^9 TCID₅₀ i.t.); and cyclophosphamide (50 mg/kg i.p.) plus Ad β -Gal (10^9 TCID₅₀ i.t.). Adenoviruses were diluted in saline (final volume 50 μ L) and i.t. injected in a single site; no leakage of material was observed after inoculations.

Tumor growth was assessed twice weekly by caliper measurement. To study protective immunity, animals that were free of disease at 4 wk after complete regression of primary tumors (8 wk after first tumor inoculation) were challenged with 5×10^5 CT26 cells on the left flank.

Age-matched naive animals received the same amount of cells to serve as controls. Mice were monitored for up to 3 months for measurement of tumor size and survival. Tumor volume (mm^3) was calculated by the formula $\pi/6 \times \text{larger diameter} \times (\text{smaller diameter})^2$.

Sample preparation for Tregs and dendritic cells flow cytometry analyses. BALB/c mice were injected with 5×10^5 CT26 cells s.c. into the right flank (day 0) and tumors were allowed to reach 85 mm^3 before treatment was started. The animals were distributed into different groups and treated with saline, cyclophosphamide, AdIL-12, cyclophosphamide plus AdIL-12, Ad β -Gal, or cyclophosphamide plus Ad β -Gal ($n = 6/\text{group}$). At day 15 (Tregs) or day 21 (dendritic cells), peripheral blood was collected by cardiac puncture and anticoagulated with EDTA. The mice were then sacrificed, spleens were excised, and single cell suspensions were prepared. The pretreatment group was constituted by splenocytes obtained from tumor-bearing mice of 8-d evolution. Peripheral blood cells or splenocytes were stained with different conjugated antibodies as it follows: anti-Foxp3 (phycoerythrin; eBioscience), and anti-CD4 (PECy5), anti-CD25 (FITC), anti-CD11c (FITC), anti-MHCII (PE), and anti-CD80 (Allophycocyanin; BD Biosciences), and their respective isotypes. Cell suspensions were then treated with RBC lysis buffer (0.15 mol/L NH_4Cl , 1 mmol/L KHCO_3 , 0.1 mmol/L $\text{Na}_2\text{-EDTA}$) and washed with PBS 1% bovine serum albumin. Then, cells were fixed with 1% paraformaldehyde and subjected to flow cytometry (FACSCalibur, Becton Dickinson-BD). Data were analyzed using WinMDI software.

Detection of IFN- γ -producing T lymphocytes. IFN- γ -producing T lymphocytes were detected and quantified using a mouse IFN- γ secretion assay-detection kit from Miltenyi Biotec (Bergisch Gladbach). BALB/c mice were inoculated with 5×10^5 CT26 cells s.c. (day 0), and injected with saline or with cyclophosphamide 50 mg/kg i.p. (day 8). Then, the mice were sacrificed and splenocytes were obtained. The pretreatment group consisted of splenocytes from CT26-bearing mice at 8 d after tumor cell injection. Splenocytes (2×10^6 cells/mL) were cultured in complete DMEM medium in 6-well plates for 24 h and then infected with AdIL-12 or Ad β -Gal at multiplicity of infection of 500 (>80% of efficacy; data not shown). Five days later, cells were harvested, washed, and labeled for 5 min at 4°C with an IFN- γ -specific high-affinity capture matrix (a bispecific antibody-antibody conjugate). Cells were then transferred into warm medium to allow secretion of cytokines for 45 min at 37°C . Cells were then washed and stained with anti-IFN- γ -PE for 10 min at 4°C . For FACS analysis, aliquots of all samples were further stained with anti-CD4-PECy5 and anti-CD8-Alexa Fluor 488 (BD Biosciences). Then, cells were fixed with 1% paraformaldehyde and subjected to flow cytometry (FACSCalibur, Becton Dickinson-BD). Data were analyzed using WinMDI software.

Measurement of IFN- γ and IL-10. Splenocytes from saline or cyclophosphamide-treated CT26 tumor-bearing mice were harvested on day 4 posttreatment and cultured at 2×10^6 cells/mL of complete RPMI medium in 6-well culture plates for 24 h. Then, cells were transduced with AdIL-12 or Ad β -Gal at a multiplicity of infection of 500. After incubation for 48 h (IL-10) or 120 h (IFN- γ) at 37°C and 5% CO_2 , culture supernatants were collected and stored at -20°C .

To measure the effect of cyclophosphamide on IL-10 production *in vivo*, BALB/c mice were inoculated with 5×10^5 CT26 cells s.c. into the right flank (day 0), and then a single dose of cyclophosphamide of 50 or 100 mg/kg i.p. was administered 8 d after tumor inoculation. A saline group was included as control. Blood samples, taken from the tail vein, were collected on days 0, 12, 15, and 26 and stored at -20°C . IFN- γ and IL-10 were measured by a sandwich ELISA assay (OptEIA, BD Biosciences Pharmingen). The assays were carried out according to the instructions provided by the manufacturer. Standards and samples were assayed in duplicates.

Cytotoxicity assay. Cytotoxicity assay was done according to standard protocols (30). Viable splenocytes (8×10^6) were stimulated *in vitro* with mitomycin C-treated CT26 cells (8×10^5 cells/well in 24-well-plates). On day 5, cells were harvested and washed, adjusted to $4 \times 10^6/\text{mL}$, and added to 96-well-plate (effector cells). To determine specific CTL cytotoxicity and NK activity three types of target cells were

used (CT26, BNL, and YAC-1) at $4 \times 10^5/\text{mL}$. After incubation for 4 h at 37°C , plates were centrifuged and cell-free supernatants were obtained. Cytotoxicity was evaluated with the LDH Cytotoxicity Detection Kit (Roche Diagnostics) following the manufacturer's instructions.

Proliferation assay. To measure the proliferation of spleen lymphocytes under different experimental conditions in response to mitomycin C-treated CT26 cells, [^3H]thymidine incorporation assay was done. *In vitro* stimulation was carried out for 5 d in 24-well plates, with each well containing 8×10^6 splenocytes and 8×10^5 mitomycin C-treated CT26 cells. Each sample was assayed in triplicate.

Histology. Tumor samples from all experimental groups were obtained 15 d after treatment and fixed in 10% phosphate-buffered formalin. Five-micrometer sections from paraffin-embedded tissues were stained with H&E for histologic examination.

