Interleukin 12-based Immunotherapy Improves the Antitumor Effectiveness of a Low-Dose 5-Aza-2'-Deoxycytidine Treatment in L1210 Leukemia and B16F10 Melanoma Models in Mice

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ABSTRACT

Purpose: Recent findings indicating that many genes related to cancer development are silenced by an aberrant DNA methylation suggest that inhibitors of this process may be effective cancer therapeutics. In this study we investigated the efficacy of low-dose 5-aza-2’-deoxycytidine (DAC), a methylation inhibitor, with interleukin (IL) 12, one of the most potent cytokines with antitumor activity.

Experimental Design: Mice inoculated with L1210 leukemia cells or with B16F10 melanoma cells were treated with 7 daily injections of low-dose DAC (0.2 mg/kg) and/or 7 daily doses of IL-12 (100 ng/dose). Scid/scid mice as well as monoclonal antibodies against CD4, CD8, and NK1.1 were used to investigate the mechanisms of the antitumor effects of the combination treatment. The activity of murine lymphocytes was measured with enzyme-linked immunospot (ELISPOT) and 51Cr release assays.

Results: Treatment with DAC or IL-12 given alone produced moderate antitumor effects. In both tumor models combined treatment resulted in potentiated antitumor effects and produced 70% long-term survivors among mice inoculated with L1210 cells. The antitumor efficacy of combined treatment was abrogated in scid/scid mice, and after depletion of CD4+ and CD8+ T cells. Mice inoculated with B16F10 melanoma cells had significantly delayed tumor growth after combined treatment with DAC and IL-12. Strong antitumor effect correlated with a significant activation of lymph node-derived CD8+ and CD4+ cells. Transient neutropenia was observed in mice under treatment of DAC alone, but remarkably this effect was not potentiated by IL-12.

Conclusions: This study provides the first evidence that antitumor effects of DAC can be strongly potentiated by IL-12 and could be beneficial in an effective low-dose-based antitumor therapy.

INTRODUCTION

It has been well established that chemoimmunotherapy using cytotoxic drugs and recombinant cytokines offers an effective approach to increase the therapeutic index in the treatment of neoplastic diseases. Moreover, some chemotherapeutics were found to exert their antitumor effects not only by direct inhibition of proliferation or cytotoxic effect on tumor cells, but also by modifying the host immune responses against tumors (1).

DAC is a potent and specific inhibitor of DNA methyltransferase able to induce gene expression and cellular differentiation by mechanism involving DNA hypomethylation (2, 3). DAC was demonstrated to restore expression of HLA class I genes frequently switched off during cancer progression and to abolish resistance of tumor cells to lysis by antigen-specific cytotoxic T lymphocytes (4, 5). Reduction of complete suppression of HLA class I expression is a relatively frequent phenomenon in human tumors, being found in 15% of melanomas, 12% of laryngeal carcinomas, 14% of colorectal carcinomas, 40% of bladder carcinomas, and 25% of breast carcinomas (6, 7). Moreover, MHC class II (8, 9) and costimulatory molecules such as intercellular adhesion molecule 1 and leukotactic factor activity 3 (5) were found to be down-regulated by hypermethylation of CpG islands inside their gene promoters and reinduced in the presence of DAC. Expression of tumor-associated antigens such as MAGE, GAGE, BAGE, SSX, and LAGE-1/NY-ESO-1 can be easily restored in tumor cells after treatment with DAC (10–15). Therefore, DAC seems to be an attractive agent for supplementation of tumor immunotherapy.

Among genes of which the expression is strongly associated with promoter methylation status and are silenced during tumor progression there is a tumor suppressor gene DAPK (16). DAPK is a serine/threonine kinase required for IFN-γ-induced

1 The abbreviations used are: DAC, 5-aza-2’-deoxycytidine; DAPK, death-associated protein kinase; IL, interleukin; ELISPOT, enzyme-linked immunospot; i.f.p., into the footpad.
apoptotic cell death (17, 18). Loss of DAPK expression correlates with suppression of apoptosis and promotes highly aggressive metastatic behavior (17, 19, 20). Re-expression of DAPK was observed in leukemic cells after DAC treatment (21, 22).

