[CANCER RESEARCH 58, 5301–5304, December 1, 1998]

*Advances in Brief*

# Realization of the Therapeutic Potential of CTLA-4 Blockade in Low-Dose Chemotherapy-treated Tumor-bearing Mice[1]

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## Abstract

**CTLA-4 blockade has been shown by other investigators [D. R. Leach, *et al.*, Science (Washington DC), *271:* 1734–1736, 1996; and Y-F. Yang, *et al.*, Cancer Res., *57:* 4036–4041, 1997] to retard tumor growth in selected tumor systems. Here, we show that CTLA-4 blockade alone was ineffective in retarding tumor growth in the murine MOPC-315 tumor system. Yet, CTLA-4 blockade offered significant therapeutic benefits to MOPC-315 tumor bearers when combined with a subtherapeutic dose of the chemotherapeutic agent melphalan, which was previously shown (L. Gorelik, *et al.*, Cancer Immunol. Immunother., *39:* 117–126, 1994) to shift the cytokine profile in the tumor bearers toward type-1 cytokines. In addition, we show here that anti-CTLA-4 monoclonal antibody enhanced antitumor cytotoxicity when the anti-CTLA-4 monoclonal antibody was added to stimulation cultures of spleen cells from low-dose melphalan-treated MOPC-315 tumor-bearing mice but not from untreated tumor-bearing mice. These results suggest that the therapeutic benefits of CTLA-4 blockade depend on the ability of drugs such as melphalan to promote an immunogenic environment by altering the cytokine profile of tumor-specific T cells.**

## Introduction

It has been recognized for some time that progression of many murine and human tumors is due to the inability of the tumors to elicit effective tumor-eradicating immunity. This in turn was attributed to multiple factors that included low immunogenicity of the tumor cells as a result of the absence or low-level expression of tumor Ag/MHC-complex (1) or the lack of expression of the costimulatory molecules B7–1 and/or B7–2 (2). In fact, many attempts to enhance the ability of tumor cells to elicit the generation of tumor-eradicating immunity focused on increasing the ability of the tumor cells to provide both signal 1 and signal 2 for T-cell activation (*e.g.*, by introducing genes for MHC class I molecules (3) or for the costimulatory molecule B7–1 or B7–2 (2, 4)). However, these manipulations provided at best some therapeutic benefits against a relatively small (barely palpable) tumor burden (4). The failure of such manipulations to provide therapeutic benefits to mice with larger tumors may have been due at least in part to the production of tumor-associated cytokines with inhibitory activity for the generation of cell-mediated antitumor immunity (5–7). In fact, recent attempts to enhance the ability of tumor bearers to mount tumor-eradicating immunity have focused on the shift in the cytokine profile at the tumor site toward cytokines with stimulatory activity for the generation of cell-mediated immunity through the use of chemotherapeutic agents such as melphalan (5, 8), cyclophosphamide (7), or bleomycin (9).

Another factor described recently (10–14) that may limit the effectiveness of tumor-eradicating immunity is CTLA-4/B7 interaction because CTLA-4, which is expressed on activated T cells (10), functions as a negative regulator of T-cell responses. In fact, CTLA-4 blockade (through the use of anti-CTLA-4 mAb[3]) was reported by Leach *et al.* (13) to lead to the inhibition of tumor growth as well as the complete regression of a few palpable tumors in the V51BLim10 colon carcinoma model. These observations by Leach *et al.* were recently extended by Yang *et al.* (14) to two other experimental tumor models, the CSA1M fibrosarcoma and the OV-HM ovarian carcinoma.

In contrast to the therapeutic benefits provided by CTLA-4 blockade in the V51BLim10, the CSA1M, and the OV-HM tumor systems, CTLA-4 blockade alone does not always provide therapeutic benefits as reported in mid August 1998 by Hurwitz *et al.* (15) in the SM1 mammary carcinoma system. Similarly, we show herein that CTLA-4 blockade alone also does not provide therapeutic benefits in the MOPC-315 tumor system. However, the therapeutic potential of CTLA-4 blockade can be realized in the MOPC-315 tumor system after administration of low-dose melphalan under conditions in which the chemotherapy promotes the *in vivo* acquisition of $CD8^+$ T-cell-mediated tumor-eradicating immunity (16). Consistent with the possibility that the therapeutic benefits of CTLA-4 blockade for low-dose melphalan-treated tumor-bearing mice are due at least in part to the ability of anti-CTLA-4 mAb to lead to enhanced antitumor immunity, the addition of anti-CTLA-4 mAb to stimulation cultures of spleen cells from low-dose melphalan-treated MOPC-315 tumor-bearing mice, but not to spleen cells from untreated tumor-bearing mice, was found to result in enhanced anti-MOPC-315 cytotoxicity.