Statistical analysis. Mann-Whitney test or Student's *t*-test (InStat, GraphPad Software) was used to statistically examine the differences between groups. $P < 0.05$ was considered statistically significant.

Results

Intratumoral injection of AdIL-12 or systemic injection of cyclophosphamide induces minor therapeutic effects. The aim of our first experiments was to find the lowest nontoxic doses of cyclophosphamide and AdIL-12 that could have significant antitumor effects when used in combined strategies. The activity of cyclophosphamide on the immune system of tumor-bearing host is highly dose-dependent. Thus, high doses of cyclophosphamide (in mice, 100-150 mg/kg) usually bring about an impairment of the host defense mechanisms along with the reduction of tumor mass, leading to severe immunosuppression. In contrast, cyclophosphamide at low doses (~ 50 mg/kg) leads to an enhancement of the immune response resulting in the inhibition of the tumor growth (31). To this aim, CT26 cells were s.c. injected into syngeneic BALB/c mice (day 0). On day 8, animals with tumors reaching 85 mm^3 in size received a single i.p. injection of cyclophosphamide: 50, 100, or 150 mg/kg. High doses of cyclophosphamide (100 and 150 mg/kg) induced a significant tumor volume reduction in comparison with the saline group (90% and 96% at day 28, respectively; $P < 0.05$) and complete tumor regression in 33% (3 of 9) and 50% (2 of 4) of animals, respectively (0% tumor regression was observed in saline group). On the other hand, a lower dose of cyclophosphamide (50 mg/kg) induced a tumor volume reduction of 72% when compared with the saline group and induced a complete tumor regression in 13% of mice (1 of 8 animals; Fig. 1A). We have previously shown in a rat B-cell lymphoma that a single low dose of cyclophosphamide is able to reduce IL-10 production by T cells and to inhibit metastases (26). Accordingly, we measured IL-10 serum levels in CT26-bearing mice after different doses of cyclophosphamide. As shown in Fig. 1B, IL-10 levels in saline-treated (control) CT26-bearing mice increased significantly on days 15 and 26 compared with healthy animals (day 0). Interestingly, treatment with 50 mg/kg cyclophosphamide (low dose) led to a significant decrease in serum IL-10, reaching the basal levels from day 15 onward. In contrast, IL-10 levels in mice treated with 100 mg/kg cyclophosphamide (high dose) were similar to the saline group on days 15 and 26.

We afterwards determined the dose of AdIL-12 to be used. When tumors reached approximately 85 mm^3 in size, different doses of AdIL-12, ranging from 10^8 to 10^9 TCID $_{50}$, were i.t. administered. AdIL-12 at 10^8 TCID $_{50}$ induced 55% of tumor

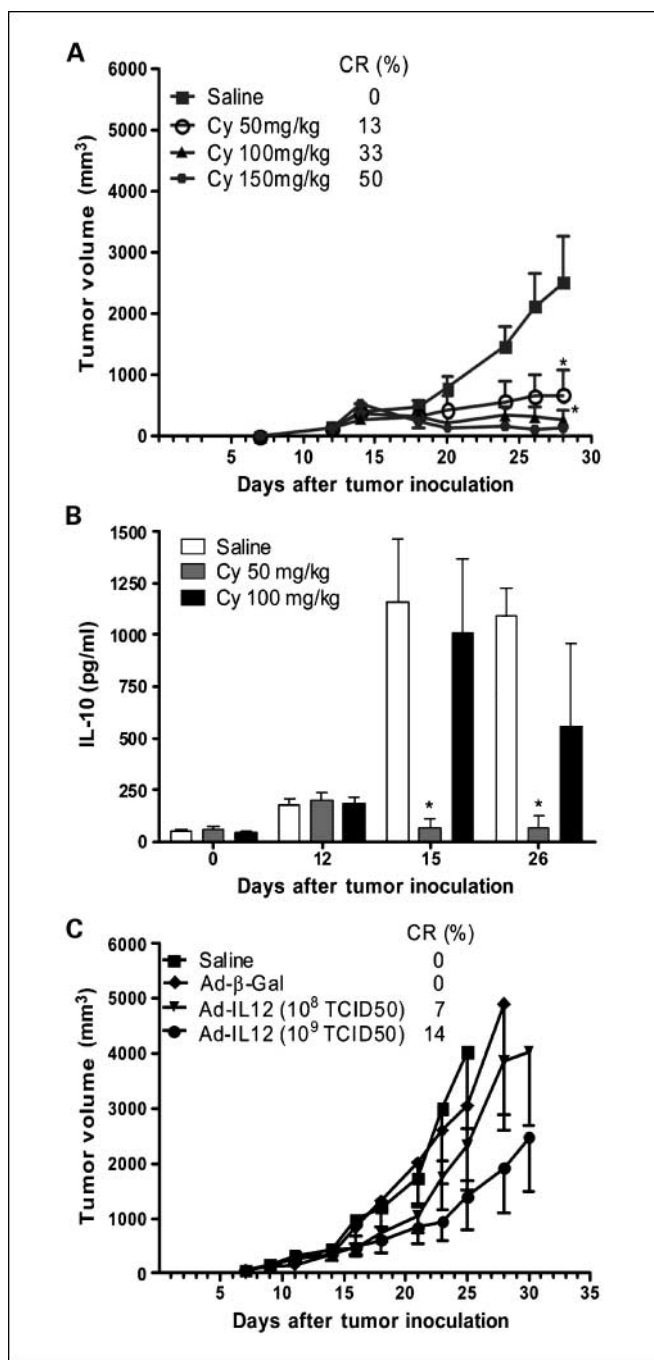


Fig. 1. A, dose-response study for cyclophosphamide. BALB/c mice were s.c. injected with 5×10^5 CT26 cells into the right flank (day 0) and tumors were allowed to reach 85 mm^3 before treatment. Animals were treated with: saline (control); cyclophosphamide i.p. 50, 100, or 150 mg/kg/body weight. Tumor growth was assessed twice a week by caliper measurement. Data are expressed as mean tumor volume (bars, SE). CR (%), percentage of animals with complete tumor regressions. *, $P < 0.05$, cyclophosphamide 100 and 150 mg/kg versus saline. B, measurement of serum IL-10 concentration in CT26 tumor-bearing mice treated with saline or a single dose of cyclophosphamide 50 or 100 mg/kg i.p. 8 d after tumor inoculation. Data are expressed as mean serum concentration (bars, SE). *, $P < 0.05$, cyclophosphamide 50 mg/kg versus saline. C, dose response study for AdIL-12. BALB/c mice were s.c. injected with 5×10^5 CT26 cells into the right flank (day 0) and tumors were allowed to reach 85 mm^3 before treatment was started. Animals were treated with: saline (control), Ad β -Gal, AdIL-12 10^8 TCID50, or AdIL-12 10^9 TCID50 i.t. Tumor growth was assessed twice a week by caliper measurement. Data are expressed as mean tumor volume (bars, SE). Student's *t* test was used to compare differences among groups.

