

Chemoimmunotherapy Reduces the Progression of Multiple Myeloma in a Mouse Model

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Abstract

Multiple myeloma (MM) is a B-cell malignancy characterized by clonal proliferation of malignant plasma cells in the bone marrow. Recently, we showed a correlation between increased ratios of functional regulatory T cells (Treg) and disease progression in a unique mouse model that mimics the human disease. Cyclophosphamide (CYC) is a cytotoxic alkylating agent widely used in chemotherapeutic regimens. Low-dose CYC was previously reported to selectively reduce Treg levels and to contribute to immunostimulation. Our objectives were (a) to determine whether treatment using a low-dose CYC could reduce MM progression and (b) to further characterize the modes of action underlying these effects. We found that both low- and high-dose CYC given to sick mice with hind limb paralysis resulted in the disappearance of the paralysis, the replacement of plasma tumor cells in the bone marrow by normal cell populations, and a significant prolongation of survival. However, only low-dose CYC treatment decreased the incidence of MM. Low-dose CYC rendered Tregs susceptible to apoptosis because of the downregulation of Bcl-xL and CTLA-4 in these cells, and a decreased production of interleukin 2 by effector CD4 cells. Moreover, using this treatment, we noted the recovery of IFN- γ -producing natural killer T cells and maturation of dendritic cells. Treatment of tumor-bearing mice with repeated administrations of low-dose CYC at longer time intervals (coinciding with the blocked renewal of Tregs) resulted in reduced tumor load, and the prevention or delay of disease recurrence, thereby breaking immune tolerance against MM tumor cells. *Cancer Prev Res*; 3(10); 1265–76. ©2010 AACR.

Introduction

The role of regulatory T cells (Treg) in tumor development has been extensively studied in recent years. CD4⁺CD25⁺Foxp3⁺ Tregs suppress T-cell proliferation, downregulate proinflammatory cytokines, and are involved in tumor tolerance (1). An increased frequency of Tregs in rodents and humans with solid tumors and hematologic malignancies has been observed (1–5), and their functional role in reducing the antitumor response has been shown in mice (6–9). In humans, the contribution of Tregs to tumor tolerance was strongly suggested by the significant correlation between Treg levels and poor survival of patients with ovarian cancer (2), and tumor relapse in those with breast cancer and non-small cell lung carcinoma (10, 11). Treg-mediated immunosuppression could be a crucial evasion mechanism that prevents the elimination of cancerous cells by the immune system.

Hence, new strategies in cancer immunotherapy aimed at reducing Tregs have been proposed (12).

Multiple myeloma (MM) is a B-cell malignancy characterized by clonal proliferation of malignant plasma cells in the bone marrow (BM), and the development of osteolytic bone lesions. Bone destruction is the main clinical consequence of MM. Our recent study showed a correlation between increased numbers of functional Tregs and disease progression in two mouse models of MM (13). Similar findings have been observed in patients with B-cell malignancies, including MM (14–16).

Cyclophosphamide (CYC) has been used as a standard anticancer alkylating chemotherapeutic agent against certain solid tumors and lymphomas because of its direct cytotoxic effect and its inhibitory activity against cycling cells. Whereas high doses of CYC might lead to the depletion of immune cells, low doses of CYC could enhance immune responses (17). A low-dose CYC has been shown to reduce the numbers and function of Tregs, and to induce antitumor, immune-mediated effects (18–20). Furthermore, administering low-dose CYC at regular intervals (the “metronomic” regimen) enabled the restoration of immune functions in patients with end-stage tumors (21). These depletion effects of CYC on Tregs were detected in normal rodents (18) and in solid tumor-bearing rodents (19, 20). However, the repeated use of this drug in low doses as a therapeutic agent has not been tested.

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doi: 10.1158/1940-6207.CAPR-10-0138

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The 5T2MM model represents the human form of the disease because its clinical characteristics involve selective malignant plasma cell localization in the BM replacing the normal BM cell populations, monoclonal gammopathy, serum M component, angiogenesis, adhesion chemokine profiles, and bone lesions in limbs and spine (22, 23). In the present study of murine MM, we found that both a single high- or low-dose CYC given to sick mice significantly prolonged their survival; however, only the low-dose CYC reduced disease incidence.

We further show that a single low-dose CYC affects Tregs to downregulate CTLA-4 and Bcl-xL and to become less resistant to apoptosis. Moreover, this treatment approach decreases the production of interleukin 2 (IL-2) by effector CD4 cells, promotes the recovery of IFN- γ -producing natural killer T (NKT) cells, and the maturation of dendritic cells (DC). All these responses could play valuable roles in inducing an efficient immune response against MM tumor cells. Our studies on Treg repopulation kinetics showed that transient gradual depletion of Tregs by treatment with low-dose CYC to 5T2MM-bearing mice was maintained beyond 42 days. Repeated treatments of low-dose CYC at 45-day intervals very markedly reduced the progression of the disease. A durable transient depletion of Tregs could facilitate the recruitment of a latent pool of compatible immune cells that would reduce tumor load and delay or prevent tumor recurrence, thus resulting in markedly reduced MM incidence.

Materials and Methods

Mice

C57BL/KaLwRij mice were purchased from Harlan CPB and bred at the Weizmann Institute Breeding Center. All experimental procedures were approved by the Weizmann Institute Animal Care Committee.

MM progression in mice

The 5T2MM mouse model (IgG_{2ak}) used in this study developed the disease spontaneously from the BM of an aging C57BL/KaLwRij mouse. This tumor can be propagated only *in vivo* by the transfer of BM from sick mice into young syngeneic mice. Briefly, 3×10^5 BM cells from 5T2MM-C57BL/KaLwRij mice (flushed contents of tibias and femurs) were injected *i.v.* into normal syngeneic female mice at the age of 8 to 10 weeks. A few foci of MM cells in spleen and BM were observed from 28 days onwards following the tumor cell challenge. The malignant clones migrated to the BM and bound to stromal cells. Larger MM foci both in spleen and BM sections were observed 40 days following the 5T2MM cell challenge. The clinical phase of the disease was observed ~60 days onwards post-tumor cell challenge by the occurrence of hind limb paralysis. From 70 days onwards, the clinical phase progressed coupled with an altered balance in osteoclasts and osteoblasts leading to increased osteoclast numbers, increased bone resorption, and decreased bone formation and hypercalcemia. Tumor cells were noted to

proliferate outside the bones into the surrounding muscles and into the vertebral canal, leading to paraplegia and increased morbidity. Increased angiogenesis involving activated vascular endothelial growth factor was also produced in the 5T2MM cells. The progression of the disease was assessed by measuring the levels of serum paraprotein (24). In 25% to 30% of sick mice (beyond 80 d) MM cells infiltrated the ovaries. At the late phase of the disease, a bulge in the cranium was sometimes observed. Sick mice were euthanized when severe limb paralysis or other signs of morbidity occurred.

Treatment with CYC

CYC (cyclophosphamide monohydrate; Sigma) was dissolved in PBS. Freshly prepared solutions were injected *i.p.* Two doses of CYC were used: a high dose (200 mg/kg, 4 mg/mouse) and a low dose (100 mg/kg, 2 mg/mouse).

Adoptive transfer of CD4⁺CD25⁺ Tregs or CD4⁺CD25⁻ T cells to 5T2MM-inflicted mice treated with CYC

After 75 days, mice inoculated with 5T2MM cells were injected with CYC (2 mg/mouse) and followed 24 hours later by adoptive transfer of either purified CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells (1.5×10^6 cells/mouse). These T cells were sorted from the spleens of normal mice. Cells were sorted using a magnetic sorting (MACS): CD4⁺CD25⁺ regulatory T-cell isolation kit (Miltenyi 130-091-041). CD4⁺CD25⁺ Treg purity was 86% and CD4⁺CD25⁻ T-cell purity was 98%.