Because DAC facilitates signal transduction through IFN-γ receptor and because of its multiple immunomodulatory actions, we decided to evaluate the antitumor effectiveness of this methylation inhibitor with IL-12, a cytokine that induces tumor regression through induction of IFN-γ secretion (23, 24). IL-12 was shown to exert potent activity in numerous murine models of solid tumors (25–27). There are also several observations that indicate the potential therapeutic activity of this cytokine in nonsolid malignancies (28–30). It was also demonstrated that strong immunomodulatory effect of IL-12 could rely on activation of cytotoxic properties of NK and T cells (31, 32).

Although many immunomodulatory properties of DAC have been revealed there was only one trial to improve the antitumor activity of DAC in combination with an immunomodulatory agent, a pyran copolymer. This combination treatment resulted in an increased life span of leukemia-bearing mice (33).

We assumed that combining immunomodulatory properties of DAC with strong antitumor effects of IL-12 might result in potentiated therapeutic action. This could allow reduction of the doses necessary to achieve stronger antitumor activity, hence, attenuation of the toxicity.

MATERIALS AND METHODS

Compounds. Murine recombinant IL-12 (specific activity of $2.8 \times 10^6$ units/mg) was kindly provided by Genetics Institute (Boston, MA), and murine recombinant IFN-γ (specific activity of $1 \times 10^7$ units/mg) was purchased from PeproTech EC Ltd. (London, United Kingdom). Cytokines were diluted with 0.1% BSA (Sigma, St. Louis, MO) in Dulbecco’s PBS (Invitrogen Corporation, Paisley, United Kingdom) for in vivo experiments. DAC was purchased from Sigma. For all of the experiments DAC was diluted from Sigma. All of the experiments were performed with the guidelines approved by the Ethical Committee at the Medical University of Warsaw.

Tumor Cells. B16F10 melanoma cells with a high metastatic potential and L1210 leukemia cells were used throughout experiments. Additionally, LLC cells were used as a tumor-specific control for ELISPOT assay. Cells were maintained in Dulbecco and RPMI 1640 (Invitrogen Corporation) supplemented with 10% heat-inactivated fetal bovine serum, antibiotics, 2-mercaptoethanol (50 μM) and L-glutamine (2 mM; all from...
Invitrogen Corporation), hereafter referred to as culture medium. Cells were cultured in 75 cm² tissue flask (Corning Costar Corp., Cambridge, MA) at 37°C in a fully humidified atmosphere of 5% CO₂ and passaged every 3–4 days.

**In Vivo Experiments.** Mice were inoculated i.p. on day 0 with 1 × 10⁷ L1210 cells in 50 μl PBS or i.f.p. on day 0 with 1 × 10⁶ B16F10 cells in 20 μl PBS. In mice bearing L1210 leukemia DAC was given i.p. at a dose of 0.2 mg/kg for 7 consecutive days beginning from day 4 and IL-12 at a dose of 0.1 μg in 50 μl i.p per injection for 7 consecutive days beginning from day 4 after inoculation of leukemia cells. Melanoma-bearing mice were injected with 0.1 μg of IL-12 per injection in the volume of 20 μl i.p for 7 consecutive days beginning from day 7, and DAC was given at the dose of 0.2 mg/kg i.p. for 7 consecutive days beginning from day 7. Appropriate volumes of the diluents (0.1% BSA-PBS for IL-12 and PBS for DAC) were given as a control. During the experiment leukemia-bearing mice were observed daily for survival. Tumor size of melanoma cell lines was measured by caliper twice weekly and tumor volume was calculated using the equation V = a²b / 2, where a and b are the smallest and largest diameters measured, respectively.