volume reduction with respect to the saline group at day 25, and complete tumor regression in 7% of mice (1 of 14). A more important tumor volume reduction was observed when 10^9 TCID50 of AdIL-12 were used instead, with a complete tumor regression in 2 of 14 animals (14%; Fig. 1C). No saline- or Ad β -Gal-treated animals showed tumor regression.

In view of previous results, we decided to evaluate the combination of the lowest dose of each treatment that displayed similar antitumor activities (equipotent doses) to analyze the *in vivo* antitumor interaction effects of the combination of both treatments. Therefore, a dose of 50 mg/kg for cyclophosphamide and of 10^9 TCID50 for AdIL-12 were chosen.

A sequential combination of a single low-dose cyclophosphamide and an i.t. injection of suboptimal doses of AdIL-12 displays synergistic antitumor effects against colorectal carcinoma. To examine the antitumor effects of the combined treatment, CT26 tumor-bearing mice were treated, on day 8 after tumor inoculation, with a single low dose of 50 mg/kg cyclophosphamide i.p., and they were injected i.t. 24 hours later with 10^9 TCID50 of AdIL-12. Animals treated with each single agent, Ad β -Gal in combination with cyclophosphamide, or saline were also included as required controls. The antitumor effects obtained with cyclophosphamide or AdIL-12 alone were similar to those obtained in the dose-response experiments [versus saline group: 77% and 76% of reduction in tumor volume, and complete tumor regression in 8% (1 of 12) and 18% (2 of 11), respectively]. In striking contrast, the combination of these suboptimal doses of cyclophosphamide and AdIL-12 consistently resulted in a marked tumor volume reduction (96% with respect to saline group, at day 24) and complete tumor regressions in 6 of 12 animals (50% versus 0% in saline group; $P < 0.05$). In addition, the combined treatment produced a significant reduction of tumor volume when compared with animals treated with each single agent from day 18 on (day 28: cyclophosphamide plus AdIL-12, $304 \pm 154 \text{ mm}^3$; cyclophosphamide, $1,534 \pm 489 \text{ mm}^3$; AdIL-12, $1,720 \pm 512 \text{ mm}^3$; $P < 0.05$; Fig. 2A). Such a potent result did not reflect transgene-unrelated effects caused by adenovirus combination with cyclophosphamide because similar doses of Ad β -Gal combined with cyclophosphamide did not show any significant change in tumor growth beyond a typical response achieved with cyclophosphamide alone. The combined cyclophosphamide plus AdIL-12 strategy was well tolerated with no signs of toxicity (data not shown). Survival of mice receiving combined therapy was significantly increased compared with mice receiving each single treatment or saline ($P < 0.001$) (Fig. 2B). Analysis of the *in vivo* interaction between both treatments was done by the fractional product method (32). Table 1 summarizes the relative tumor volume of different groups on three different time points. On day 6 after treatment, in the combination group there was a 1.1-fold improvement in the treatment antitumor efficacy when compared with the expected additive effect. Moreover, on days 13 and 22, the combination of cyclophosphamide and AdIL-12 showed a 1.3-fold increase in the inhibition of tumor growth over an additive effect (expected fractional tumor volume). These results allow us to conclude that the cyclophosphamide (50 mg/kg)-AdIL-12 (10^9 TCID50) combined therapy has a synergistic effect on tumor-growth inhibition.

Microscopic examination of tumor sections obtained 15 days after treatment showed gross areas of necrosis only in tumors treated with AdIL-12 plus cyclophosphamide (Fig. 2C). In

addition, tumors treated with the combination showed a marked infiltration of inflammatory mononuclear cells (not shown).

Long-term antitumor immunity induced by combined treatment. To determine whether combined treatment can induce lasting immunologic memory, we rechallenged animals that were free of tumors with a new s.c. inoculation of CT26 tumor cells (in the contralateral flank) at 4 weeks after complete regression of primary tumors. A rejection in 6 of 6 animals (100%) was observed after the second challenge with CT26 cells (Fig. 2D). In contrast, 5 of 5 mice treated with saline developed tumors and by day 28 they had died. These data indicate that therapy using a combination of cyclophosphamide and AdIL-12 can induce long-term protection against colorectal carcinoma recurrence.

Assessment of CD4+ CD25+Foxp3+ regulatory T cells in peripheral blood and spleen. The prevalence of CD4-positive T cells coexpressing CD25 and Foxp3 (Tregs) was determined in pe-

ripheral blood and spleen using triple-staining flow cytometry. The ratio CD4+CD25+Foxp3+/CD4+ in the peripheral blood and spleen of healthy mice was found to be 5.5% and 1.3%, respectively. During colorectal carcinoma progression, this ratio was significantly increased, reaching about 8.7% and 5.3%, respectively (pretreatment groups; $P < 0.05$; Fig. 3A and B). When the proportion of CD4+CD25+Foxp3+ cells was analyzed at day 7 of treatment (15 days after tumor inoculation), the saline group showed a further increase in Tregs (14.1% and 18.4% in peripheral blood and spleen, respectively) when compared with pretreatment groups ($P < 0.01$). The administration of cyclophosphamide as single therapy induced a significant decrease in the percentage of Tregs in comparison with saline group (peripheral blood, 6.1%; spleen, 6.7%; $P < 0.05$), and similar results were found after AdIL-12 treatment in spleen (8.8%; $P < 0.05$). The combined strategy of cyclophosphamide and AdIL-12 induced a decrease in the percentage of Tregs in both peripheral