## Materials and Methods

**Tumors.** The MOPC-315 plasmacytoma was maintained *in vivo* in female BALB/c AnNCrlBR mice 7–10 weeks old (Charles Rivers Breeding Laboratories, Wilmington, MA). Unless otherwise stated, mice were inoculated s.c. with $1 \times 10^6$ viable tumor cells, a dose that is at least 300-fold higher than the minimal lethal tumor dose.

**Chemotherapy.** A fresh stock solution of melphalan (Sigma Chemical Co., St. Louis, MO) was prepared as described previously (17). A dose of 1.5–2.0 mg melphalan/kg body weight (low-dose) was given i.p. to BALB/c mice bearing large (~20 mm) tumors that resulted from the s.c. inoculations of $1 \times 10^6$ MOPC-315 tumor cells 10 days earlier.

**Antibody Treatments.** For CTLA-4 blockade, mice received a daily i.p. injection of 100 μg (per mouse) of the affinity-purified, hamster IgG anti-CTLA-4 mAb (UC10–4F10–11; Ref. 10) for up to 10 injections or until the tumor nodules reached 25 mm in diameter. This mAb was produced in our laboratory and purified by Genetic Institute (10). As a control, mice received affinity-purified NIgG (Sigma Chemical Co.).

**Spleen Cell Suspensions.** Spleens used for the preparation of single-cell suspensions were derived from two sources: (*a*) mice bearing a large (20–22-

Received 9/2/98; accepted 10/19/98.

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[1] Supported by Research Grant CA-76532 from the National Cancer Institute.

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[3] The abbreviations used are: mAb, monoclonal antibody; LU, lytic units per $1 \times 10^7$ effector cells wherein 1 LU is defined as the number of effector cells producing 20% lysis of $1 \times 10^4$ target cells; NIgG, normal IgG.

mm) s.c. tumor that resulted from the inoculation of $1 \times 10^6$ MOPC-315 tumor cells 10–12 days earlier; and (*b*) mice that were treated with low-dose melphalan 4 days earlier when the mice bore an ~20-mm s.c. tumor that resulted from the inoculation of $1 \times 10^6$ MOPC-315 tumor cells 10–12 days earlier.

***In Vitro* Generation of Anti-MOPC-315 Cytotoxicity.** Spleen cells were stimulated *in vitro* with mitomycin C-treated MOPC-315 tumor cells according to the method we have described previously (5, 17) for the *in vitro* generation of CTL activity by $CD8^+$ spleen cells from untreated or low-dose melphalan-treated MOPC-315 tumor bearers. Briefly, spleen cells were cultured at 37°C for 5 days with mitomycin C-treated MOPC-315 tumor cells in DMEM supplemented with 5% fetal bovine serum, $5 \times 10^{-5}$ M 2-mercaptoethanol (Sigma Chemicals), 1% nonessential amino acids, 50 units/ml penicillin, 50 μg/ml streptomycin, and 15 mM HEPES buffer (Life Technologies, Gaithersburg, MD).

**Antitumor Cytotoxicity Assay.** The level of antitumor cytotoxicity exhibited by *in vitro* stimulated spleen cells was determined by the $^{51}Cr$ release assay. Briefly, $1 \times 10^4$ $^{51}Cr$-labeled MOPC-315 tumor cells were incubated with effector cells at three different *E*:*T* ratios. The percentage of specific $^{51}Cr$ release was calculated by the following formula:

$$E^{cpm} - S^{cpm}/M^{cpm} - S^{cpm} \times 100$$

where $E^{cpm}$ represents the $^{51}Cr$ released by target cells incubated with effector cells, $S^{cpm}$ represents the spontaneous release and $M^{cpm}$ represents the maximal release obtained by the addition of 2% NP40 detergent (Particle Data Corp., Elmhurst, IL) solution. Some variations were noted in the levels of antitumor cytotoxicity between different experiments, however, the pattern of results remained consistent. The level of antitumor cytotoxicity of a representative experiment is presented as the mean percentage of $^{51}Cr$ release of triplicate samples ± SE. In addition, to illustrate the reproducibility of our observations, the data from all of the experiments addressing the same question were converted to LU/1 $\times 10^7$ effector cells and are presented as LU ± SE.

