Cancer Immunol Immunother (2002) 51: 200–208
DOI 10.1007/s00262-002-0271-9

ORIGINAL ARTICLE

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Anti-metastatic activity of hapten-modified autologous tumor cell vaccine in an animal tumor model

Received: 15 November 2001 / Accepted: 9 January 2002 / Published online: 9 April 2002

Abstract We used mice from which the primary 410.4 mammary carcinoma had been surgically excised to assess the anti-metastatic activity of low-dose cyclophosphamide (CY) followed by vaccination with dinitrophenyl (DNP)-modified, irradiated, autologous tumor cells (ATC) admixed with bacille Calmette–Guérin (BCG). Our studies revealed that CY treatment of mice followed by vaccination with DNP-modified. irradiated, ATC admixed with BCG improved the relapse-free survival compared to the survival of mice receiving either CY followed by vaccination with unmodified, irradiated, ATC admixed with BCG, or saline (control group). In addition, our studies demonstrated the importance of CY administration in eliciting the therapeutic effect of DNP-modified ATC vaccine against metastatic disease. The therapeutic effect of CY followed by DNP-modified ATC vaccine was abrogated by depletion of $CD4^+$ or $CD8^+$ T-cells, illustrating the importance of both T-cell subsets for the anti-metastatic effect of this therapeutic protocol. In addition, neutralizing anti-IFN-γ monoclonal antibody (mAb), or neutralizing anti-tumor necrosis factor (TNF) mAb reduced the relapse-free survival of mice treated with CY followed by DNP-modified ATC vaccine, indicating the importance of both cytokines for the realization of the anti-metastatic effect of this therapeutic protocol. Since the therapeutic protocol used in our studies was similar to that employed by Berd et al. as postsurgical adjuvant therapy in cancer patients and yielded a comparable anti-metastatic effect, the information obtained from the current studies with our clinically relevant experimental tumor model is expected to shed light on the mechanism(s) by which the anti-metastatic effect of this post-surgical adjuvant therapy is realized in cancer patients.

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Keywords Autologous tumor cell vaccine · DNP-modified tumor cell · T-cell-mediated anti-metastatic activity

Abbreviations *BCG* bacille Calmette–Guérin · *CY* cyclophosphamide · *DNP* dinitrophenyl · *mAb* monoclonal antibody · *TCR* T-cell receptor

Introduction

Although immunotherapy is actively being tested in animal tumor models against primary tumors and experimental metastases, very few animal studies have examined the effectiveness of immunotherapy against spontaneously occurring metastatic disease [9, 15, 17, 31, 38]. However, the latter models [9, 15, 17, 31, 38] are likely to be more relevant for the assessment of the potential of an immunotherapeutic protocol for the treatment of cancer patients, as the primary tumors are excised before the patients receive immunotherapy in an attempt to eradicate or slow down the progression of metastases. Given this concern, we employed the 410.4 murine mammary carcinoma model, which resembles human tumors in terms of metastatic characteristics [23, 30, 41], to assess the effectiveness of an immunotherapeutic protocol against metastatic disease after excision of the primary tumor [38].

The immunotherapeutic protocol employed in our studies was based on that developed by Berd et al. [3, 4, 5] as postsurgical adjuvant therapy for cancer patients [5, 6, 7]. Specifically, after excision of the primary 410.4 mammary tumor, the mice were given low-dose cyclophosphamide (CY) followed by vaccination with dinitrophenyl (DNP)-modified, irradiated, autologous tumor cells admixed with bacille Calmette–Guérin (BCG). Similar to the observations of Berd et al. on patients with stage III melanoma [5, 6, 7], or with stage III ovarian cancer [6, 7], in the present study we show that CY followed by DNP-modified autologous tumor cell (ATC) vaccine also provides therapeutic benefits

against established metastases in the murine 410.4 tumor model.

Initially, we determined whether CY administration prior to vaccination with DNP-modified, irradiated, ATC admixed with BCG was important in eliciting the anti-metastatic activity of this therapeutic protocol. One of the reasons that CY was incorporated in the original protocol was that numerous investigators had demonstrated that alkylating agents (e.g. CY, melphalan or BCNU) could potentiate the acquisition of anti-tumor immunity in tumor bearers [8, 28] and in patients with malignancies [1, 2]. Since the initiation of the current studies, some new and interesting information has become available regarding the mechanisms through which the alkylating agents may mediate their immunopotentiating effects. Specifically, it has been shown that alkylating agents can lead to the rapid expression of the B7-1 co-stimulatory molecule [37], as well as to the rapid production of type I interferon (IFN) [14, 35], which in turn can promote the activation of dendritic cells (DC) [22, 33] and the development of a pro-inflammatory response [14, 33].