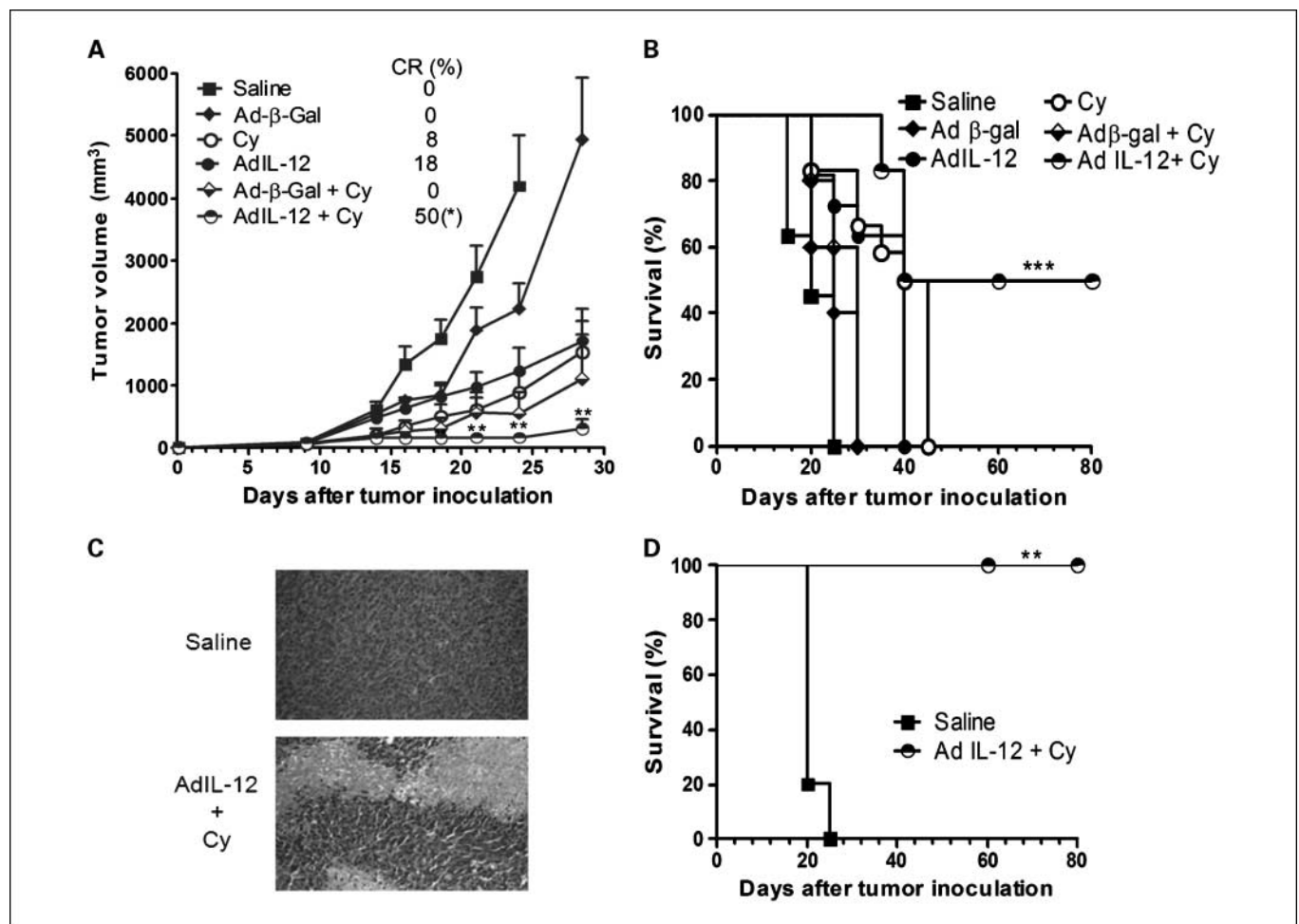


Fig. 2. A, antitumor activity of cyclophosphamide, AdIL-12, and their combination in CT26 tumor model. BALB/c mice were s.c. injected with 5×10^5 CT26 cells into the right flank (day 0) and tumors were allowed to reach 85 mm³ before treatment was started. Animals were distributed into different groups: saline, cyclophosphamide (50 mg/kg i.p., day 8), AdIL-12 (10^9 TCID₅₀ i.t., day 9), cyclophosphamide (50 mg/kg i.p.) plus AdIL-12 (10^9 TCID₅₀ i.t.), Adβ-Gal (10^9 TCID₅₀ i.t.), and cyclophosphamide (50 mg/kg i.p.) plus Adβ-Gal (10^9 TCID₅₀ i.t.). Data are expressed as mean tumor volume (bars, SE). **, $P < 0.01$, cyclophosphamide and AdIL-12 versus AdIL-12 plus cyclophosphamide, from day 18 onward. Student's *t* test was used to compare tumor sizes among different groups. B, survival of animals after treatment with each single treatment and the combination (Kaplan-Meier, log rank test, $P < 0.001$). C, histologic examination of tumor samples from mice treated with saline and combination of AdIL-12 plus cyclophosphamide. Representative H&E-stained sections. D, long-term protection against CT26 cells rechallenge at distant sites. CT26 cells (5×10^5 cells) were s.c. inoculated on the left flank of animals that had been cured after treatment of established tumor with the combination of cyclophosphamide and AdIL-12 (4 wk after complete regression). Age- and sex-matched naive animals inoculated with CT26 cells were also included as control. Deaths of animals were documented, and results were expressed as the percentage of animal survival (Kaplan-Meier, log rank test, $P < 0.01$).

Table 1. Combined therapy with cyclophosphamide and AdIL-12**Fractional tumor volume* relative to untreated controls**

Day [†]	Combination treatment			Expected [‡]	Observed	R [§]
	Cy (50 mg/kg)	AdIL-12 (10 ⁹ TCID50)				
6	0.343	0.769		0.264	0.249	1.1
13	0.225	0.359		0.081	0.061	1.3
22	0.235	0.212		0.050	0.038	1.3

Abbreviation: Cy, cyclophosphamide.

*FTV, (experimental mean tumor volume)/(control mean tumor volume).

[†]Day after treatment onset.[‡](Cyclophosphamide mean FTV) × (AdIL-12 mean FTV).[§]R = Expected FTV/Observed FTV. A ratio >1 indicates a synergistic effect, and a ratio <1 indicates a less than additive effect.

blood and spleen similar to that of cyclophosphamide-alone application (7.9% and 6.0%, respectively; $P < 0.05$ versus saline).