**Statistical Analysis.** The significance of differences in the fraction of mice surviving after different treatments was determined by the generalized Savage (Mantel-Cox) test. For all of the other statistical analyses, Student's *t* test was used. A *P* value of ≤ 0.05 was considered significant in both tests.

## Results and Discussion

In light of reports that anti-CTLA-4 mAb treatment of mice bearing a V51BLim10 (13), CSA1M (14), or OV-HM (14) tumor leads to a substantial reduction in tumor growth and even regression of a few of the tumors, experiments were carried out to determine whether anti-CTLA-4 mAb treatment would offer some therapeutic benefits also to mice bearing the MOPC-315 plasmacytoma. As seen in Fig. 1, anti-CTLA-4 mAb treatment initiated on day 7 after MOPC-315 tumor inoculation, when the mice bore an ~10 mm s.c. tumor, did not retard MOPC-315 tumor growth (Fig. 1*A*). Because the tumor burden at the time of initiation of the anti-CTLA-4 mAb treatment was larger in the MOPC-315 tumor system than in the V51BLim10, CSA1M, or OV-HM tumor systems (13, 14) and because, in the V51BLim10 tumor system, anti-CTLA-4 mAb treatment initiated at the time of tumor inoculation was also effective in inhibiting tumor growth and even preventing tumor establishment (13), we examined whether anti-CTLA-4 mAb treatment initiated at the time of MOPC-315 tumor inoculation could offer any "therapeutic" benefits. In this study, mice were inoculated s.c. with either $1 \times 10^6$ or $2 \times 10^5$ MOPC-315 tumor cells. As seen in Fig. 1, anti-CTLA-4 treatment did not inhibit significantly tumor growth when administered to mice inoculated with either $1 \times 10^6$ (Fig 1*B*) or $2 \times 10^5$ (Fig.1*C*) MOPC-315 tumor cells. Thus, in contrast to the therapeutic benefits offered by CTLA-4 blockade in the V51BLim10, CSA1M, and OV-HM tumor systems, CTLA-4 blockade alone did not offer any therapeutic benefits in the MOPC-315 tumor system.

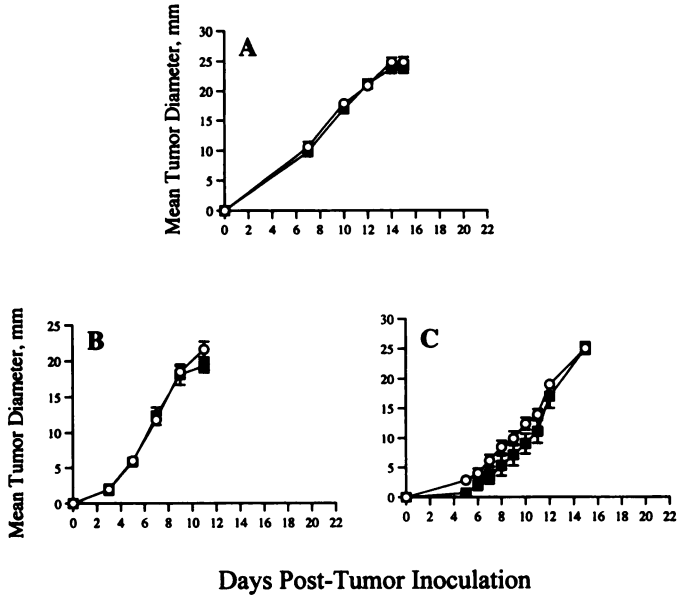


Fig. 1. The effect of anti-CTLA-4 mAb treatment on tumor progression in mice inoculated s.c. with MOPC-315 tumor cells. Mice inoculated with either $1 \times 10^6$ (*A* and *B*) or $2 \times 10^5$ (*C*) were treated daily with 100 μg of anti-CTLA-4 mAb (■) or NIgG (○) beginning on day 7 after tumor inoculation (*A*) or at the time of tumor inoculation (*B* and *C*). The mean tumor diameter of the anti-CTLA-4 mAb-treated mice ($n$ = 5–7 mice/group) was not significantly different from the mean tumor diameter of mice treated with NIgG (4–7 mice/group).