As part of the current studies, we also initiated experiments to elucidate the mechanism by which CY followed by DNP-modified ATC vaccine mediates its anti-metastatic activity in the 410.4 tumor model. Specifically, we determined whether $CD4^+$ and/or $CD8^+$ T-cells were important in vivo for the realization of the anti-metastatic effect of this immunotherapeutic protocol. In addition, we investigated the importance of IFN-γ for the realization of the anti-metastatic effect of CY followed by DNP-modified ATC vaccine, in light of the demonstration by Lattime et al. [20] that CY followed by DNP-modified ATC vaccine led to the upregulated expression of IFN-γ mRNA in subcutaneous metastases of melanoma patients who developed inflammation as a result of the treatment. Finally, we examined the role of tumor necrosis factor (TNF) for the realization of the anti-metastatic effect of CY followed by DNP-modified ATC vaccine, in the light of our previous observations in another experimental tumor system subjected to a different therapeutic modality, that TNF is essential for the acquisition of $CD8^+$ T-cell-dependent tumor-eradicating immunity [11, 39]. The information obtained from the current studies with regard to the mechanism by which the anti-metastatic effect of CY followed by DNP-modified ATC vaccine is realized in our clinically relevant experimental tumor model is expected to shed light on the mechanism involved in the anti-metastatic effect of this immunotherapeutic protocol in cancer patients.

Materials and methods

Tumor cells

The highly metastatic 410.4 tumor cell line [23, 25, 30] that originated from a spontaneously arising murine mammary carcinoma [13, 24] was used throughout our studies. Tumor cells were maintained in vitro at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 mg/ml streptomycin.

In vivo tumor model

In vitro grown 410.4 tumor cells were detached with trypsin–EDTA (Life Technologies Inc., Grand Island, N.Y.), and 3×10^5 410.4 tumor cells were injected into the mammary fatpads of the left breast of female 7- to 10-week-old Balb/cAnNCrlBR mice (Charles Rivers Breeding Laboratories, Wilmington, Mass.). Approximately five weeks after tumor inoculation, when the primary tumors reached 6 to 8 mm in diameter, the tumors were excised and the mice were randomized into treatment cohorts. The animals were monitored once to twice a week for local tumor recurrence and for the appearance of palpable metastases in the other breast and in the regional lymph nodes. Similar to the observations of Kown et al. [17] in the TRAMP-C2 prostate tumor model, in the 410.4 tumor system, local tumor recurrence also accounted for approximately 10%, and metastatic cancer progression for the remaining 90%, of the treatment failure after excision of the primary tumor.

Vaccine preparation

On the day of vaccination, in vitro grown 410.4 tumor cells were detached with 0.02% EDTA solution (without trypsin) (Sigma Chemical, St Louis, Mo.) and forceful pipetting. The tumor cells were then subjected to irradiation (2,500 cGy from a cesium-137 source; J. L. Sepherd and Associates model 143-68 irradiator), and an aliquot of the irradiated 410.4 tumor cells was DNP-modified by exposure to dinitrofluorobenzene (DNFB; Sigma Chemical, St. Louis, Mo.), according to the protocol of Berd et al. [5]. Each vaccine consisted of $3–5\times10^6$ unmodified or DNP-modified, irradiated, tumor cells admixed with $0.5–4\times10^6$ colony-forming units (CFU) of BCG (Tice strain).

Study design

Four to six days after excision of the primary tumor, the mice were given an i.p. injection of 15 mg/kg CY (Mead Johnson/Bristol-Myers Squibb, Princeton, N.J.). Three days after the low-dose CY treatment, the mice received an s.c. injection either of unmodified or DNP-modified, irradiated, ATC vaccine close to the site of tumor excision. This protocol was repeated every 10 days for the duration of the experiment. In one set of experiments, one group of mice received DNP-modified, irradiated, ATC vaccine without CY pretreatment. In all experiments, the mice were monitored once to twice a week for tumor recurrence at the primary site as well as for the appearance of palpable metastases in the other breast and in the regional lymph nodes. Once metastases were evident, tumor progression was followed for the duration of the experiments or until the mice showed signs of distress and were sacrificed. The results are presented as percentage relapse-free survival among all mice subjected to the same treatment protocol.

In vivo depletion of $CD4^+$ or $CD8^+$ T-cells

The in vivo depletion of $CD4^+$ or $CD8^+$ T-cells was carried out as previously described [18, 39]. Briefly, mice were given an i.p. injection 1 mg/mouse of anti-CD4 (derived from hybridoma GK1.5) or anti-CD8 (derived from hybridoma 2.43) monoclonal antibody (mAb), starting one day before the first vaccination and repeated every five to seven days thereafter for the duration of the experiment. This anti-CD4 and anti-CD8 mAb treatment protocol was found by indirect immunofluorescence staining followed by flow-cytometric analysis to lead to $>95\%$ depletion of $CD4^+$ cells or $>90\%$ depletion of $CD8^+$ cells respectively, with no decrease but actually with some increase in the percentage of the other T-cell subset.

IFN-γ secretion

On day 3 following the second injection of unmodified or DNP-modified ATC vaccine to mice in which the primary tumor had been excised, the draining lymph nodes were removed from three animals per group and single cell suspensions were prepared. The lymph node cells (5×10^5) were cultured for 48–72 h in a 96-well tissue culture plate, after which the level of IFN-γ secreted into the culture supernatant was determined by enzyme-linked immunosorbent assay (ELISA). For this purpose, we used the R4-6A2 rat anti-mouse IFN-γ mAb as capture Ab and the XMG1.2 rat anti-mouse IFN-γ mAb (BD Pharmingen, San Diego, Calif.) as detection Ab [26]. The sensitivity of this ELISA was 30 pg.