Effect of the combination of cyclophosphamide and AdIL-12 on dendritic cells. Recent experimental evidence suggests that Tregs might modulate the maturation and/or functional status of dendritic cells (33). Considering our results regarding Tregs, we decided to investigate the effects of the AdIL-12 plus cyclophosphamide combination on possible changes in the proportion and maturation status of dendritic cells in the spleen of mice with colorectal carcinoma. Fourteen days after treatment, a significantly higher proportion of spleen CD11c+ MHC-II+CD80+ cells was found in cyclophosphamide-treated animals (3.5%) when compared with the saline group (1.8%; $P < 0.05$). Interestingly, and although the percentage of dendritic cells in the group treated with AdIL-12 alone (1.8%) was similar with that in the saline group, the combination of AdIL-12 with cyclophosphamide induced a further increase in the percentage of mature dendritic cells (4.8%; Fig. 3C).

Cyclophosphamide pretreatment increased IFN- γ -producing T lymphocytes after gene transfer of IL-12. The AdIL-12 plus cyclophosphamide combination is able to eradicate colorectal carcinoma tumors through synergistic induction of efficient immune responses. To confirm this possibility, we analyzed the proportion of IFN- γ -secreting T lymphocytes in the spleen of CT26 tumor-bearing mice. In this experiment, the specific production of IFN- γ by CD4-positive or CD8-positive splenic T lymphocytes of cyclophosphamide-treated CT26 tumor-bearing mice was quantified after *in vitro* transduction with AdIL-12. Administration of cyclophosphamide alone increased the number of IFN- γ -secreting CD4-positive T lymphocytes (2.4%) as compared with the saline group (1.6%; $P < 0.05$; Fig. 4A and B). A similar result was observed in non-cyclophosphamide-treated mice after *in vitro* transduction with AdIL-12 (2.8%; $P < 0.05$). However, the combined treatment (cyclophosphamide *in vivo* plus AdIL-12 *in vitro*) led to a ~3-fold increment in the number of IFN- γ -secreting CD4-positive T lymphocytes (6.3%; $P < 0.01$, versus cyclophosphamide and AdIL-12). No changes in the percentage of IFN- γ -secreting CD8-positive T lymphocytes were detected in between treatments (data not shown).

Effects of combined treatment on cytokine production profile. As expected, the observed changes in the number of IFN- γ -

secreting CD4-positive T lymphocytes were found to correlate with the levels of IFN- γ in supernatants from cultured splenocytes. Splenocytes from mice bearing colorectal carcinoma tumors at the beginning of treatment (pretreatment group) produced low levels of IFN- γ *in vitro* (18 ± 3 pg/mL). Each single treatment (cyclophosphamide or AdIL-12) induced a slight increase in IFN- γ levels (150 ± 50 pg/mL and 227 ± 99 pg/mL, respectively) as compared with the saline group (33 ± 13 pg/mL; $P < 0.01$). When AdIL-12 was applied in combination with cyclophosphamide, a ~5-fold increase in IFN- γ production was found when compared with each single therapy ($1,332 \pm 173$ pg/mL; $P < 0.01$; Fig. 4C). In addition, cyclophosphamide alone or combined with AdIL-12 significantly reduced IL-10 production. As shown in Fig. 4D, IL-10 concentration increased with tumor growth from day 8 (pretreatment, 102 ± 36 pg/mL) to day 12 (saline, 287 ± 84 pg/mL; $P < 0.05$). Cyclophosphamide alone or cyclophosphamide plus AdIL-12 induced a significantly decreased IL-10 production (59 ± 20 pg/mL and 53 ± 17 pg/mL, respectively) in comparison with the saline group ($P < 0.05$). AdIL-12 alone did not significantly affect the concentration of IL-10 (205 ± 52 pg/mL). This result suggests that cyclophosphamide is responsible for the inhibition of IL-10 production and that AdIL-12 does not significantly influence IL-10 production levels when applied in combination with cyclophosphamide.

Low-dose cyclophosphamide and gene transfer of IL-12 synergize to induce tumor-specific CTL activity. We next investigated whether the antitumor effect induced by combined treatment with cyclophosphamide and AdIL-12 was mediated by activation of NK and/or CTLs. As shown in Fig. 5A, treatment of tumor-bearing mice with AdIL-12 or cyclophosphamide as single therapy was found to elicit a mild cytotoxic activity against CT26 cells. However, splenocytes harvested from mice receiving the combined treatment displayed a significantly higher lytic activity against CT26 after 5 days of *in vitro* restimulation ($P < 0.05$). The specificity of cytotoxicity was confirmed against BNL cells and the NK-sensitive target YAC-1. Our data strongly suggest that cyclophosphamide was able to enhance the specific CTL-promoting activity of suboptimal gene transfer of IL-12 against CT26 colorectal carcinoma tumor cells. In addition, the AdIL-12 plus cyclophosphamide combined treatment was the only condition found