Antibodies and reagents

Anti-CD4-PE (clone GK1.5), anti-CD4-APC (clone L3T4), anti-CD25-FITC (clone 7D4), anti-CTLA-4-PE (clone 1B8), anti-CD8-FITC (clone 53-6.7), anti-NK1.1-PE (clone PK136), and their matched isotype controls were obtained from Southern Biotechnology Associates. Anti-CD45RB-PE (clone 16A), anti-CD62L-PE (clone MEL-14), anti-CD44-FITC (clone IM7), and their matched isotype controls were purchased from PharMingen. Anti-CD11c-APC (clone N418), anti-Foxp3-FITC (clone FJK-16s Set), anti-IL-2-FITC (clone JES6-5H4), anti-IFN- γ -FITC (clone XMG1.2), anti-I-A/I-E-PE (clone M5/114.15.2), and anti-CD86-PE (clone GL1) were purchased from eBioscience. Anti-Bcl-xL-PE (clone H-5) and its isotype control were purchased from Santa Cruz Biotechnology. Fixation and permeabilization solutions were obtained from Serotec.

Flow cytometry analysis

Splenocytes (10^6 cells) were incubated with the relevant antibody and analyzed by fluorescence-activated cell sorting (Becton Dickinson). For intracellular staining, the cells were incubated with a fixation solution, washed, and then resuspended in a permeabilization solution (Serotec). Actual gates of CD25^{high} cell populations were determined based on intensity of CD25 staining greater than 10 squares, where staining of Foxp3 was positive in more than 85% of gated cells. Total cell numbers were calculated as the number of total spleen cell count

(after RBC depletion) multiplied by (%CD4⁺) and by (%CD25^{high} of CD4⁺).

Detection of apoptosis

The viability of the cells was determined by staining with 7-amino-actinomycin D, which was purchased from PharMingen and used according to the protocols of the manufacturer. Apoptosis of cells was detected by staining with Annexin V and propidium iodide using the phosphatidyl serine detection kit (IQ Products), according to the protocol supplied by the manufacturer. Cells were analyzed by fluorescence-activated cell sorting.

Serum paraprotein levels

Analysis of the mouse serum proteins was done using capillary zone (Capillarys 2 Sebia France) electrophoresis (24) at the Department of Immunology, Central Laboratory of Maccabi Health Services in Rehovot, Israel.

Histology

Tissues for light microscopy studies were fixed in 10% formalin in PBS. Femurs and tibias were first decalcified

using the formic acid sodium citrate method. H&E staining on paraffin sections were complemented. An Olympus BX50 microscope (objective, $\times 20$; ocular, $\times 10$) and a Nikon Digital DXM 1200 camera were used.

Statistical analysis

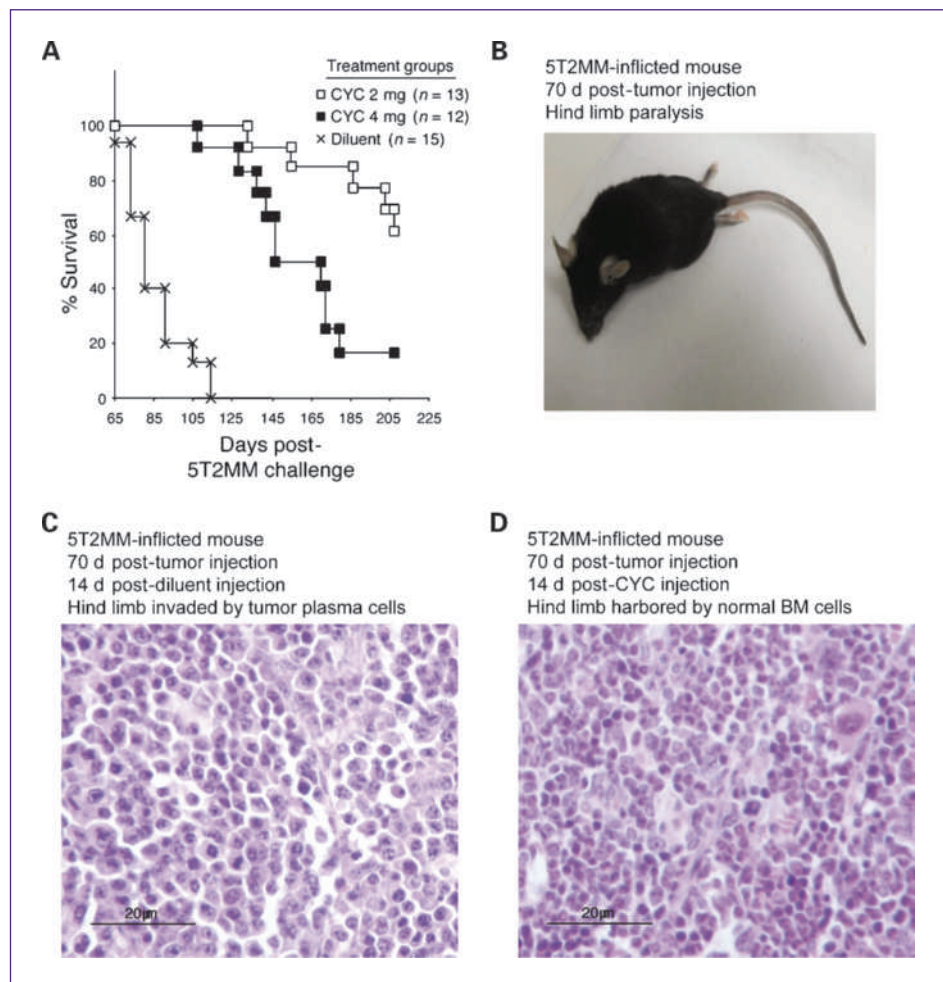
Significance was assessed by using unpaired Student's *t* test. *P* < 0.05 values were considered significant.

Results

Effects of a single low- and high-dose CYC treatment on 5T2MM progression in mice

CYC is an anticancer alkylating cytotoxic drug. We compared the effects of two doses of CYC in a murine model of MM. Toward this end, a pool of 40 mice was injected i.v. with 3×10^5 BM cells from sick mice with 5T2MM. At 65 days after MM cell challenge, the serum paraprotein level was 0.89 to 1.32 g/dL, and all mice developed hind limb paralysis, owing to the compression of the sciatic nerve by enlarged sciatic nodes in the back (Fig. 1B). These mice were divided into three groups as follows: group 1

Fig. 1. Myeloma progression following treatment with a low- or a high-dose CYC. Sixty-five days after 5T2MM cell challenge (i.v. injection of 3×10^5 /mouse), the mice were divided into three treatment groups as follows: a single i.p. injection of either a low-dose CYC (2 mg/mouse; *n* = 13), a high-dose CYC (4 mg/mouse; *n* = 12), or the diluent (*n* = 15). A, a follow-up of the mice survival was carried out for ~200 d. All diluent-treated mice (15 of 15) died within a mean latency of 85 d. Mice injected with a low-dose CYC exhibited reduced MM incidence to 38% (5 of 13) and prolonged survival (a 185-d mean latency). Mice injected with a high-dose CYC also exhibited prolonged survival (a 145-d mean latency) but a high incidence of 83% (10 of 12) of sick mice was observed. Representative results of three independent experiments are shown. B, hind limb paralysis in a 5T2MM mouse, 70 d post-tumor injection. Histology preparations of BM sections taken from 5T2MM mice 70 d after tumor injection and 14 d after treatment with diluent (C) or CYC (D).