In vivo neutralization of IFN-γ and TNF

The in vivo neutralization of IFN-γ or TNF was carried out as previously described [10, 11]. Briefly, mice were given an i.p. injection 1 mg/mouse of anti-IFN-γ mAb (derived from hybridoma R4-6A2) [10] or anti-TNF mAb (derived from hybridoma 2E2) [11], which neutralizes both TNF-α and TNF-β [19], starting one day before the first vaccination. The mAb treatment was repeated every five to seven days thereafter for the duration of the experiment.

Statistical analysis

The relapse-free survival of mice subjected to different treatment protocols was compared by the use of the *Z*-score, which compares two independent samples [16, 27]. For all other statistical analyses, Student's *t*-test was used. A P value of ≤ 0.05 was considered significant in both tests.

Results

Assessment of the anti-metastatic effect elicited by DNP-modified ATC vaccine with or without CY pretreatment in the murine 410.4 tumor model

Experiments were carried out to determine whether the therapeutic protocol developed by Berd et al. [5, 6, 7] as postsurgical adjuvant therapy for cancer patients also provided therapeutic benefits against established metastases in an experimental tumor model. Specifically, we assessed the therapeutic effect of CY followed by vaccination with DNP-modified, irradiated, ATC admixed with BCG against metastatic disease in mice in which the primary 410.4 tumor had been excised when it reached 6 to 8 mm in diameter. The relapse-free survival among mice subjected to this therapeutic protocol was compared to that of primary tumor-excised mice treated with saline alone or with CY followed by vaccination with unmodified (instead of DNP-modified), irradiated, ATC admixed with BCG. A total of six experiments were carried out, yielding similar results. The cumulative results of the six experiments with 59 or 60 mice per group are provided. As seen in Fig. 1, CY followed by DNP-modified, irradiated, ATC vaccine provided anti-metastatic benefits to 410.4-tumor bearers relative to the saline treatment group. Moreover, this therapeutic protocol was also superior in its anti-metastatic effect to CY followed by unmodified, irradiated, ATC vaccine. Thus, DNP modification is important for the anti-metastatic effect elicited by CY followed by vaccination with irradiated ATC admixed with BCG.

Experiments were next undertaken to determine whether CY administration prior to vaccination with DNP-modified, irradiated, ATC admixed with BCG was important in eliciting the anti-metastatic effect of this therapeutic protocol. As seen in Fig. 2, administration of DNP-modified ATC vaccine led to a worse relapse-free survival in non-CY-pretreated mice than in CY-pretreated mice. Thus, CY administration is important in eliciting the therapeutic benefits of DNP-modified ATC vaccine against metastatic disease. Therefore, CY pretreatment was included in all subsequent experiments.

Assessment of the importance of $CD4^+$ and/or $CD8^+$ T-cells for the realization of the anti-metastatic effect CY followed by DNP-modified ATC vaccine

Experiments were carried out to determine whether $CD4^+$ and/or $CD8^+$ T-cells were important for the anti-metastatic effect elicited by CY followed by vaccination with DNP-modified, irradiated, ATC admixed with BCG. This was done by determining the effect of in vivo depletion of $CD4^+$ (Fig. 3) or $CD8^+$ (Fig. 4) T-cells, with the aid of the corresponding mAb, on the therapeutic benefits provided by CY followed by

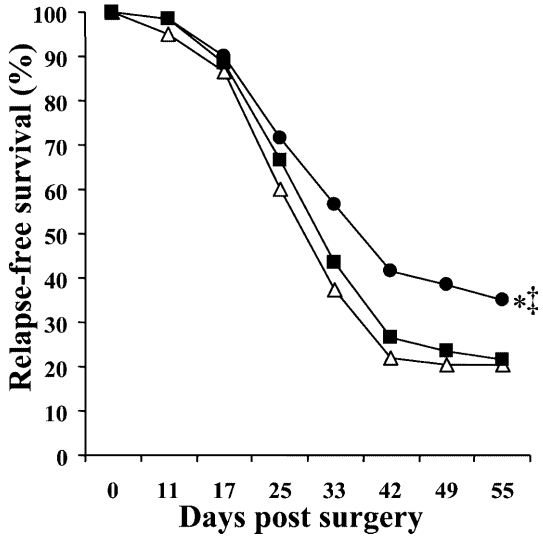

Fig. 1. Therapeutic benefits of CY followed by DNP-modified autologous tumor cell vaccine against metastatic disease in the murine 410.4 mammary tumor model. Relapse-free survival of 410.4 primary tumor-excised mice subjected to CY followed by vaccination with DNP-modified, irradiated, ATC admixed with BCG (60 mice; ●), or CY followed by unmodified, irradiated, ATC admixed with BCG (60 mice; ■). As reference, we provide the relapse-free survival of 410.4 primary tumor-excised mice treated with saline alone (59 mice; Δ). *Significantly better relapse-free survival than the saline treatment group, ‡significantly better relapse-free survival than mice treated with CY followed by unmodified ATC vaccine