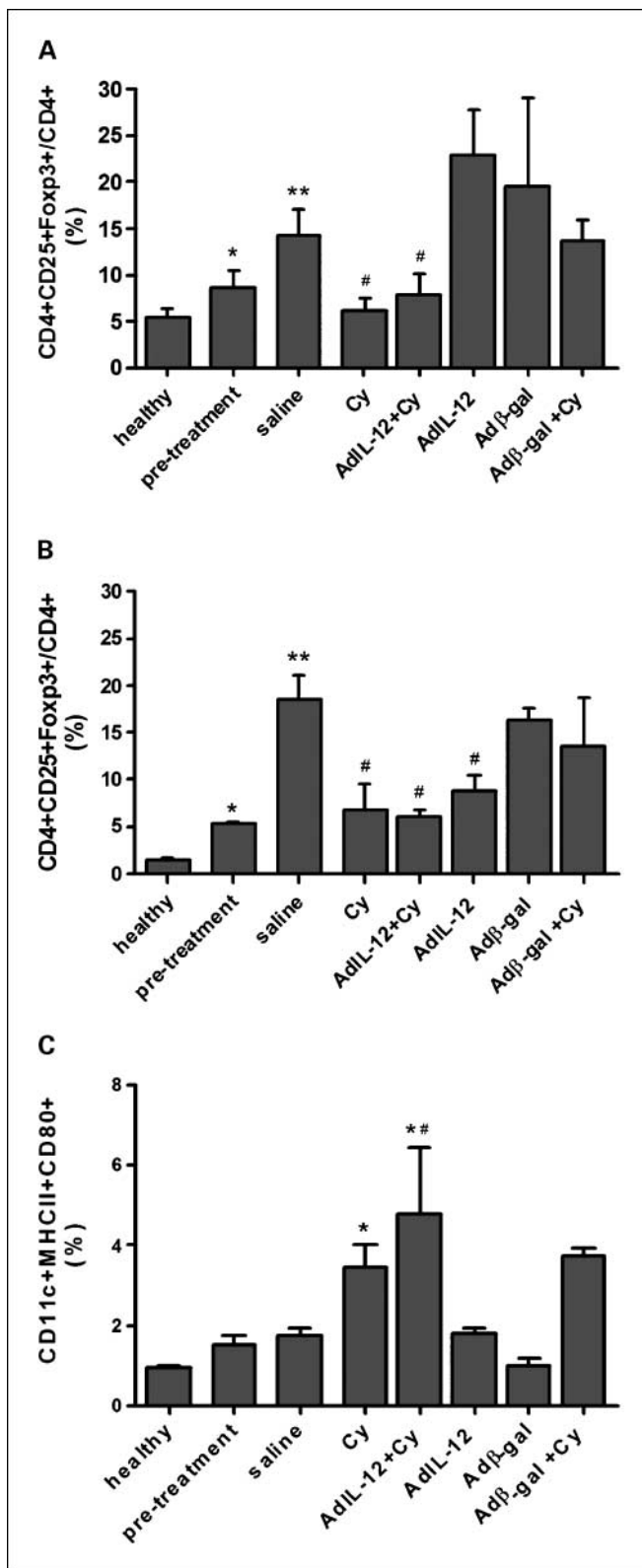


Fig. 3. Percentage of CD4+CD25+Foxp3+/CD4+ cells (Tregs) in peripheral blood (A) or spleen (B). *, $P < 0.05$, pretreatment versus healthy; **, $P < 0.05$, saline versus pretreatment; #, $P < 0.05$, cyclophosphamide, AdIL-12, and AdIL-12 plus cyclophosphamide versus saline. C, percentage of splenic CD11c+MHCII+CD80+ cells. *, $P < 0.05$, cyclophosphamide and AdIL-12 plus cyclophosphamide versus saline; #, $P < 0.05$, AdIL-12 plus cyclophosphamide versus cyclophosphamide. Mann-Whitney test was used to compare differences among groups.

to significantly increase the proliferation rate of splenocytes (Fig. 5B).

Discussion

Multiple immunotherapy strategies for colorectal carcinoma are currently under preclinical and clinical evaluation (34). Some of them involve local administration of immunomodulatory molecules, such as cytokines, with the objective to induce immunity against cancer cells (4, 5). Currently, one of the most common approaches to transfer immunostimulatory molecules inside tumors consists in the *in vivo* intratumoral/peritumoral injection of vectors/cells expressing cytokines and/or costimulatory molecules genes (6, 35). Immunotherapy approaches usually have to face strong mechanisms of immune escape induced by tumors (3). To increase the efficacy of passive or active immunotherapy strategies it seems reasonable to explore therapeutic interventions to intercourse tolerogenic processes, such as those induced by Tregs in tumor-bearing hosts (36). In this scenario, it has been shown that a low dose of cyclophosphamide not only decreases the number, but also inhibits the immunosuppression activity of the residual Tregs (37, 38). Therefore, we decided to investigate the antitumor activity of these two agents, IL-12 and cyclophosphamide, as a new therapeutic combination for colorectal carcinoma. As expected, we herein show that a low dose of cyclophosphamide and suboptimal doses of AdIL-12 cause a significant inhibition of a protolerogenic response induced by colorectal carcinoma. Interestingly, combined therapy was found to induce a significant antitumoral response, as shown by higher i.t mononuclear cell infiltrates, the induction of a potent specific CTL response and of long-term immunity, and the increase in IFN- γ levels and in the number of IFN- γ -secreting CD4-positive T lymphocytes. The antitumoral immunologic response elicited by the combined treatment was found to be highly efficient because >50% of the mice showed tumor regression. In addition, animal survival was significantly increased when compared with that of single-agent applications. Furthermore, our study shows that the combination of a single low dose of cyclophosphamide with AdIL-12 results from the synergistic potentiation of the effect of both agents with regard to their antitumoral activities.

From all different dose combinations tested, a single administration of 50 mg/kg cyclophosphamide 24 hours before i.t. injection of 10^9 DICT50 of AdIL-12 was the optimal strategy, both in dose levels and schedule. These results strongly suggest that the reduction in numbers of CD4+CD25+FoxP3+ Treg cells and the increased activation of dendritic cells, which might require the application of cyclophosphamide prior to AdIL-12, is necessary to achieve the enhanced Th1-type response observed.

Sequential systemic injection of cyclophosphamide followed by intratumoral AdIL-12, in suboptimal doses, was able to induce tumor regressions in ~50% of the cases. This observation is of particular importance for three reasons: (a) in our CT26 tumor model, AdIL-12 by itself failed to induce complete regressions in >80% of the cases; (b) high doses of IL-12 can induce severe toxicity; and (c) the synergistic effect achieved suggest the possibility that a low dose of IL-12 could reach significant antitumoral effects without toxicity.

Previous studies have shown that cyclophosphamide potentiates the antitumor activities of a number of immunotherapeutic

strategies (25, 39). The immunomodulatory effects of cyclophosphamide are not fully understood, but several mechanisms have been proposed, including enhanced expansion of antigen-specific T cells (40); stimulation of innate immune response, in particular the activation of dendritic cells (41); induction of type I IFN (42); Th2/Th1 shift in cytokines profile (27); and elimination/inhibition of regulatory T cells (43). We herein show that the AdIL-12 plus cyclophosphamide combined treatment strongly reduced the number of CD4+CD25+Foxp3+ T cells in both peripheral blood and spleen. The Tregs inhibition observed was similar to that of cyclophosphamide-alone treatment, indicating that cyclophosphamide is responsible for this effect. Consistently, the reduction in the type-2 cytokine production profile dominated by the presence of IL-10 was mainly dependent on cyclophosphamide effects, causing a Th2/Th1 shift, characterized by significantly higher levels of IFN- γ . The induction of a similar cytokine profile change was previously observed by our group in a different tumor model (26, 27). Taken together, these data suggest that cyclophosphamide pretreatment might modulate the immune system and perhaps the

tumor microenvironment, being able to potentiate the enhancement of the antitumoral activity mediated by IL-12 subsequent application.