**Fig. 3** Effect of DAC and/or IFN-γ on cell survival. Tumor cells were incubated with serial dilutions of DAC and/or IFN-γ for 96 h and 72 h, respectively. Data represent means of triplicate samples; bars, ±SD. Results were shown as relative viability (% of control survival) when tested in L1210 (A) and B16F10 (B) cell lines. *, P < 0.05 (Student’s t test) in comparison with all other groups. Isobologram analysis of drug interaction on L1210 (C) and B16F10 (D) cell lines. Confidence interval = 95%.

**Fig. 4** Antitumor effects of the combined treatment with DAC and/or IFN-γ in L1210 leukemia-bearing scid/scid mice. Kaplan-Meyer plots of the survival of the L1210 leukemia-bearing mice.
ma-bearing mice was measured every 2 days. Tumor volume was determined according to the formula: tumor volume = (shorter diameter × shorter diameter × longer diameter/2).

In Vivo Lymphocyte Depletion Studies. Hybridoma cell lines PK-136 (anti-NK1.1), GK-1.5 (anti-CD4), and 53–6.72 (anti-CD8), obtained from American Type Culture Collection (Rockville, MD), were used as a source of monoclonal antibodies. Production of antibodies was carried out using a CellMax Quad System (Cellco Incorporated, Germantown, MA) according to the manufacturer’s protocol. Sterile and cell-free supernatants were used directly for in vivo depletion. Protocols yielding depletion of 90% of CD4+/H11001, CD8+/H11545, and NK1.1 cells subsets (23, 25) consisted of three (every 12 h) i.p. injections of 500 μl (1 mg/ml) of anti-CD4, anti-CD8, or anti-NK1.1 on day 3 after inoculation of leukemia cells and 12 h before initiation of treatment.

Isolation of CD4+*, CD8+*, and NK Cells. On day 7 of treatment animals were sacrificed. Popliteal and inguinal lymph nodes were isolated and homogenized gently with a tissue grinder (Sigma) under aseptic conditions. Cells were washed twice with chilled PBS, counted, and resuspended in a warm culture medium. To obtain specific subsets of lymphocytes magnetic cell-separating system (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) was used according to the manufacturer protocol.

ELISPOT Assay. To determine antigen-specific anti-tumor response ELISPOT assay was performed. A 96-well MaxiSorp flat-bottomed NuncImmuno microtiter plate (Nunc, Roskilde, Denmark) was coated with antimouse-IFN-γ or antimouse-IL-4 monoclonal antibodies (both from BD PharMingen, San Diego, CA) and incubated for 24 h at 4°C. Plates were washed with PBST (1% Tween 20; ICN Biomedicals, Aurora, OH; in phosphate buffered saline without Ca2+ and Mg2+) and blocked with 0.5% BSA (Sigma) in culture medium. Target cells were added to the wells at a concentration of 1 × 104 cells/ml. Effector cells were added to the wells at E:T ratio 100:1. Plates were incubated for 24 h. After washing with PBST, biotinylated secondary antibodies were added (anti-IFN-γ or anti-IL-4, both from BD PharMingen). Color reaction was developed using Alkaline Phosphatase Substrate Kit III (Vector Laboratories Inc., Burlingame, CA) and Streptavidin-Alkaline Phosphatase (In-vitrogen Corporation). Treatment groups were coded, and two independent investigators counted dots. Cell activity was estimated as releasing lymphocytes according to the formula: releasing lymphocytes = (experimental dots formation – effector cells spontaneous dots formation – target cells spontaneous dots formation) × 100.