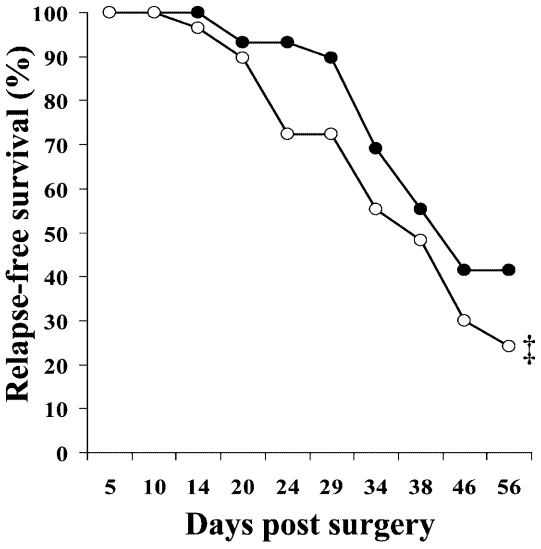

Fig. 2. Importance of CY for the realization of the anti-metastatic effect of the DNP-modified ATC vaccine. Relapse-free survival of 410.4 primary tumor-excised mice subjected to vaccination with DNP-modified, irradiated, ATC admixed with BCG with (29 mice; ●) or without (29 mice; ○) CY pretreatment. ‡ Indicates significantly worse relapse-free survival than the CY pretreatment group

DNP-modified ATC vaccine against metastatic disease. As seen in Figs. 3A and 4A, and in confirmation of the results presented in Fig. 1, treatment of 410.4 primary tumor-excised mice with CY followed by DNP-modified ATC vaccine led to a significantly better relapse-free survival than treatment of mice with CY followed by unmodified ATC vaccine. However, the relapse-free survival of animals treated with CY followed by DNP-modified ATC vaccine was significantly reduced when the mice were depleted of $CD4^+$ (Fig. 3B) or $CD8^+$ (Fig. 4B) T-cells. In fact, the relapse-free survival of mice depleted of $CD4^+$ T-cells and treated with CY followed by DNP-modified ATC vaccine was not significantly different to that of mice depleted of $CD4^+$ T-cells and treated with CY followed by unmodified ATC vaccine (Fig. 3C). Similarly, the relapse-free survival of mice depleted of $CD8^+$ T-cells and treated with CY followed by DNP-modified ATC vaccine was not significantly different to that of mice depleted of $CD8^+$ T-cells and treated with CY followed by unmodified ATC vaccine (Fig. 4C). Thus, both $CD4^+$ and $CD8^+$ T-cells are important for the realization of the therapeutic effect of CY followed by DNP-modified ATC vaccine against metastatic disease.

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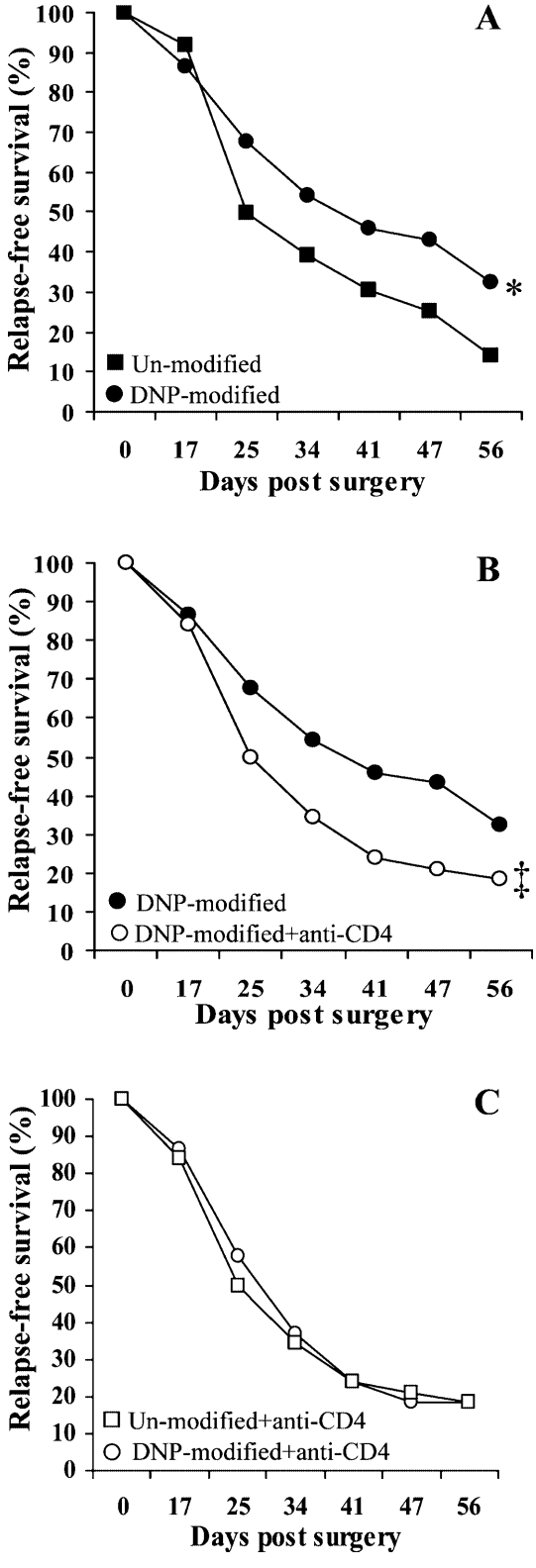