The response of tumor-bearing hosts to cyclophosphamide-based therapy is heterogeneous, and no one mechanism alone can entirely explain its immunomodulatory effects. It has been recently shown that Tregs might induce tolerance status through modulation of dendritic cell number and/or activity. It was shown that Tregs render dendritic cells inefficient as antigen-presenting cells and this effect was accompanied with increased transforming growth factor- β and IL-10 secretion and reduced expression of costimulatory molecules on dendritic cells (33). We observed that cyclophosphamide alone as well as in combination with AdIL-12 was able to increase the percentage of activated dendritic cells. This effect might play a role in the observed antitumor response induced by the combination. Hence, we propose that Tregs inhibition induced by cyclophosphamide may interfere with the negative feedback between Tregs and dendritic cells likely potentiating the antitumor activity of AdIL-12.

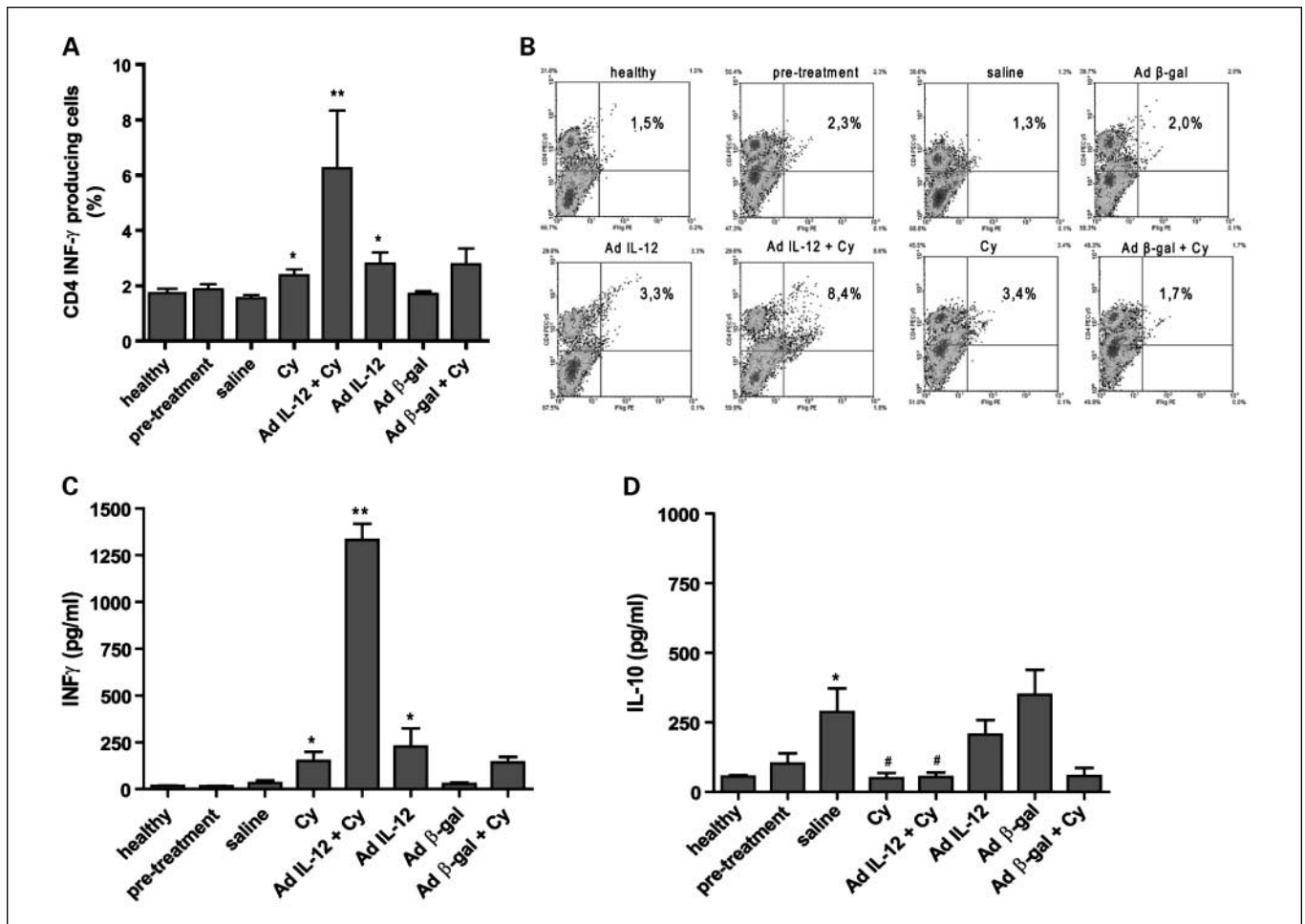


Fig. 4. A, percentage of splenic IFN- γ -secreting CD4-positive T lymphocytes. *, $P < 0.05$, cyclophosphamide and AdIL-12 versus saline; **, $P < 0.01$, AdIL-12 plus cyclophosphamide versus AdIL-12 and cyclophosphamide. B, representative density plots for A. C, IFN- γ quantification. *, $P < 0.05$, cyclophosphamide and AdIL-12 versus saline; **, $P < 0.01$, AdIL-12 plus cyclophosphamide versus cyclophosphamide and AdIL-12. D, IL-10 quantification. *, $P < 0.05$, saline versus pretreatment and healthy; #, $P < 0.05$, cyclophosphamide and AdIL-12 plus cyclophosphamide versus saline. Mann-Whitney test was used to compare differences among groups.