Cytotoxic Assay. Cytotoxic assay was performed as described earlier (34). B16F10 melanoma cells were used as targets. Lymph node leukocytes were added at a 100:1 ratio to each well. Monoclonal antibody anti-CD8a (BD PharMingen) or an isotypic control, rat IgG2a (Sigma) was added to a final concentration of 50 μg/ml. Cells were incubated at 37°C for 18 h. Maximum 51Cr release was determined in target cells treated with Triton X-100 at a final concentration of 0.5%. Effector cell cytotoxicity was estimated as a cell lysis percentage according to the formula: cell lysis % = [(experimental release – spontaneous release)/(maximum release – spontaneous release)] × 100.

Cell Survival Assay. The cytostatic/cytotoxic effects of IFN-γ and/or DAC in vitro were tested in a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described (35). Serial dilutions of DAC were added to the wells at a final concentration range of 0.3125–5 μg/ml. After 24 h IFN-γ was added to the plates at concentration ranges of 3–24.
or 12.5–100 units/ml for L1210 or B16F10 cells, respectively. The means and SDs were determined for triplicate samples. The cytostatic/cytotoxic effects of DAC and/or IFN-γ/H9253 were expressed as relative viability (percentage of controls) and were calculated as shown: relative viability = \((\text{experimental absorbance} - \text{background absorbance})/\text{absorbance of untreated controls} - \text{background absorbance}) \times 100\%.

**Drug Interaction Analysis.** To examine drug interactions, isobolanalysis was used as described by Berenbaum (36). Briefly, inhibition of cell proliferation was determined as described above. Efficacious concentrations (concentration of either drug, alone or in combination, that gave equivalent inhibition of cell growth as compared with untreated controls at \(P \leq 0.05\) (Student’s \(t\) test) were analyzed. The interaction index for the combination of the two drugs was computed according to the equation: interaction index = \((\text{DAC}_A/\text{DAC}_B)/(\text{IFN}_A/\text{IFN}_B)\), where DAC\(_A\) and IFN\(_A\) are concentrations of DAC and IFN-γ, respectively, that produce some specified effect when used alone, and DAC\(_B\) and IFN\(_B\) are concentrations of DAC and IFN-γ, respectively, that produce the same effect when used in combination.

Synergy occurs when the interaction index is <1.0. Confidence interval of 95% was used to assess results as significant.

**Analysis of Peripheral Blood Hemoglobin Concentration and Neutrophil Counts.** Mice were treated as described in the above experiments. Blood was collected from tail vein of anesthetized mice as described on scheme (Fig. 1), and peripheral blood cells were assessed using a Sysmex-820 cell counter (Sysmex, Kyoto, Japan) adapted for the analysis of rodent cells.

**Statistical Analysis.** Kaplan-Mayer plots were generated, and survival time of animals was analyzed for significance by log-rank survival analysis. Results were considered significant when \(P < 0.05\). Differences between groups in B16F10 melanoma experiments and cytotoxic assay were determined using analysis of variances and \(P\) values assigned using Student’s \(t\) test. Results were considered significant when \(P < 0.05\). ELISPOT experiments analysis was performed as described else-
where (37). Analysis was performed using Mann-Whitney $U$ test and nonparametric Kruskal-Wallis ANOVA test. Results were considered significant when $P < 0.05$. All of the statistical tests are two-sided.

RESULTS

Effects of DAC and IL-12 Treatment on the Survival of Mice Inoculated with L1210 Cells. In all of the experiments mice from the control groups inoculated with L1210 cells died up to the 12th day of the experiment. Daily administrations of IL-12 resulted in a significant, albeit modest prolongation of the survival time. Administration of DAC also prolonged mouse survival time, but none of the results were statistically significant. It has been determined that mice that survive for >60 days after inoculation of L1210 cells might be regarded as long-time survivors. Combined treatment significantly prolonged mouse survival time and resulted in 71% of animals surviving >60 days (Fig. 2A). After 150 days, long-time survivors were reinfected with 10-times higher number of tumor cells i.e., $1 \times 10^9$ L1210 cells. These animals rejected inoculated L1210 leukemia with no signs of ascites development. The complete protective effect was observed until day 360 of the experiment when mice were sacrificed. In all of the rechallenge experiments we used age-matched controls that uniformly died within 10 days after inoculation of L1210 cells.