Next, we considered the possibility that the failure of anti-CTLA-4 mAb to provide any therapeutic benefits to MOPC-315 tumor bearers was due to the fact that tumor progression in the MOPC-315 tumor system (5), as in many other murine and human tumors (6, 7, 9), activates not only T cells that produce type-1 cytokines and promote the development of cell-mediated immunity but also T cells that produce type-2 cytokines, and have the potential to inhibit the generation of cell-mediated immunity. Consequently, CTLA-4 blockade would be expected to promote the activity of both kinds of activated T cells as we (18, 19) and others (20) have shown—*in vivo* in nontumor systems—that CTLA-4 blockade can sustain not only the production of type-1 cytokines but also the production of type-2 cytokines. Consistent with such a scenario, anti-CTLA-4 mAb treatment was found in the studies by Yang *et al.* (14) to offer therapeutic benefits to mice at early stages of CSA1M or OV-HM tumor growth, and spleen cells from mice bearing small tumors were found to produce elevated levels of interleukin 2 and IFN-γ *in vitro* upon culture with anti-CTLA-4 mAb. At the same time, anti-CTLA-4 mAb treatment did not offer any therapeutic benefits to mice that bore tumors larger than 5 mm in diameter, and spleen cells from mice bearing larger tumors did not produce elevated levels of interleukin 2 and IFN-γ *in vitro* upon culture with anti-CTLA-4 mAb.

As a first step toward the testing of our hypothesis, we carried out studies to determine whether the therapeutic potential of CTLA-4 blockade can be realized in the MOPC-315 tumor system when the balance is shifted through external manipulations toward T cells that are involved in the generation/exertion of tumor-eradicating immunity. For this purpose, we took advantage of our previous observations, which demonstrated that the administration of low-dose melphalan to mice bearing large (~20 mm in diameter) MOPC-315 tumors shifts the cytokine profile in favor of type-1 cytokines (5, 8) and leads to the development of potent $CD8^+$ T-cell-dependent antitumor immunity that in turn eradicates a large tumor mass (16, 17). In the current studies, we used a suboptimal dose of melphalan (1.5

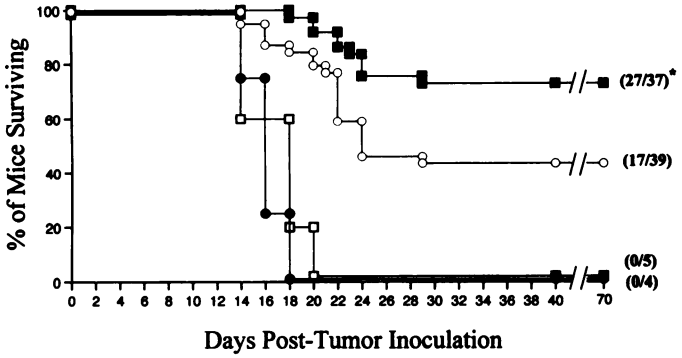


Fig. 2. The effect of anti-CTLA-4 mAb on the curative effectiveness of low-dose melphalan for mice bearing a large s.c. MOPC-315 tumor. Mice bearing a large (~20 mm in diameter) day-10 s.c. tumor were treated with 1.5 mg/kg melphalan plus 100 μg of anti-CTLA-4 (■) or NIgG (○) daily beginning 2 h after the chemotherapy. As a reference point, we provide information regarding the survival time of mice treated with NIgG (●) or anti-CTLA-4 mAb (□) but without chemotherapy. All of the mice that were alive on day 70 after low-dose chemotherapy were tumor-free. *Numbers in parentheses*, number of mice surviving out of the total mice studied; *, statistically significant extension in survival time relative to that of mice treated with melphalan plus NIgG.