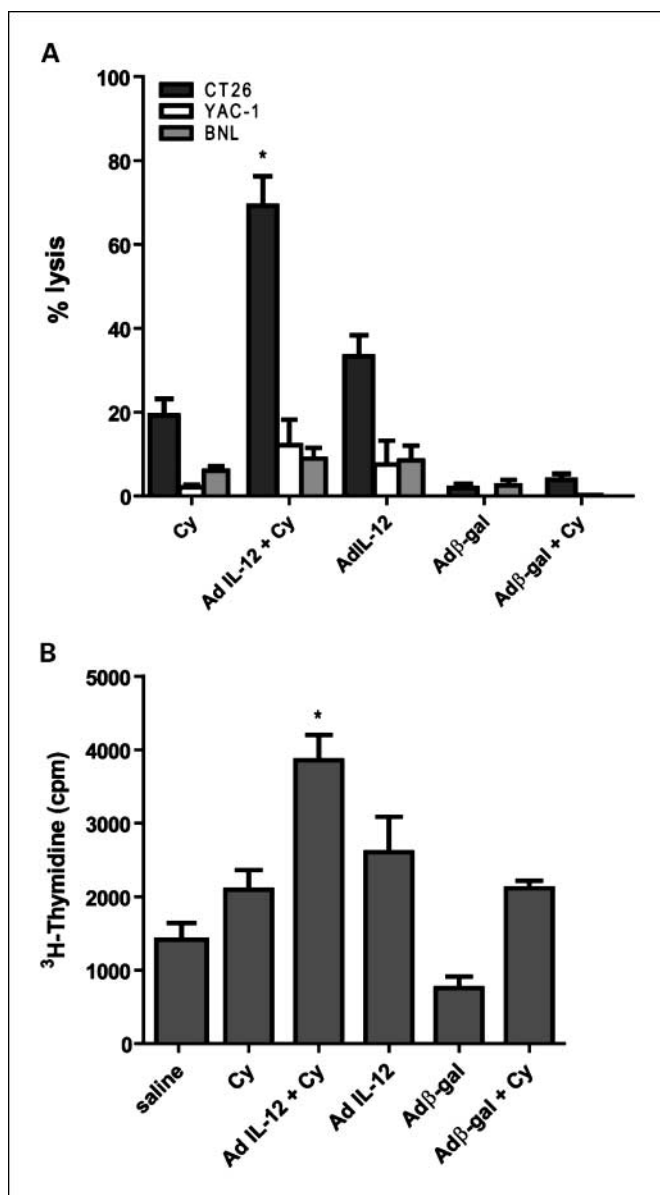


Fig. 5. A, specific CTL activity. Splenocytes from different experimental groups were stimulated *in vitro* with mitomycin-C-treated CT26 cells for 5 d. Specificity of CTL cytotoxicity and NK activity were evaluated using BNL and YAC-1 cells, respectively. Cytotoxicity was evaluated with the LDH Cytotoxicity Detection Kit. The percentage of specific cytotoxicity was calculated according to the following formula: [(abs 492 nm experimental - abs 492 nm background)/(abs 492 nm maximum - abs 492 nm background)] × 100. Data represent the mean of triplicate cultures. *, $P < 0.05$, AdIL-12 plus cyclophosphamide versus AdIL-12 and cyclophosphamide. B, cell proliferation. Splenocytes from different experimental groups were stimulated *in vitro* with mitomycin-C-treated CT26 cells for 5 d. Cell proliferation was evaluated by ³H-thymidine incorporation assay at day 4. *, $P < 0.05$, AdIL-12 plus cyclophosphamide versus AdIL-12 and cyclophosphamide. Mann-Whitney test was used to compare differences among groups.

IL-12 has shown considerable antitumor activity in preclinical models (7). However, the immunosuppressive mechanisms frequently found in advanced tumors may likely limit the efficacy of IL-12 and other kinds of immunotherapies (3). Thus, we decided to investigate if immunomodulatory preconditioning of cyclophosphamide could enhance the IFN- γ production induced by AdIL-12. Our results show that combined therapy

significantly increased IFN- γ secretion by CD4-positive T lymphocytes. The role of IFN- γ -producing CD4-positive T cells would likely be the stimulation of tumor cell killing by macrophages (44) and of dendritic cells able to prime CTLs (45). These results, together with the measurable effects on the macroscopic tumor growth, were strengthened with histologic examination of excised tumors. The regression of CT26 tumors treated with the combination was associated with extensive areas of necrosis and inflammatory infiltrates. This phenomenon was not observed using each agent alone nor was in seen in nontreated tumors.

Altogether, our results suggest that the expansion of CD4+ CD25+Foxp3+ regulatory T cells and the increase in Th2 cytokines production (particularly IL-10) during tumor progression might be responsible for the limited anticancer effect of AdIL-12 as well as for other type of immunotherapy. In our hands, depletion of CD4+CD25+Foxp3+ regulatory T cells and a switch towards Th1 response can be achieved by a single injection of low-dose cyclophosphamide. This effect has the ability to potentiate the efficacy of gene therapy with AdIL-12. This immunomodulatory approach seems to be an attractive strategy to overcome the tolerance induced by colorectal carcinoma and to tip the balance towards the generation of antitumor immunity. In this regard, we show herein a remarkable increase in anti-CT26 CTL activity upon combined treatment application, which is probably related to an ongoing Th1 response induced by cyclophosphamide-mediated activation of CD4-positive T cells as well as by a reduction in CD4+ CD25+Foxp3+ cells. Nevertheless, other potential effects of the combination could favor CTL generation such as the observed necrosis of malignant tissue that could release tumor antigens in a way suitable for presentation by dendritic cells (46).

These data support that a successful immunotherapy against tumors requires the combination of two sequential manipulations of the immune system: (a) immunomodulation, to make the initial host immune response adequate against tumor cells, and then (b) immunostimulation, to amplify anticancer immune response into a strong T cell-mediated antitumor response. The use of low-dose cyclophosphamide plus AdIL-12 in this study satisfies both requirements, possibly by cyclophosphamide-mediated elimination of immunosuppressive agents and a further IL-12-mediated amplification of a Th1-type immune response with the result of the generation of a potent antitumor CTL activity.

Regardless of the specific mechanisms involved in the observed antitumor effects, some of which have been herein uncovered, we postulate the sequential administration of cyclophosphamide followed by adenoviral gene transfer of IL-12 as a potent tool for treatment of experimental colorectal carcinoma in mice. Furthermore, our results suggest this approach as a potential tool for clinical treatment of colorectal carcinoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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