($n = 13$), i.p. injection of a low dose of CYC (2 mg/mouse); group 2 ($n = 12$), i.p. injection of a high dose of CYC (4 mg/mouse); and group 3 ($n = 15$), i.p. injection of the diluent. A follow-up of their survival was carried out for about 200 days, and the results are presented in Fig. 1A.

All mice in the control groups (15 of 15) died within a mean latency of 85 ± 12 days. Both doses of CYC caused the disappearance of paralysis within 14 to 30 days when the serum paraprotein levels were reduced to those determined for the normal control (0.13-0.2 g/dL). Thus, a reduction in the initial tumor load was observed. The tumor cells observed in both hind limbs 65 days after 5T2MM injection (Fig. 1C) were replaced with normal BM cell populations (Fig. 1D).

The single injection of a low-dose CYC reduced the incidence of the diseased mice to 38% (5 of 13) and prolonged their survival (177 ± 26 d versus 85 ± 12 d in the control group; $P < 0.0001$). As shown in Fig. 1A, treatment with the high-dose CYC also delayed the development of the disease (mean latency, 150 ± 18 d) as compared with the control group (mean latency, 85 ± 12 d; $P < 0.0007$), but a high percentage of mice (83%) ultimately developed MM. The longer survival among the low-dose CYC-treated mice versus the high-dose is significant ($P < 0.005$). These results were observed in three independent experiments. Thus, a low-dose CYC was more effective than a high-dose CYC in reducing the development of the disease.

Effects of a single injection of CYC on Tregs in 5T2MM mice

Following our observation that elevated Treg cell levels were associated with MM progression (13), we studied the kinetics of Tregs in 5T2MM mice following a single treatment of a low-dose versus a high-dose CYC. Spleen-derived cells from individual 5T2MM mice in each treatment group ($n = 5$ /group) were analyzed 25 days following the single injection of CYC using flow cytometry. As shown in Fig. 2A, treatment using the two doses of CYC resulted in a decrease in absolute counts of CD4 and CD8 T cells in comparison with the injection of diluent; however, the diminishing effect of a high-dose CYC on absolute counts of the latter cells was more prominent.

The effect of a single injection of CYC on CD4⁺CD25^{high}Foxp3⁺ cells is presented in Fig. 2B. As shown, the low, and to a lesser extent, the high doses of CYC decreased the percentages of CD4⁺CD25^{high}Foxp3⁺ cells. However, the low dose, and to a greater extent, the high dose of CYC resulted in a pronounced reduction in the absolute counts of CD4⁺CD25^{high}Foxp3⁺ cells. These results were observed in three independent experiments.

Because the low- and the high-dose CYC could effectively eliminate CD4⁺CD25^{high}Foxp3⁺ cells (Fig. 2A and B), and because the clinical ameliorative effect of a low-dose CYC was superior to a high-dose CYC (Fig. 1A), we also studied the long-term effects of a low-dose CYC on CD4⁺CD25^{high}Foxp3⁺ cells. Toward this objective, 65 days after the 5T2MM cell challenge, the mice were injected

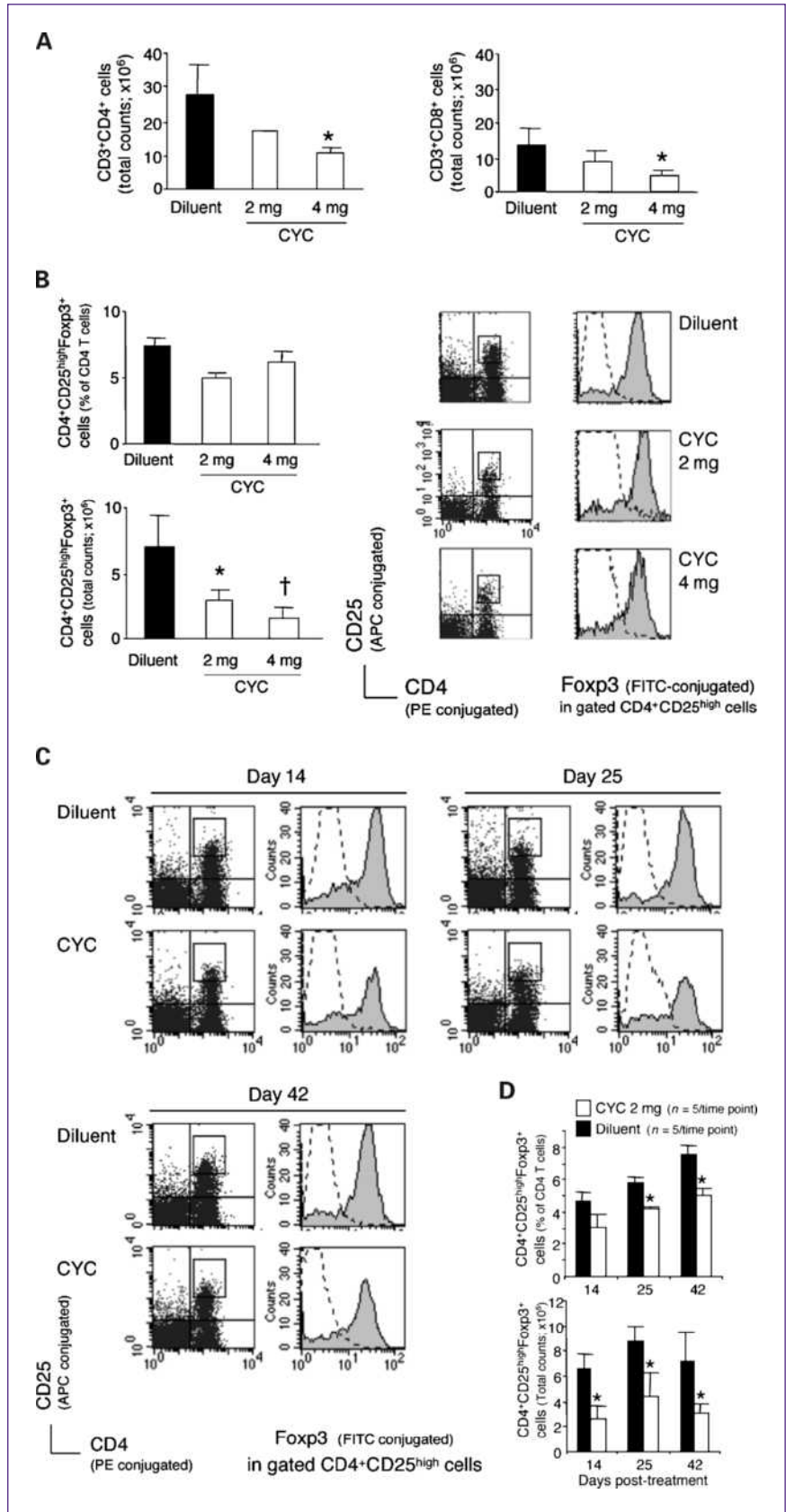
with a single dose of either a low-dose CYC ($n = 15$) or the diluent ($n = 15$), and the percentages and absolute counts of CD4⁺CD25^{high}Foxp3⁺ cells were determined 14, 25, and 42 days later. Figure 2C presents representative dot plots depicting staining with CD4 and CD25, as well as histograms of Foxp3 expression in CD4⁺CD25^{high}-gated cells. Figure 2D presents the mean (\pm SD) percentages and absolute counts of CD4⁺CD25^{high}Foxp3⁺ cells of individual mice per treatment group during the follow-up period. Accordingly, the percentages of CD4⁺CD25^{high}Foxp3⁺ cells were increased gradually at 25 and 42 days relative to that observed at 14 days following injections in both groups (Fig. 2D); however, decreased levels of CD4⁺CD25^{high}Foxp3⁺ cells were observed in CYC-injected mice in comparison with those injected with the diluent at all time points tested. Moreover, the absolute counts of CD4⁺CD25^{high}Foxp3⁺ cells were steadily reduced in the CYC-injected mice in comparison with the relatively consistently elevated counts of these cells from diluent-injected mice.