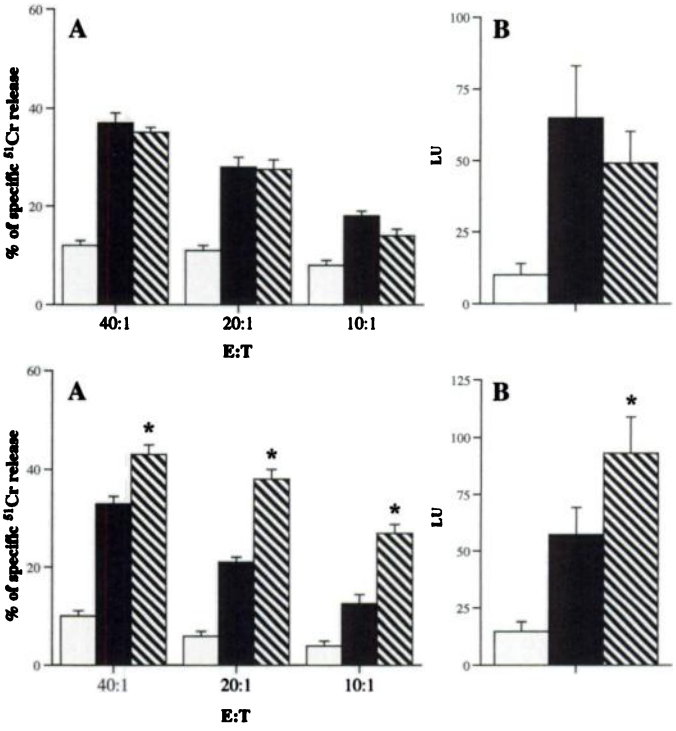


Fig. 3. The effect of CTLA-4 blockade on the level of anti-MOPC-315 cytotoxicity exhibited by *in vitro* stimulated spleen cells from untreated or low-dose-melphalan-treated MOPC-315 tumor bearers. Spleen cells derived from untreated mice bearing a large (~20 mm) s.c. tumor (*top panels*) or mice that were treated with low-dose (2.0 mg/kg) melphalan when they bore an ~20-mm s.c. MOPC-315 tumor (*bottom panels*) were stimulated *in vitro* with mitomycin C-treated MOPC-315 tumor cells in the presence of 50 μg/ml anti-CTLA-4 mAb (▨) or NIgG (■). Five days after the initiation of the stimulation cultures, the spleen cells were assessed for their anti-MOPC-315 cytotoxicity by the 3.5-h $^{51}$Cr release assay. The results of a representative experiment out of a total of 10 experiments are presented in the *A panels* as % of $^{51}$Cr release. As a reference point, we provide information regarding the level of anti-MOPC-315 cytotoxicity exhibited by spleen cells cultured *in vitro* in the absence of added stimulator tumor cells (□). The cumulative data from all of the experiments were converted to LU and are presented in the *B panels*. *, a significantly higher level of antitumor cytotoxicity than the level exhibited by spleen cells stimulated in the presence of NIgG. The level of antitumor cytotoxicity exhibited by tumor-bearer spleen cells that were stimulated *in vitro* in the presence of anti-CTLA-4 mAb was not significantly different ($P = 0.14$) from the level exhibited by tumor-bearer spleen cells stimulated in the presence of NIgG.

mg/kg), which is curative for only 25–50% of the MOPC-315 tumor bearers because of the appearance of insufficient CD8$^+$ T-cell-mediated antitumor immunity (8, 16), and determined whether anti-CTLA-4 treatment would offer any therapeutic benefits to these mice. As seen in Fig. 2, treatment of MOPC-315 tumor bearers with anti-CTLA-4 mAb improved significantly the curative effectiveness of the suboptimal dose of melphalan, with ~70% of the mice alive and tumor-free at the end of a 70-day observation period, as compared with only ~40% of the mice in the melphalan-plus-NIgG treatment group.

Experiments were next carried out to determine whether CTLA-4 blockade could lead to enhanced anti-MOPC-315 cytotoxicity when added to stimulation cultures of spleen cells from low-dose-melphalan-treated MOPC-315 tumor bearers but not when added to stimulation cultures of spleen cells from untreated tumor-bearing mice. Specifically, spleen cells from untreated MOPC-315 tumor-bearing mice or from low-dose-melphalan-treated tumor-bearing mice were stimulated *in vitro* for 5 days with MOPC-315 tumor cells in the presence or absence of anti-CTLA-4 mAb, and subsequently the spleen cells were evaluated for their antitumor cytotoxicity by the $^{51}$Cr release assay. As seen in Fig. 3, the addition of anti-CTLA-4 mAb to stimulation cultures of spleen cells from untreated tumor-bearing mice did not lead to an enhanced (but actually led to a somewhat suppressed) anti-MOPC-315 cytotoxicity. In contrast, the addition of anti-CTLA-4 mAb to stimulation cultures of spleen cells from low-dose-melphalan-treated MOPC-315 tumor bearers led to enhanced anti-MOPC-315 cytotoxicity.

In summary, the results presented herein illustrate that in a tumor system in which CTLA-4 blockade alone is unable to offer any therapeutic benefits, the therapeutic benefits of CTLA-4 blockade can still be realized through the use of melphalan. In addition, our data suggest that melphalan allows for the realization of the therapeutic benefits of CTLA-4 blockade in the MOPC-315 tumor system, at least in part, by allowing for the realization of the ability of CTLA-4 blockade to lead to enhanced antitumor immunity. Thus, CTLA-4 signaling is one of multiple immunosuppressive activities in a tumor-bearing host, and future immunotherapies should be targeted not only at CTLA-4 blockade but also at the removal/neutralization of other factors with inhibitory/down-regulatory activity for effective antitumor immune responses.

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*Cancer Res* 1998;58:5301-5304.