Fig. 3. Importance of $CD4^+$ T-cells for the realization of the anti-metastatic effect of CY followed by DNP-modified ATC vaccine. Relapse-free survival of 410.4 primary tumor-excised mice subjected to CY followed by vaccination with DNP-modified, irradiated, ATC admixed with BCG in conjunction with (38 mice; ○) or without (37 mice; ●) anti-CD4 mAb. As reference, we provide the relapse-free survival of 410.4 primary tumor-excised mice subjected to CY followed by vaccination with unmodified, irradiated, ATC admixed with BCG in conjunction with (38 mice; □) or without (36 mice; ■) anti-CD4 mAb. *Significantly better relapse-free survival than that of the other treatment group in the same panel, ‡significantly worse relapse-free survival than that of the other treatment group in the same panel

Assessment of IFN-γ production by lymph node cells from 410.4 tumor-bearing mice treated with CY followed by DNP-modified ATC vaccine

In light of the report of Lattime et al. [20] on elevated expression of IFN-γ mRNA in subcutaneous metastases of melanoma patients who developed inflammation as a result of treatment with CY followed by DNP-modified ATC vaccine, experiments were carried out to determine whether elevated expression of IFN-γ was evident at the protein level in the draining lymph nodes of 410.4 primary tumor-excised mice treated with CY followed by DNP-modified ATC vaccine. As part of this study, we investigated the importance of DNP modification for the elevated production of IFN-γ by comparing the level of IFN-γ present in culture supernatants of cells derived from the draining lymph nodes of mice treated with CY followed by DNP-modified ATC vaccine relative to that present in the supernatants of cells derived from the draining lymph nodes of mice treated with CY followed by unmodified ATC vaccine. For this purpose, the draining lymph nodes were removed from mice three days following the second injection of either DNP-modified or unmodified ATC vaccine and cultured for two or three days without additional stimulation. The level of IFN-γ present in the supernatants of these cultures was determined by ELISA, and compared to that present in the culture supernatants from lymph node cells of 410.4 primary tumor-excised mice injected with saline. A total of six experiments were carried out, yielding similar results. The results of a representative experiment with lymph node cells from three mice per group are presented. As seen in Fig. 5, culture supernatants from lymph node cells of 410.4 primary tumor-excised mice treated with CY followed by either DNP-modified or unmodified ATC vaccine contained significantly more IFN-γ than culture supernatants from lymph node cells of 410.4 primary tumor-excised mice injected with saline. However, culture supernatants from lymph node cells of 410.4 primary tumor-excised mice treated with CY followed by DNP-modified ATC vaccine contained significantly more IFN-γ than culture supernatants from lymph node cells of 410.4 primary tumor-excised mice treated with CY followed by unmodified ATC vaccine. Thus, lymph node cells from mice treated with CY followed by DNP-modified ATC vaccine produce elevated levels of IFN-γ, indicating that the DNP modification is important for the IFN-γ production.