The beneficial effects of a low-dose CYC on 5T2MM mice are due to disrupted survival of CD4⁺CD25^{high} cells

Next, we studied the mechanisms contributing to the elimination of CD4⁺CD25^{high}Foxp3⁺ cells by treatment with CYC. 5T2MM mice were treated with a single injection of a low dose of CYC or a diluent, and 25 days later, CD4⁺CD25^{high} cells from individual mice within a group were analyzed by flow cytometry. The results, shown in Fig. 3, revealed that the expression of the survival molecule, Bcl-xL, was significantly downregulated ($P = 0.0004$) in CD4⁺CD25^{high} cells from CYC-injected mice in comparison with those cells from diluent-injected mice (Fig. 3A). Indeed, CD4⁺CD25^{high} cells from CYC-injected mice exhibited increased rates of apoptosis, as detected by staining with Annexin V/propidium iodide (Fig. 3A). Of note, low-dose CYC had no significant apoptotic effects on other cell populations including macrophages and DCs (data not shown). Furthermore, the expression of CTLA-4 in CD4⁺CD25^{high} cells was significantly downregulated ($P = 0.0008$) in CYC-injected mice in comparison with that noted in diluent-injected mice (Fig. 3A). Furthermore, the production of IL-2 by CD4 T cells was significantly reduced ($P = 0.0009$) in response to a single injection of low-dose CYC relative to the effect of the diluent of these cells (Fig. 3B).

To confirm our results indicating that the ameliorative effects of a low-dose CYC on 5T2MM mice are associated with the suppressive effects of CYC on CD4⁺CD25^{high} cells, we did adoptive transfer experiments. Mice carrying 5T2MM cells were treated with a low-dose CYC (given 75 d after tumor cell challenge). Twenty-four hours later, when the cytotoxic effects of CYC were already absent (due to the 30-min half-life of the drug; refs. 25, 26), the CYC-treated mice ($n = 4$ /group) were adoptively transferred with either purified CD4⁺CD25^{high} cells (1.5×10^6 cells/mouse) or purified CD4⁺CD25⁻ cells (1.5×10^6 cells/mouse), and a follow-up was conducted. The results show

Fig. 2. Effects of a single injection of a low- versus a high-dose CYC on Tregs in 5T2MM-inflicted mice. Forty days following a 5T2MM cell challenge, the mice were divided into three treatment groups ($n = 5/\text{group}$) as follows: a single injection of 2 mg CYC, 4 mg CYC, or the diluent alone. Twenty-five days following the treatment, spleen-derived cells were analyzed individually for all mice within a group using flow cytometry. Results from three independent representative experiments are shown. A, columns, mean absolute counts of CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells; bars, SD. B, columns, mean percentages and absolute counts of CD4⁺ T cells that are CD4⁺CD25^{high}Foxp3⁺ cells; bars, SD. Representative dot plots for CD4 and CD25 staining and representative histograms depicting the expression of Foxp3 in CD4⁺CD25^{high}-gated cells are also shown. C, a single injection of 2 mg of CYC or the diluent was given to mice ($n = 15/\text{group}$) challenged with 5T2MM cells 42 d earlier. Kinetic measurements of spleen-derived CD4⁺CD25^{high}Foxp3⁺ cells are shown by representative dot plots for staining with CD4 and CD25, and histograms depicting Foxp3 expression in CD4⁺CD25^{high}-gated cells, 14, 25, and 42 d following a single injection of 2 mg CYC or the diluent ($n = 5/\text{time point}/\text{group}$). D, columns, mean percentages and absolute counts of CD4⁺CD25^{high}Foxp3⁺ cells of individual mice within a group; bars, SD (*, $P < 0.05$; †, $P < 0.005$).