Effects of DAC and IL-12 Treatment on the Growth of B16F10 Melanoma in Mice. To evaluate the effect of a combined therapy with DAC and IL-12 in solid tumors we chose as a target a poorly differentiated melanoma cell line, B16F10. In all of the experiments mice inoculated with B16F10 melanoma cells developed progressively growing tumors (Fig. 2B). Daily administrations of IL-12 resulted in a significant reduction of tumor volume ($P < 0.05$ from day 17 of the experiment versus all of the other treatment groups). Administration of DAC alone did not produce any statistically significant antitumor effects. However, combined treatment with DAC and IL-12 resulted in a significant reduction of tumor volume ($P < 0.05$ from day 11 of the experiment versus all of the single agent-treated groups and controls).

In Vitro Cell Viability Assay and Synergy Analysis. To analyze whether the effect of the combined treatment regimen in vivo results from a direct influence of IL-12-induced IFN-γ and DAC on tumor cell viability we performed the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. We found that combined treatment with IFN-γ and DAC exerted strong cytostatic/cytotoxic effect against L1210 leukemia cells in vitro (Fig. 3A). Isobologanalysis of this combined treatment revealed strong synergistic interaction between DAC and IFN-γ (Fig. 3B). Similar analysis was performed with B16F10 melanoma cells. Although these experiments revealed potentiation of the antitumor effectiveness of DAC by IFN-γ (Fig. 3C), this effect was not as strong as in the case of L1210 leukemia cell line (Fig. 3D). All of the in vitro experiments with DAC were performed with assumption of effective drug dose in serum of 5 μg/ml.

Effects of DAC and IFN-γ Treatment on the Survival of scid/scid Mice Inoculated with L1210 Cells. To verify whether the synergistic cytostatic/cytotoxic effects of DAC and IFN-γ observed in the previous in vitro experiments with L1210 cells might be responsible for such strong antitumor effects of the combination therapy in mice, scid/scid mice were inoculated with L1210 cells and treated with 5000 units of IFN-γ and 0.2 mg/kg DAC daily for 7 consecutive days. All of the mice in this experiment died of leukemia within 21 days after inoculation of leukemia cells, and there were no significant differences in the survival between any of the experimental groups (Fig. 4).

Analysis of Immune Response by ELISPOT Assay. Strong antitumor effectiveness of the combined therapy with DAC and IL-12, and a long-lasting antitumor response prompted us to analyze the activation of the immune system. These experiments were performed with B6D2F1 mice inoculated with B16F10 melanoma cells. On day 7 of treatment mice were sacrificed, and regional lymph nodes isolated and gently homogenized. Lymphocytes were added to the plate and incubated with target B16F10 cells. Cells were analyzed for secretion of IFN-γ (Th1-like response) and IL-4 (Th2-like response). Quantification of the immune response by ELISPOT assay revealed that treatment with combined regimen resulted in strong and significant induction of B16F10-specific IFN-γ-secreting cells (Fig. 5A). There were no significant differences in IL-4 secretion among all of the treatment groups (Fig. 5B). No response was detected against LLC cells (data not shown).

Analysis of CD4+, CD8+, or NK Response Quantified by ELISPOT Assay. Several reports demonstrated that antitumor properties of IL-12 are dependent on the activation of cytotoxic lymphocytes (38, 39). Because, our observations indicate that there is no induction of IL-4 release by B16F10-specific lymphocytes, we decided to analyze the activity the IFN-γ releasing populations of CD4+ and CD8+ T cells, and NK cells. We observed that combined regimen treatment strongly stimulates specific CD4+ and CD8+ but not NK cells to secrete significant amounts of IFN-γ quantified by ELISPOT assay (Fig. 6, A–C).