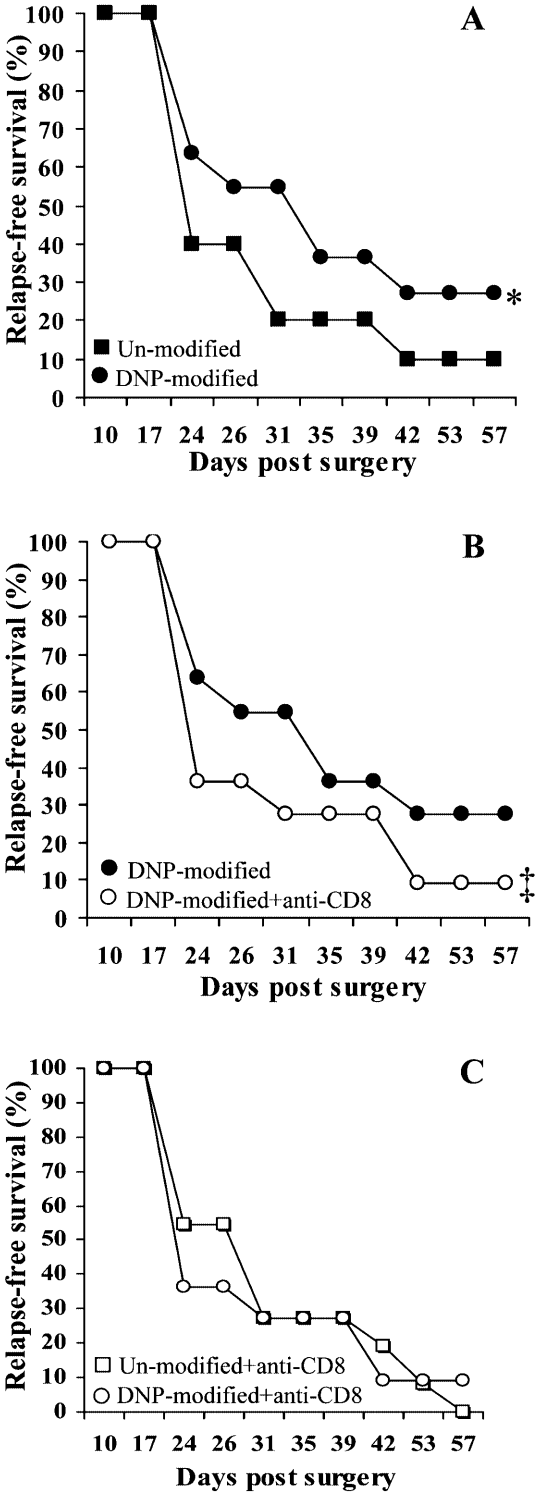

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Fig. 4. Importance of $CD8^+$ T-cells for the realization of the anti-metastatic effect of CY followed by DNP-modified ATC vaccine. Relapse-free survival of 410.4 primary tumor-excised mice subjected to CY followed by vaccination with DNP-modified, irradiated, ATC admixed with BCG in conjunction with (11 mice; ○) or without (11 mice; ●) anti-CD8 mAb. As reference, we provide the relapse-free survival of 410.4 primary tumor-excised mice subjected to CY followed by vaccination with unmodified, irradiated, ATC admixed with BCG in conjunction with (11 mice; □) or without (10 mice; ■) anti-CD8 mAb. *Significantly better relapse-free survival than that of the other treatment group in the same panel, ‡significantly worse relapse-free survival than that of the other treatment group in the same panel

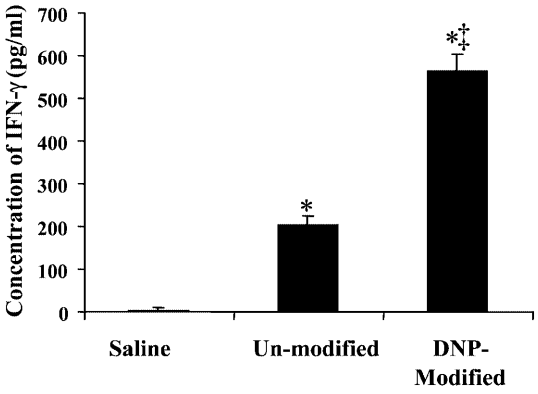

Fig. 5. Elevated production of IFN-γ by cells from the draining lymph nodes of primary tumor-excised mice subjected to CY followed by vaccination with DNP-modified ATC vaccine. Draining lymph nodes were removed from 410.4 primary tumor-excised mice three days following the second injection of DNP-modified, irradiated, ATC vaccine (*DNP-modified*), or unmodified, irradiated ATC vaccine (*unmodified*), and cultured for two days without additional stimulation. Subsequently, ELISA was used to determine the level of IFN-γ present in the supernatants of these cultures as well as in the culture supernatants from lymph node cells of 410.4 primary tumor-excised mice injected with saline (*saline*). *Presence of significantly more IFN-γ than in the culture supernatants of lymph node cells from the saline treatment group, ‡presence of significantly more IFN-γ than in the culture supernatants of lymph node cells from mice treated with CY followed by unmodified ATC vaccine

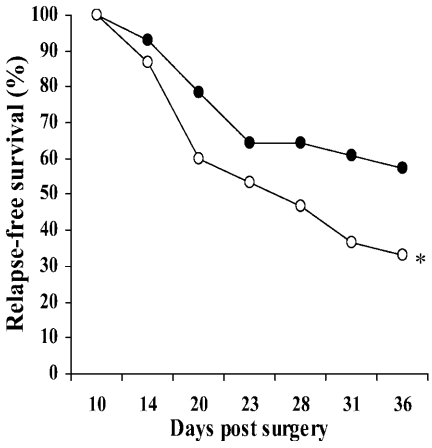

Fig. 6. Importance of IFN-γ for the realization of the anti-metastatic effect of CY followed by DNP-modified ATC vaccine. Relapse-free survival of 410.4 primary tumor-excised mice subjected to CY followed by DNP-modified, irradiated, ATC vaccine admixed with BCG in conjunction with (30 mice; ○) or without (28 mice; ●) anti-IFN-γ neutralizing mAb. *Significantly worse relapse-free survival than without IFN-γ neutralization

Assessment of the importance of IFN-γ for the realization of the anti-metastatic effect elicited by CY followed by DNP-modified ATC vaccine

Experiments were undertaken to determine whether IFN-γ was important for the realization of the anti-metastatic effect elicited by CY followed by DNP-modified ATC vaccine. This was done by determining the effect of neutralizing anti-IFN-γ mAb on the therapeutic benefits provided by CY followed by DNP-modified ATC vaccine against metastatic disease. A total of three experiments were carried out, yielding similar results. In one of these experiments, rat IgG mAb raised against an unrelated antigen was used as control and was found not to reduce the anti-metastatic effect of CY followed by DNP-modified ATC vaccine (data not shown). The cumulative results of the three experiments assessing the effect of anti-IFN-γ mAb on the anti-metastatic effect of CY followed by DNP-modified ATC vaccine are presented. As seen in Fig. 6, anti-IFN-γ mAb reduced the relapse-free survival of mice treated with CY followed by DNP-modified ATC vaccine. Thus, IFN-γ is important for the realization of the therapeutic effect of CY followed by DNP-modified ATC vaccine against metastatic disease.