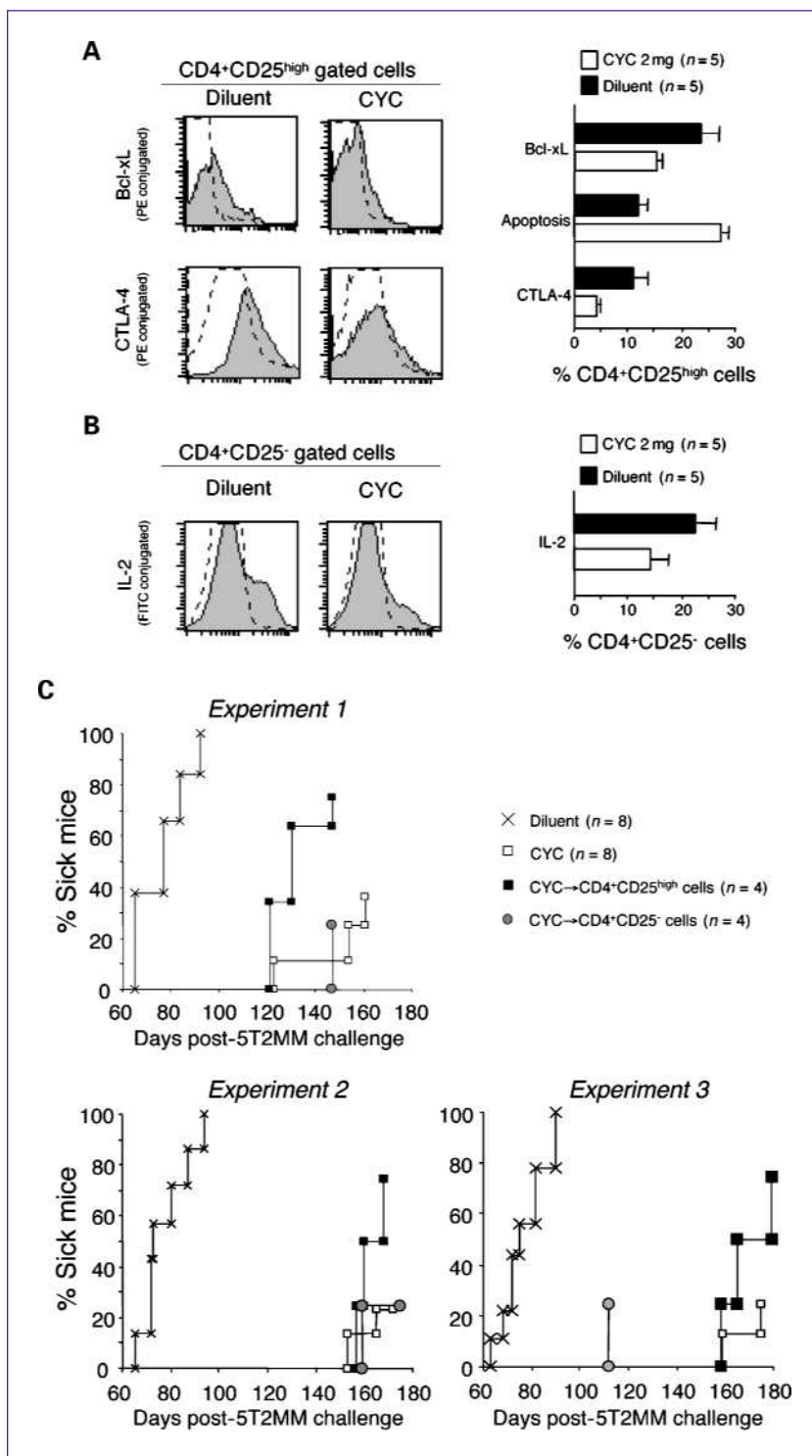


Fig. 3. CYC affects molecules essential for the survival and function of CD4⁺CD25⁺Foxp3⁺ cells in ameliorating 5T2MM-inflicted mice. 5T2MM-inflicted mice were treated with a single injection of a low-dose CYC or a diluent and 25 d later, spleen-derived cells were analyzed using flow cytometry. A, left, representative histograms showing the effect of CYC on the expression of Bcl-xL and CTLA-4 in CD4⁺CD25^{high}-gated cells. Right, columns, mean percentages of Bcl-xL, apoptosis (using Annexin V/propidium iodide), and CTLA-4 in spleen-derived CD4⁺CD25^{high} cells from individual mice within a group; bars, SD. B, left, representative histograms for IL-2 production by CD4 T cells. Right, columns, mean percentages of spleen-derived CD4⁺IL-2⁺ cells from individual mice within a group; bars, SD. C, seventy-five days after 5T2MM cell challenge, mice were treated with a single injection of 2 mg of CYC alone (n = 8), or in combination 24 h later with an adoptive transfer of purified CD4⁺CD25^{high} cells (1.5 × 10⁶ cells/mouse; n = 4) or CD4⁺CD25⁻ cells (1.5 × 10⁶ cells/mouse; n = 4). A fourth group of 5T2MM-inflicted mice were treated with the diluent only (n = 8). Results of three independent experiments are shown (*, P < 0.05; †, P < 0.005).

(Fig. 3C) that all the mice (eight of eight) challenged with 5T2MM cells and injected with the diluent, developed the disease at a mean latency of 78 ± 8 days. Treatment with low-dose CYC reduced the development of the disease by up to 38% (three of eight) and prolonged the sur-

vival of the mice (145 ± 37 d; P < 0.0001). Restoration of CD4⁺CD25⁺ T cells abolished the antitumor effect of the low-dose CYC as 75% (three of four) of mice in this group developed the disease (136 ± 7 d). In contrast, maintenance of the CYC effect was observed in 75% (three of

four) of mice following the transfer of CD4⁺CD25⁻ cells. Percentages of mice that developed MM in a second experiment were as follows: 100% (seven of seven) in the control group (72 ± 4 d mean latency), 43% (three of seven) in CYC-treated mice (160 ± 17 d, *P* < 0.00008), 75% (three of four) in CYC-treated mice restored with CD4⁺ CD25⁺ T cells (157 ± 5 d), and 25% (one of four) in CYC-treated mice restored with CD4⁺CD25⁻ cells (172 ± 20 d). Percentages of mice that developed MM in a third experiment were as follows: 100% (nine of nine) in the control group (75 ± 8 d), 38% (three of eight) in CYC-treated mice (182 ± 23 d, *P* < 0.00005), 75% (three of four) in CYC-treated mice restored with CD4⁺CD25⁺

T cells (165 ± 22 d), and 25% (one of four) in CYC-treated mice restored with CD4⁺CD25⁻ cells (112 d).

The beneficial effects of a low-dose CYC on 5T2MM mice are associated with an increase in IFN-γ-producing NKT cells and maturation of DCs

In parallel with decreased levels of CD4⁺CD25^{high} Foxp3⁺ cells following a low-dose CYC, we analyzed the effects of the drug on NKT cells and DCs. These cells could be affected as a result of Treg cell reduction (27, 28). Spleen-derived cells from individual 5T2MM mice treated with a single injection of low-dose CYC (*n* = 5) or the diluent (*n* = 5) were stained for NK1.1 and CD3,

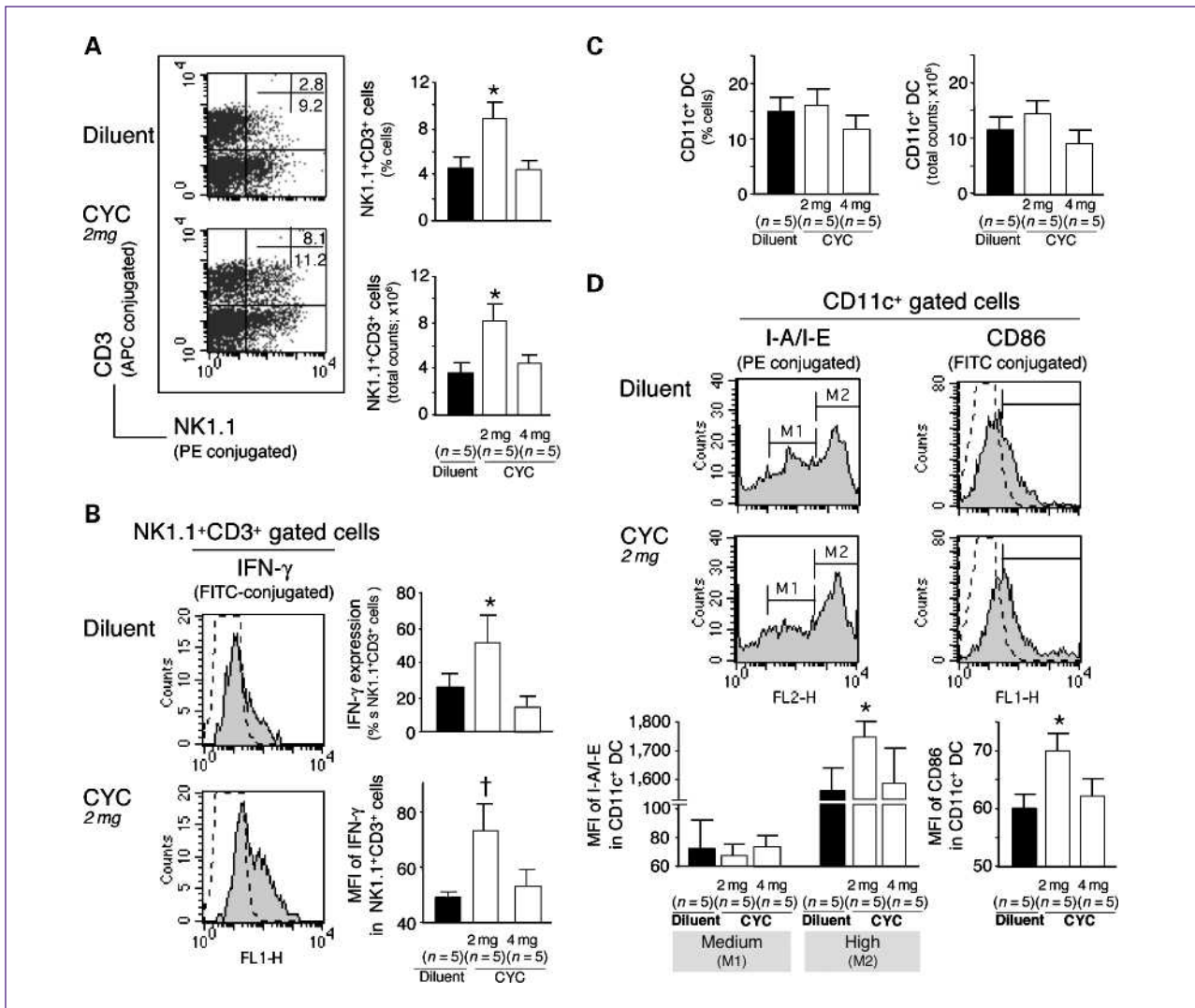


Fig. 4. IFN-γ-producing NKT cells are upregulated in association with DC activation in 5T2MM-inflicted mice following treatment with CYC. Forty-two days after a challenge of mice with 5T2MM cells, a single injection of CYC (at a dose of 2 mg/mouse or 4 mg/mouse), or the diluent was given (*n* = 5/group). Twenty-five days afterwards, spleen-derived cells were analyzed by flow cytometry. Columns, mean of individual mice within a group; bars, SD. A, representative dot plots for staining with NK1.1 and CD3. Columns, mean percentage and absolute counts of NK1.1⁺CD3⁺ cells; bars, SD. B, representative histograms of IFN-γ production in NK1.1⁺CD3⁺-gated cells. Columns, mean percentages of NK1.1⁺CD3⁺ cells that express IFN-γ and MFI of IFN-γ in NK1.1⁺CD3⁺-gated cells; bars, SD. C, columns, mean percentage and absolute counts of CD11c⁺ DC; bars, SD. D, representative histograms; columns, mean MFI for the expression of I-A/I-E and CD86 in CD11c⁺ DC; bars, SD (*, *P* < 0.05; †, *P* < 0.005).