Analysis of Lymphocyte Cytotoxicity with Anti-CD8a Antibody. To study the role of CD8+ cytotoxic T lymphocytes in the antitumor effectiveness of the combined treatment we performed a cytotoxicity assay with $^{51}$Cr-labeled B16F10 cells as targets. To specifically block the activation of CD8+ lymphocytes, anti-CD8a antibodies were used. In accordance with our earlier studies no significant cytotoxicity was observed in untreated (control) group or in any group incubated with a single agent alone. Lymph node lymphocytes obtained from mice treated with DAC and IL-12 showed statistically significant antitumor activity versus all of the other groups. When these lymphocytes were incubated with an anti-CD8a antibody, their antitumor activity was completely abrogated (Fig. 7).

Antileukemic Effects of IL-12 and DAC in scid/scid Mice and NK.1 Cell-depleted Mice. To get additional insight into the mechanisms of the antileukemic effects of IL-12 and DAC we have undertaken experiments in scid/scid mice that lack T and B cells but have normal NK cells. Both IL-12 and DAC when administered alone exerted slight, nonsignificant antitumor effects as compared with controls. There was no potentiation of the antileukemic efficacy in animals treated with both agents simultaneously (Fig. 8A). Similarly, administration of IL-12 and DAC in mice depleted of NK.1 cells showed an abrogation of the antileukemic effects and resulted in long-term...
survival in 75% of animals (Fig. 8B). These results indicate that the presence of NK cells is not sufficient to induce antitumor effects in L1210 leukemia.

Effects of Treatment with IL-12 and DAC in Leukemia-bearing Mice Depleted of CD4+ and CD8+ Cells. The results of previous ELSIPOT and 51Cr release assays together with the experiments in scid/scid and NK1.1+ cell-depleted mice indicate that the antileukemic response mediated by IL-12 and DAC is dependent on the adaptive immune response. Therefore, in the next series of experiments we have evaluated the antileukemic effects of the combination treatment in mice depleted of major subpopulations of T cells. Treatment with IL-12 and DAC in control antibody-injected animals resulted in 75% of long-term survivors. This effect was abrogated in animals depleted of CD4+ or CD8+ cells (Fig. 9). These results indicate that T cells are necessary for the complete eradication of leukemic cells.

Analysis of Peripheral Blood Hemoglobin Concentration and Neutrophil Counts. Because DAC was shown to generate reversible myelosuppression and IL-12 was also shown to be myelotoxic we analyzed peripheral blood for hemoglobin concentration (Table 1) and neutrophil counts (Table 2) during and after therapy with DAC and IL-12. Our experiments revealed that combined treatment did not augment neutropenia nor decreased the hemoglobin concentration versus IL-12 or DAC alone, either during treatment or after it. Treatment regimen with either drug alone or in combination resulted in a statistically significant (P < 0.05 versus controls) decrease in peripheral blood values on days 7 and 10 of the experiment. The myelosuppressive influence of therapy with low-dose DAC and/or IL-12 was completely reversible, and neutrophil count and hemoglobin concentration normalized on day 13 of the experiment.