Assessment of the importance of TNF for the realization of the anti-metastatic effect elicited by CY followed by DNP-modified ATC vaccine

Our current observation that $CD8^+$ T-cells are important for the realization of the anti-metastatic effect of CY followed by DNP-modified ATC vaccine, coupled with our previous observation that TNF production is essential for the acquisition of $CD8^+$ T-cell-mediated tumor-eradicating immunity in another tumor system subjected to a different therapeutic modality [11, 39] prompted us to examine the importance of TNF for the realization of the anti-metastatic effect of CY followed by DNP-modified ATC vaccine in the 410.4 tumor system. Accordingly, we determined the effect of neutralizing anti-TNF mAb on the therapeutic benefits provided by CY followed by DNP-modified ATC vaccine against metastatic disease. As seen in Fig. 7, anti-TNF mAb led to a significant reduction in the relapse-free survival of mice treated with CY followed by DNP-modified ATC vaccine. Thus, TNF is important for the realization of the therapeutic effect of CY followed by DNP-modified ATC vaccine against metastatic disease.

Discussion

Berd et al. [5, 6, 7] have previously shown that administration of CY followed by DNP-modified ATC vaccine can constitute an effective postsurgical adjuvant treatment for melanoma patients with bulky regional lymph node metastases. More recently, Berd et al. [6, 7] have reported that this therapeutic approach is also applicable to ovarian cancer, supporting his claim that this technology can theoretically be applied to other types of human cancer [7]. Here we extended the observations of Berd et al. [5, 6, 7] to an animal tumor model, by demonstrating the effectiveness of this novel therapeutic approach for the treatment of established metastases in 410.4 primary tumor-excised mice. Moreover, we extended the above findings [5, 6, 7] by providing insight into the mechanism through which the anti-metastatic

effect of CY followed by DNP-modified ATC vaccine is realized. The information obtained from our studies on this clinically relevant system is expected to shed light on the mechanism through which the anti-metastatic effect of this immunotherapeutic protocol is realized in cancer patients.

Here we show that treatment of 410.4 primary tumor excised mice with CY followed by DNP-modified ATC vaccine was superior, in terms of anti-metastatic effect, not only to the saline treatment but also to CY followed by unmodified ATC vaccine. These observations illustrate the importance of the DNP modification for the realization of the anti-metastatic activity of CY followed by ATC vaccine. Moreover, the studies described herein show that the anti-metastatic activity induced as a result of the DNP modification is dependent on T-cells, as depletion of T-cells (either $CD4^+$ or $CD8^+$) completely abrogates the anti-metastatic effect elicited by CY followed by DNP-modified ATC vaccine. At present the mechanism by which the DNP modification leads to the realization of the T-cell-dependent anti-metastatic effect against unmodified tumor cells is unknown. However, studies by Martin et al. [21] suggest a potential mechanism. Specifically, these studies show that T-cells bearing low affinity receptors for self peptides can be activated by peptide–hapten complexes, thereby allowing for recall responses to unmodified self peptide to occur.

Consistent with the possibility that $CD8^+$ T-cells are important for the anti-metastatic effect of CY followed by DNP-modified ATC vaccine, Berd et al. [3, 4] noticed that the inflammatory response induced by their treatment regimen in melanoma metastases consisted predominately of $CD8^+$ T-cells. Moreover, these infiltrating $CD8^+$ T-cells preferentially utilized a particular T-cell receptor (TCR)–Vβ gene segment (Vβ14) [36], suggesting selective clonal expansion of these $CD8^+$ T-cells in response to DNP-modified ATC vaccine. Furthermore, T-cell lines derived from two infiltrated skin metastases and enriched in TCR–Vβ14 T-cells were capable of lysing in vitro unmodified autologous melanoma cells in an HLA class I-restricted manner. Here we extend the observations of Berd et al. [3, 4, 36] by demonstrating that $CD8^+$ T-cells are actually important for the realization of the anti-metastatic effect of CY followed by DNP-modified ATC vaccine. Specifically, we show that selective depletion of $CD8^+$ T-cells completely abrogated the ability of CY followed by DNP-modified ATC vaccine to provide any anti-metastatic benefits to 410.4 primary tumor-excised mice.