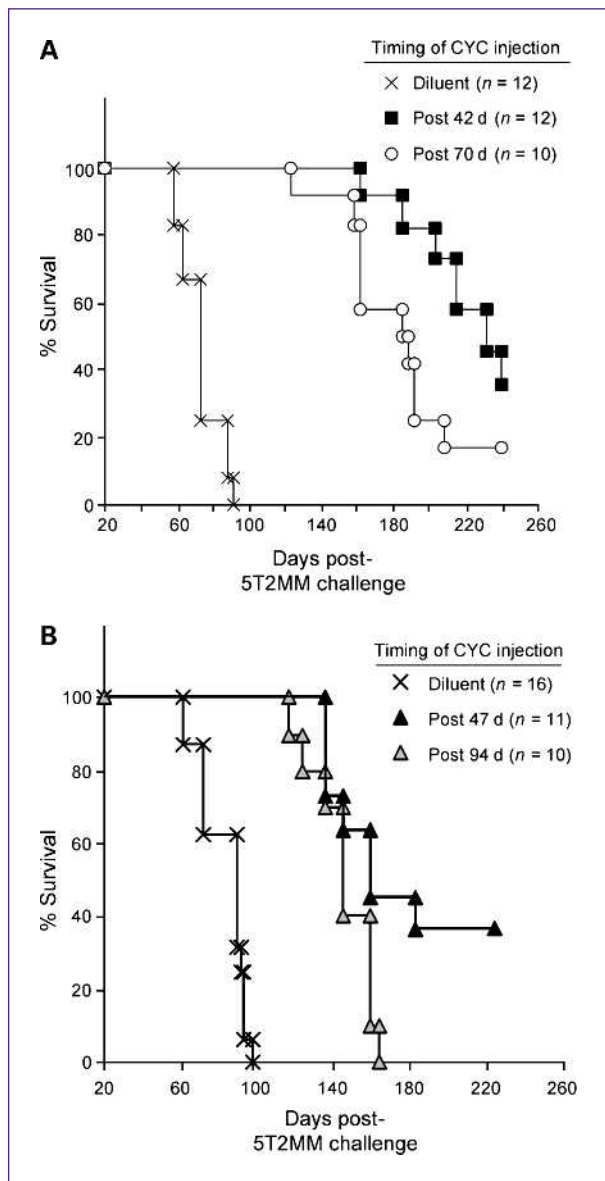


Fig. 5. Effects of a single injection of a low-dose CYC depending on tumor load. A, a single i.p. injection of a low-dose CYC (2 mg/mouse) was given to 5T2MM-inflicted mice at 42 ($n = 12$) and 70 d ($n = 10$) after tumor challenge. A third group of control mice ($n = 12$) received the diluent. All control mice died at a mean latency of 73 ± 9 d. The tumor incidence in mice treated with CYC after 42 d was reduced to 66% (8 of 12) with a mean latency of 207 ± 23 d. Administration of CYC at 70 d following MM cell challenge reduced MM incidence to 83% (10 of 12) and prolonged the survival (mean latency of 173 ± 23 d). Results were reproduced in two independent experiments. B, the effect of a low-dose CYC treatment in 5T2MM-inflicted mice 47 d (low tumor load) or 94 d (high tumor load) after tumor cell challenge. In mice bearing low tumor load and treated with low-dose CYC, 63% developed MM (7 of 11) at a mean latency of 156 ± 13 d. In mice bearing high tumor load and treated with low-dose CYC, 100% (10 of 10) developed MM, at a mean latency of 145 ± 15 d. In a control group of mice injected with the diluent, 100% (16 of 16) developed MM at a mean latency of 79 ± 13 d. Prolonged survival in comparison with the control group was observed in both CYC-treated groups ($P < 0.005$) but reduced MM incidence (63%) was observed only in mice carrying low tumor load.

characteristic markers of NKT cells (e.g., NK1.1⁺CD3⁺). As shown in Fig. 4A, the mean (\pm SD) percentages and the mean (\pm SD) absolute counts of NKT cells were significantly increased ($P = 0.0006$) in response to a single injection of a low-dose CYC as compared with the injection of the diluent. Furthermore, the NKT cells from CYC-treated mice were highly activated as the production of IFN- γ was significantly intensified ($P = 0.005$) in these cells (Fig. 4B).

The effect of a low-dose CYC on CD11c⁺ DC was also studied. Despite the comparable mean percentages and absolute counts of CD11c⁺ DC observed in both diluent- and CYC-treated mice (Fig. 4C), only CD11c⁺ DC from the 5T2MM mice that received CYC exhibited an activated phenotype. This effect was evident from the intensified expression of MHC class II and CD86 costimulatory molecules (Fig. 4D). The effects of a high-dose CYC on NKT cells and CD11c⁺ DC were not as prominent or significant as those of a low-dose CYC.

Clinical effect of a single injection of a low-dose CYC depends on tumor load

Three groups of 5T2MM mice were treated with a single injection of the diluent (group 1) or a low-dose CYC (2 mg/mouse) at 42 days (group 2) or 70 days (group 3) after tumor cell challenge. The levels of paraprotein associated with MM development were as follows: 0.14 to 0.28 g/dL in group 1, 0.66 to 0.98 g/dL in groups 2, and 1.10 to 1.52 g/dL in group 3. The progression of the disease was followed for 240 days after the initial tumor cell transfer. The results are summarized in Fig. 5A. In control group 1, 100% (12 of 12) of the mice developed MM, with a mean latency of 73 ± 9 days. In group 2, the single CYC injection 42 days after 5T2MM cell challenge prolonged survival (mean, 207 ± 23 d) and reduced tumor incidence (8 of 12, 66%). Giving a single CYC injection to paralyzed mice at day 70 following the initial 5T2MM cell challenge (group 3) prolonged the survival (mean latency, 173 ± 23 d) of the 5T2MM mice and reduced the incidence of disease to 83% (10 of 12). Hence, in mice with a reduced tumor load, CYC gave the best results, showing a decrease in tumor incidence (66%) and a significantly prolonged survival ($P < 0.001$ compared with group 3, and $P < 0.0001$ compared with the control group).

We also tested the effect of CYC treatment on very sick mice (i.e., 94 d as compared with 47 d following 5T2MM tumor cell injection). The serum paraprotein levels were 0.57 to 0.98 g/dL in the 47-day MM mice (group 1) and 2.55 to 2.99 g/dL in the 94-day sick mice (group 2). Treatment with CYC did not change the state of paralysis in the 94-day MM mice. Among the diluent-injected mice (group 3), 100% (16 of 16) died at a mean latency of 79 ± 13 days, as compared with 63% (7 of 11) at a mean latency of 156 ± 18 days in group 1, and 100% (10 of 10) at a mean age of 145 ± 15 days in group 2 (Fig. 5B). Thus, the high tumor load in the very sick mice prevented the ability of CYC to reduce tumor incidence

but prolonged survival in comparison with the control group ($P < 0.0001$).