DISCUSSION

Promoter hypermethylation of genes important in the regulation of cell proliferation and differentiation is a frequent abnormality observed in primary neoplasms and tumor cell lines (40, 41). Thus, the application of pharmacologic inhibitors of DNA methylation provides an attractive and rational approach to re-establish the antiproliferative and differentiation-inducing signals silenced by hypermethylation (42). However, the preliminary results of studies with methylation inhibitors in the clinical trials were not encouraging. Initial trials of 5-azacytidine in patients with sickle cell disease and β-thalassemia (43, 44) were abandoned because of an apparent increase in the incidence of tumors in rats treated with this agent (45). In 1988, a novel methylation inhibitor DAC was reported to be nontumorigenic and was shown to demonstrate antitumor effects in animal models (46). Moreover, studies of methylation inhibitors in patients with myelodysplastic syndrome and acute myeloid leukemia revealed limited systemic toxicity when treatment was conducted at low concentrations of DAC (30 mg/m2) versus
high concentrations (150 mg/m²). In our studies combination treatment with IL-12 and DAC used at a low dose did not result in any major toxicity in treated animals, such as weight loss, diarrhea, hunched habitus, and so forth. Importantly, the dose and regimen of treatment with DAC was close to that used in patients with sickle cell disease, where the only observed drug-related adverse change was a transient neutropenia, which was not accompanied by any clinical sequelae (47). In our study, analysis of peripheral blood revealed a significant decrease in hemoglobin concentration and neutrophil count on day 10 of experiment in mice treated with DAC alone and in combination with IL-12 versus controls (Tables 1 and 2). However, no significant difference was observed between these two groups suggesting that IL-12 did not augment its myelosuppressive actions. Furthermore, we demonstrated that antitumor activity of DAC was highly potentiated by IL-12 resulting in >70% complete responses in L1210 leukemia-bearing mice and a statistically significant retardation of tumor growth in B16F10 melanoma-bearing mice (Fig. 2, A and B). In L1210 leukemia the combined treatment resulted in a long lasting (for at least 150 days) immune memory evaluated in rechallenge experiments.

Because of the multiple biological effects assigned to IL-12 and wide antitumor properties of DAC, the exact mechanisms underlying therapeutic effects of the combined therapy are difficult to pinpoint. IL-12 was shown to exert its antitumor activity through induction of IFN-γ secretion from T and NK cells (23, 24). Moreover, it potently enhances the proliferation and cytotoxicity of these cells (26). Numerous studies have revealed that different cell types, depending on the tumor model or treatment regimen used, ensure optimal antitumor effectiveness of IL-12 (27).

It was shown in previous studies (28) that IL-12 is a potent inducer of IFN-γ secretion in L1210 leukemia-bearing mice. Therefore, to get insight into the mechanism of antitumor action of the combination treatment we have studied the in vitro effects of DAC combined together with IFN-γ. Although we could observe a pronounced potentiation of DAC-mediated cytostatic/cytotoxic effects by IFN-γ against L1210 leukemia in vitro, there were no antileukemic effects in acid/scid mice treated with this combination of drugs. These results prompted us to investigate the immune response of mice treated with DAC and IL-12. Previous reports revealed that T, NK, and NK T cells, macrophages, or even neutrophils could be involved in the antitumor responses generated by IL-12 used alone or in combination with other antitumor drugs (48–50). In our studies we have observed that the antitumor efficacy of the combination treatment with IL-12 and DAC relies on the activation of CD4+ cells and CD8+ cells. The antitumor effectiveness of this drug combination is significantly decreased after elimination of T cells and is completely abrogated in severe combined immuno-deficiency mice. Moreover, T cell-dependent tumor-specific immune responses correlate with the expression of MHC class I molecules on B16F10 cells treated with DAC and IFN-γ (data not shown). The combined therapy results in the induction of long-lasting immunological memory in mice inoculated with L1210 cells. Although we demonstrated the induction of immune response both in mice bearing leukemia and melanoma, the overall therapeutic effect of the combined treatment seems to be tumor type-dependent.

It is not completely clear why the combination of IL-12 and DAC is more effective against L1210 leukemia than against B16F10 melanoma. Importantly, this is the first combination of IL-12 with a chemotherapeutic that is effective in both tumor models. Previous combinations of IL-12 with doxorubicin, mitoxantrone, or 5-fluoruracil effective in the L1210 leukemia model were not showing antitumor activity in melanoma nor in Colon-26 carcinoma models in mice (51–53). On the other hand, Taxol, cisplatin, and cyclophosphamide were effectively potentiating antitumor effectiveness of IL-12 in melanoma and sarcoma models but were completely unsuccessful in the treatment of L1210 leukemia (52–54). Therefore, there are some specific