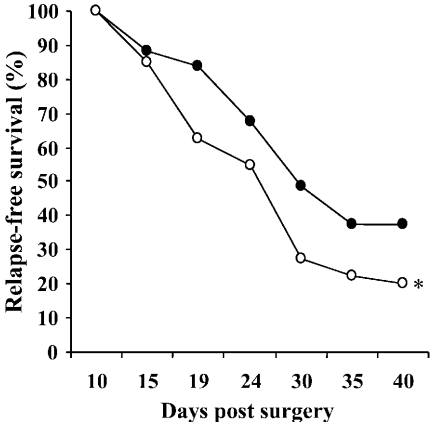

Fig. 7. Importance of TNF for the realization of the anti-metastatic effect of CY followed by DNP-modified ATC vaccine. Relapse-free survival of 410.4 primary tumor-excised mice subjected to CY followed by DNP-modified, irradiated, ATC admixed with BCG in conjunction with (40 mice; ○) or without (43 mice; ●) anti-TNF neutralizing mAb. *Significantly worse relapse-free survival than without TNF neutralization

$CD4^+$ T-cells, not only $CD8^+$ T-cells, are shown here to be important for the realization of the anti-metastatic effect of CY followed by DNP-modified ATC vaccine. At first glance this may appear to be in conflict with the findings of Berd et al. [4], illustrating that $CD4^+$ T-cells represent only a minor component of the inflammatory response induced in metastatic melanoma tumors by CY followed by DNP-modified ATC vaccine. However, very few $CD4^+$ T-cells may be sufficient to elicit the anti-metastatic effect of this therapeutic protocol. Moreover, the $CD4^+$ T-cells are probably important for the activation of $CD8^+$ T-cells, and this activation most likely does not take place at the metastatic sites, but rather in secondary lymphoid organs [40].

Lattime et al. [20] have previously reported an elevated expression of IFN-γ mRNA in subcutaneous metastases of melanoma patients who developed inflammation as a result of treatment with CY followed by DNP-modified ATC vaccine. However, the cell type responsible for the elevated expression of IFN-γ mRNA was not identified, although in a later publication these authors stated that inflammatory T-cells produce IFN-γ in situ [7]. Here we extend the observations of Lattime et al. by demonstrating that administration of CY followed by DNP-modified ATC vaccine leads to the elevated production of the IFN-γ protein by cells from the draining lymph nodes of 410.4 primary tumor-excised mice. Specifically, we show that culture supernatants from draining lymph node cells of 410.4 primary tumor excised mice treated with CY followed by DNP-modified ATC vaccine contained significantly more IFN-γ than culture supernatants from draining lymph node cells of 410.4 primary tumor-excised mice treated with CY followed by unmodified ATC vaccine, indicating the importance of the DNP modification for the elevated production of the IFN-γ protein. Moreover, we show the importance of IFN-γ production for the realization of the anti-metastatic effect of this therapeutic protocol by demonstrating that neutralization of IFN-γ reduces the anti-metastatic effect of CY followed by DNP-modified ATC vaccine in the 410.4 tumor system.

In addition to assessing the importance of IFN-γ for the realization of the anti-metastatic effect of CY followed by DNP-modified ATC vaccine in the 410.4 tumor system, we also assessed the importance of TNF for this purpose. We focused our attention on the impor-

tance of TNF for several reasons. First, in the current studies $CD8^+$ T-cells were found to be important for the realization of the anti-metastatic effect of CY followed by DNP-modified ATC vaccine, and TNF production was found in our previous studies to be critical for the acquisition of $CD8^+$ T-cell-mediated tumor-eradicating immunity in another tumor system subjected to a different therapeutic modality [11, 39]. Second, Neurath et al. [29] have recently shown that TNF production is important for the development of autoimmune colitis following the application of 2, 4, 6-trinitrobenzene sulfonic acid (TNBS) to the colonic mucosa of mice. Finally, Wu et al. [41] have recently reported that inhibition of TGF-β production through the use of antisense TGF-β is required to elicit the anti-metastatic activity of a 410.4 subline transfected with the IFN-γ gene in primary tumor-excised mice, and TNF has been shown in our previous studies [12] as well as in studies by others [32], to overcome the inhibitory activity of TGF-β for the generation of CTL activity by $CD8^+$ T-cells. Here we show that indeed neutralization of TNF reduces the anti-metastatic effect of CY followed by DNP-modified ATC vaccine in the 410.4 tumor system.

Taken together, the studies described herein demonstrate that a treatment protocol consisting of CY followed by DNP-modified ATC vaccine, which has previously been shown to provide therapeutic benefits in postsurgical adjuvant settings to patients with melanoma [5, 6, 7] or ovarian cancer [7], also has a therapeutic effect against metastatic disease in mice from which the primary mammary tumor has been excised. In addition, the current studies provide some insight into the mechanism by which the anti-metastatic effect of this therapeutic protocol is realized in a clinically relevant experimental tumor model. This information is expected to shed light on the mechanism through which CY followed by DNP-modified ATC vaccine elicits an anti-metastatic effect in cancer patients.

Acknowledgements We would like to thank D. Berd from the Thomas Jefferson University for helpful and insightful discussions as well as for a constructive review of the manuscript. In addition, we would like to thank A. Fine for expert technical assistance. This study was supported by a grant from Avax Technologies.

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