Optimal time schedules of repeated administrations of low-dose CYC treatment for a minimal disease occurrence

Our studies indicated that low-dose CYC treatment could maintain Treg depletion beyond 42 days (Fig. 2D). Populations involved in antitumor immune responses could effectively be recruited while Treg renewal was blocked. Here, we analyzed the effect of repeated CYC injections at different time intervals. To this end, mice bearing 5T2MM cells were treated 60 or 70 days after tumor cell challenge (paraprotein levels then were 0.59-1.25 g/dL and 1.10-1.63 g/dL, respectively) with a low-dose CYC according to the following protocols: the first experiment involved a comparison of two injections of CYC at either a 21- or a 45-day interval (Fig. 6A). In the second experiment, the effect of a single injection versus four CYC injections at 7- or 45-day intervals was tested (Fig. 6B). Figure 6A presents the results showing the significance of prolonged intervals between CYC injections. In the diluent-treated control group, 100% (13 of 13) developed MM at a latency of 60 ± 12 days. Two repeated CYC injections at 21-day intervals resulted in 83% (10 of 12) of mice that developed the disease at a mean latency of 159 ± 28 days. Prolonging the interval to 45 days between two CYC injections resulted in a rather decreased incidence of the disease, e.g., 46% (7 of 15) at a mean latency for disease of 183 ± 15 days ($P < 0.005$ when comparing with group 2, and $P < 0.0001$ when comparing with the control). These results were obtained in three independent experiments. In the second set of experiments, results of different treatment protocols of low-dose CYC are presented (Fig. 6B). In the control group, 100% (15 of 15) developed MM at 82 ± 11 days mean latency. A single CYC injection yielded 53% (8 of 15) MM at a prolonged latency of 176 ± 22 days ($P < 0.0005$). Repetition of four CYC injections at 7-day intervals resulted in a high MM incidence (85%, 12 of 14) but with a prolonged survival, 190 ± 18 days mean latency ($P < 0.0007$ as compared with the control group). Four CYC treatments at 45-day intervals yielded a very low incidence of 16% (2 of 12) at 201 and 240 days. The results were obtained in three independent experiments.

Discussion

The main findings of the current study are that mice with MM were shown to benefit from repeated treatments of a low-dose CYC at longer time intervals. This approach reduced the occurrence of disease and improved the survival rates. The mechanisms underlying the treatment protocol seem to involve effects associated with transient depletion of Tregs in the diseased mice; CYC renders these cells susceptible to apoptosis. Moreover, the therapy induces a recovery of IFN- γ -producing NKT cells as well as the maturation of DCs. Both of

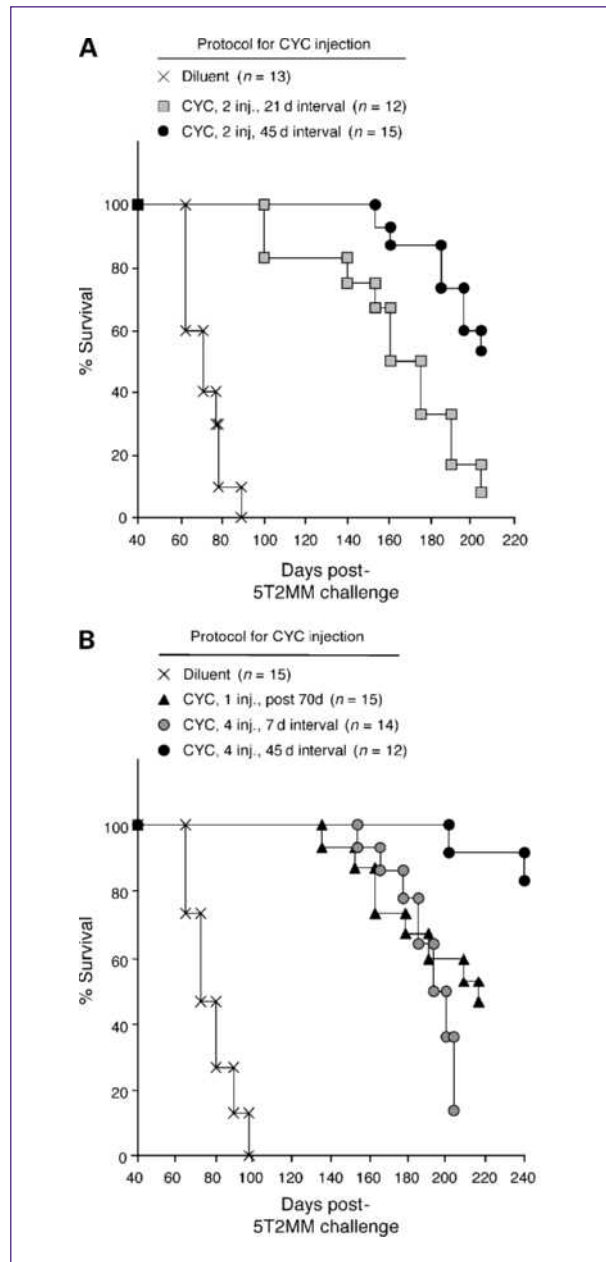


Fig. 6. Effect of repeated injections of a low-dose CYC. A, 5T2MM mice were treated 60 d after tumor cell challenge with CYC (2 mg/mouse) as follows: group 1 ($n = 13$), received the diluent, and all died at a mean latency of 60 ± 12 d; group 2 ($n = 12$), a 21-d interval between two injections of CYC; group 3 ($n = 15$), a 45-d interval between two injections of CYC. The incidence of MM in the latter two groups was 83% (10 of 12) and 46% (7 of 15), and the mean latency period was 159 ± 28 and 183 ± 15 d, respectively. Representative results of three independent experiments are shown. B, 5T2MM mice were treated 70 d after tumor cell challenge with CYC (2 mg/mouse) as follows: group 1 ($n = 15$) injected with diluent, 100% developed MM at a mean latency of 82 ± 11 d; group 2 ($n = 15$) receiving a single CYC injection, 53% (8 of 15) developed MM at a mean latency of 176 ± 22 d; group 3 ($n = 14$) received four CYC injections at 7-d intervals, 85% (12 of 14) developed MM at 190 ± 18 d mean latency; and group 4 ($n = 12$) received four CYC injections at 45-d intervals (16%, 2 of 12) developed MM at 201 and 240 d. Representative results of three independent experiments.

these effects are essential for an efficient immune response against the tumor.

Both low- and high-dose CYC given only once to 5T2MM mice in the early clinical phase resulted in the disappearance of hind limb paralysis and in a significant prolonged survival (Fig. 1). Shortly after CYC treatment, serum paraprotein levels were reduced to reference ranges and transformed plasma cells in the hind limbs were replaced by normal BM populations for as long as 80 days (of follow-up) post-CYC injection (Fig. 1B and C). Prolonged survival following a single high- or low-dose CYC treatment might be related to the eradication of plasma tumor cells from the BM. Homing of MM cells in the BM is important for their interaction with stromal cells inducing a microenvironment for their survival and growth signals (29). MM cells could still be detected in sick mice following treatment with CYC but only in the spleen (a hematopoietic organ in mice), lymph nodes, and sometimes also in the liver. Previously, it had been shown that tumor cell death in response to anthracyclines could elicit an antitumor effect, and that DCs played a critical role in this process (30). Recently, it has been shown that CYC treatment increased the potency of DCs in antigen presentation, cytokine secretion, and partial inhibition of the suppressor activity of Tregs (31). Perhaps the rapid cytotoxic-necrotic process of CYC-induced cell death might have an immunogenic effect contributing to the delay in tumor growth and longer survival of the mice.

The main difference between the low- and high-dose CYC treatments in 5T2MM mice was on the ultimate development of disease. A high percentage of diseased mice (85%) was observed in 5T2MM mice treated with a high dose of CYC versus a low percentage (38%) in mice receiving a low dose of CYC (Fig. 1A). The cytotoxic effect of high-dose CYC is less selective concerning cell populations with antitumor properties, and thus could consequently lead to the recovery of residual 5T2MM cells and to yield a high MM incidence. In contrast, treatment with the low-dose CYC was associated with selective transient depletion of Tregs in diseased mice leading to the restoration of peripheral T-cell proliferation and immune functions resulting in lower MM incidence.