### Table 1 Hemoglobin concentration in peripheral blood of mice treated with DAC and/or IL-12

| Day of treatment | Controls | Day 1 | | Day 3 | | Day 7 | | Day 10 | | Day 13 | | Day 16 |
|-----------------|----------|------|---|------|---|------|---|------|---|------|---|------|---|
|                 | 15.9 ± 1.5 | 16.2 ± 2.0 | 16.6 ± 1.1 | 15.2 ± 1.9 | 15.3 ± 0.7 | 15.2 ± 0.4 | 15.0 ± 0.8 | 14.5 ± 0.8 | 14.5 ± 1.5 | 15.0 ± 0.6 |
| DAC             | 17.0 ± 1.3 | 18.2 ± 1.5 | 18.5 ± 1.6 | 17.0 ± 1.7 | 16.3 ± 1.8 | 16.1 ± 1.9 | 15.8 ± 1.7 | 15.3 ± 1.6 | 15.0 ± 1.4 | 14.8 ± 1.5 |
| IL-12           | 17.2 ± 0.8 | 18.0 ± 1.3 | 18.2 ± 1.5 | 17.0 ± 1.6 | 16.3 ± 1.7 | 16.0 ± 1.8 | 15.7 ± 1.6 | 15.3 ± 1.5 | 15.0 ± 1.4 | 14.8 ± 1.5 |
| DAC and IL-12   | 16.4 ± 1.6 | 17.2 ± 1.7 | 17.8 ± 1.8 | 16.6 ± 1.9 | 16.0 ± 2.0 | 15.7 ± 2.1 | 15.4 ± 2.0 | 15.1 ± 1.9 | 14.8 ± 1.8 | 14.6 ± 1.7 |

* Measurements of peripheral blood values were performed according to the scheme on Fig. 1. Data represent mean value ± SD.

### Table 2 Neutrophil counts in peripheral blood of mice treated with DAC and/or IL-12

| Day of treatment | Controls | Day 1 | | Day 3 | | Day 7 | | Day 10 | | Day 13 | | Day 16 |
|-----------------|----------|------|---|------|---|------|---|------|---|------|---|------|---|
|                 | 2.15 (1.3–5.3) | 3.55 (0.7–6.1) | 2.7 (1.3–6.5) | 2.7 (2.1–3.6) | 2.65 (1.2–4.2) | 2.2 (1.3–3.7) | 2.1 (1.3–3.7) | 2.0 (1.2–4.0) | 2.0 (0.7–4.1) | 1.1 (0.5–5.7) | 1.4 (1.0–2.9) | 3.1 (2.1–4.6) | 2.1 (1.3–2.4) |

* Measurements of peripheral blood values were performed according to the scheme on Fig. 1. Data represent median value and range (min–max).
combinations of IL-12 and chemotherapeutics that are effective in some but not in other tumor models.

We cannot exclude other mechanisms that could participate in the potentiated antitumor activity of DAC and IL-12. It has been demonstrated that a single agent treatment with either DAC or IFN-γ inhibits progression through the cell cycle and induces apoptosis of tumor cells (55–58). Several reports indicate that both DAC and IL-12 exhibit strong antiangiogenic activities (26, 59–61) that could be potentiated in mice treated with both drugs simultaneously. It is noteworthy that antitumor strategies using the antiangiogenic effects of IL-12 in combination with other agents capable of inhibiting new blood vessel formation have already proved effective (62, 63).

In conclusion, we demonstrate that IL-12 can be successfully used as an adjuvant treatment to the DNA methylation inhibiting therapeutics such as DAC. Combined treatment with these agents results in activation of tumor-specific CD4+ and CD8+ lymphocytes, and is capable of generating long-term immunological memory. We hope that the results of our studies could be of benefit to therapy of cancer patients.

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