The inhibitory effects of CYC on Tregs have been shown in previous studies (18–20); however, the mechanisms underlying these effects remain largely unknown (17). Our current study provides mechanistic pathways to explain the susceptibility of Tregs to CYC. A low-dose CYC resulted in downregulated expression of the survival molecule, Bcl-xL, in CD4⁺CD25^{high} cells, which consequently led to increased apoptosis rates of the cells (Fig. 3A). In agreement, Roux and colleagues have shown that expression of Foxp3 could confer susceptibility of Tregs to apoptosis after treatment with low-dose CYC (32). Elevated levels of Bcl-xL have been shown in patients and mice with MM (33, 34), which influenced both the development of the disease and the response to treatment. Furthermore, Bcl-xL has been implicated in the development of Tregs

(35). In addition, the expression of CTLA-4 in CD4⁺CD25^{high} cells was significantly reduced in 5T2MM mice that were treated with a low-dose CYC (Fig. 3A). This effect may also contribute to the decreased expression of Bcl-xL in Tregs (36), and also interrupt the *in vivo* development and suppressive function of the Tregs (37).

Notably, a low-dose CYC affected the production of IL-2 as well (Fig. 3B); it was significantly decreased in CD4 effector cells in treated mice. This effect could also lead to reduced numbers of CD4⁺CD25^{high} cells because these cells do not produce IL-2. Moreover, these cells highly consume IL-2 for homeostasis maintenance (38). These inhibitory effects of a low-dose CYC on Tregs are essential for accomplishing antimyeloma activities because the latter were abolished by restoring Treg cells in mice (Fig. 3C). In accordance, Ercolini et al., in studying mammary tumor development, also showed antitumor immune response following the depletion of cycling populations of Tregs by a low-dose CYC; restoration of Tregs abolished the antitumor effect (20). Also, Ghiringhelli et al. (21) used the metronomic CYC regimen for a month on patients with advanced cancer, and reported a decrease in the number of functional Tregs, which led to a restoration of peripheral proliferation and innate killing activities, but only when a low-dose CYC was used.

The CYC-induced depletion of CD4⁺CD25^{high}Foxp3⁺ cells is unlikely to account for all the ameliorative effects of the drug regarding disease manifestations. Importantly, NKT cells are capable of controlling tumor growth *in vivo* (39); they are affected reciprocally by Tregs (27, 28). Indeed, we found that 5T2MM mice treated with a low-dose CYC, in contrast with a high-dose CYC, significantly upregulated the numbers of NKT cells (Fig. 4A). Furthermore, treatment with a low-dose CYC resulted in a significant upregulation in the production of IFN- γ following the increased number of NKT cells (Fig. 4B). The latter abilities are known to be disrupted in patients with progressive MM (40), and antitumor properties of NKT cells are linked to their ability to produce IFN- γ (41). Therefore, the IFN- γ -producing NKT cells should be considered as a major beneficial constituent of a low-dose CYC regimen in MM disease.

The differentiation and function of DCs in patients with MM is abnormal and impaired (42). We found that treatment with a low-dose CYC did not affect the number of DCs; however, it promoted the development of a mature phenotype of DCs, as manifested by an intensified expression of MHC class II and CD86 costimulatory molecules (Fig. 4C and D). Therefore, their function as antigen-presenting cells is increased. The loss of MHC class II molecules might allow cancer cells to escape host immunity (43); strategies using DCs for MHC class II presentation of myeloma-related peptides derived from secreted immunoglobulins were shown to induce antimyeloma effects (44). Therefore, the ability of a low-dose CYC to promote terminal maturation of DCs in MM is a valuable response. IFN- γ enhances the expression of MHC class II molecules (45), and NKT cells could support DC maturation (46).

Thus, the insufficient induction of NKT cells with their production of IFN- γ could partially explain the nonsatisfactory effect on DC maturation in response to a high dose of CYC as opposed to a low dose of CYC treatment. Recently, Roux and colleagues have shown that low-dose CYC in combination with Bacillus Calmette-Guérin resulted in functional DCs that could eradicate tumor cells via the induction of tumor necrosis factor-related apoptosis-inducing ligand (32). Thus, it is possible that low-dose CYC not only promotes the terminal maturation of DCs but also enables the cytotoxic activity of these cells against tumor cells.

The efficiency of a single low-dose CYC to reduce MM progression was shown to be dependent on tumor load, as reflected in the serum paraprotein level at the time of drug administration (Fig. 5). Approximately 40% to 60% of MM incidence following CYC treatment was observed in 5T2MM mice with a lower tumor load versus 80% incidence in mice with increased tumor load. MM incidence was not affected by CYC in mice with very high serum paraprotein levels. Nevertheless, a substantial prolonged survival was observed in 5T2MM mice treated with CYC in comparison with those injected with the diluent irrespective of the tumor load level.

Optimal reduction of MM development is dependent on low CYC doses and timing of repeated CYC administration. CYC has a direct immune augmenting effect, enhancing T-cell functions associated with Treg depletion. In 5T2MM sick mice, transient Treg depletion following low-dose administration was maintained beyond 45 days thus involving the "window of opportunity" during the recovery phase. More frequent injections of low-dose CYC at 7- or 21-day intervals did not improve the therapeutic effect because it resulted in a high incidence of MM (85%) among the treated mice (Fig. 6B). In contrast, mice treated at 45-day intervals developed a significantly lower MM incidence (16-46%) thus tipping the balance toward effector T cells for a more prolonged period of time.

In our previous study (13), we described increased rates of Tregs in peripheral lymphoid tissues of MM-inflicted mice 28 and 42 days post-tumor cell challenge. Moreover, further increases in Tregs were noted with tumor progression and with the development of paralysis at 65, 90, and

104 days (in spleen, lymph nodes, thymus, BM, and peripheral blood). Here, we found that giving both low and high doses of CYC significantly reduced the numbers of CD4⁺CD25^{high}Foxp3⁺ cells (Fig. 2C). Importantly, the inhibitory effect of low-dose CYC on Tregs was maintained even 45 days afterwards (Fig. 2C). The latter effect is of a major importance because the number of Tregs is increased in patients and in mice with MM (2–5, 13), and depletion of Tregs has been shown to promote antitumor T-cell responses (6, 47, 48).

Collectively, our study suggests that the use of high doses of CYC against myeloma cells might be less effective in immunocompetent individuals because the cytotoxic effects of the drug are nonselective and might reduce immune cells with potential antitumor properties. In contrast, giving lower doses of CYC at longer intervals results in a significant decrease in Tregs without the major cytotoxic effects of a high-dose CYC. Therefore, this treatment presents a window of opportunity for the appearance of cell subsets such as NKT cells and mature DCs that will help induce tumor regression. Hence, the inclusion of repeated injections of low-dose CYC at longer time intervals as a protocol for treating MM patients could be an efficient strategy for breaking immune tolerance towards the MM tumor cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We are grateful to Prof. Jay A. Levy of the University of California, San Francisco, CA, for his support, valuable discussions, helpful suggestions, and critical reading of this manuscript. We are thankful to Prof. Edna Mozes of the Weizmann Institute of Science, Rehovot, Israel for critical review of this manuscript. Many thanks for the most helpful assistance of Dr. Miriam Parizade of Central Maccabi Health Service in Rehovot for analyzing the sera of 5T2MM mice.

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Received 06/22/2010; revised 08/04/2010; accepted 08/11/2010; published OnlineFirst 08/18/2010.

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