

Combination of Docetaxel and Recombinant Vaccine Enhances T-Cell Responses and Antitumor Activity: Effects of Docetaxel on Immune Enhancement

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Abstract **Purpose:** Taxanes comprise some of the most widely used cancer chemotherapeutic agents. Members of this drug family, including docetaxel, are commonly used to treat breast, prostate, and lung cancers, among others. This study was designed to determine if this taxane has the ability to modulate components of the immune system independent of antitumor activity and to investigate the potential synergistic activities of the combination of docetaxel and vaccine therapy. **Experimental Design:** We examined the *in vivo* effects of docetaxel on immune-cell subsets and on the function of CD4⁺, CD8⁺, and regulatory T-cell (Treg) populations in response to antigen-specific vaccination. We also examined the antitumor effects of the combination of docetaxel and vaccine in a preclinical model in which docetaxel has no observable effect on tumor growth. **Results:** These studies show for the first time that (a) docetaxel modulates CD4⁺, CD8⁺, CD19⁺, natural killer cell, and Treg populations in non-tumor-bearing mice; (b) unlike cyclophosphamide, docetaxel does not inhibit the function of Tregs; (c) docetaxel enhances CD8⁺ but not CD4⁺ response to CD3 cross-linking; (d) docetaxel given after vaccination provides optimal enhancement of immune response to recombinant viral vaccines; (e) docetaxel combined with recombinant viral vaccine is superior to either agent alone at reducing tumor burden; and (f) docetaxel plus vaccine increases antigen-specific T-cell responses to antigen in the vaccine, as well as to cascade antigens derived from the tumor. **Conclusions:** These findings suggest potential clinical benefit for the combined use of docetaxel and recombinant cancer vaccines.

Various types of cancer immunotherapy are under active investigation in preclinical and clinical settings. Whereas nonviral target cancer vaccines have yet been approved by the Food and Drug Administration, several randomized phase II trials are showing evidence of clinical benefit, particularly in increased patient survival (1). Many cancer vaccine clinical protocols have been used in patients who have failed prior therapy or, in some cases, multiple therapies. To date, however, little attention has been given to the use of vaccines in combination with chemotherapy.

Previous preclinical and clinical studies have shown that cancer vaccines that target tumor-associated antigens can enhance immune response to those tumor-associated antigens. We have previously described recombinant poxviral vaccine

vectors that contain transgenes for specific tumor antigens in addition to a triad of T-cell costimulatory molecules (B7-1, ICAM-1, and LFA-3; designated TRICOM; ref. 2). Optimal immune responses are achieved with s.c. vaccinations in a diversified prime-and-boost regimen using vaccinia vectors as the prime and fowlpox vectors as boosts (2, 3). Addition of granulocyte macrophage colony-stimulating factor (GM-CSF) to the vaccine has been shown to further enhance immune responses (4). In clinical studies, TRICOM-based vaccines seem to have evidence of increased survival in patients with low tumor burden, such as prostate cancer patients with biochemical progression after definitive local therapy (1, 5). Although these trials were not designed to show survival, the data seem promising. However, combining cancer vaccines with conventional therapies may prove to be the most effective strategy for patient benefit.

Traditionally, the goal of chemotherapy has been direct cytotoxicity and induction of tumor cell death. Taxanes, which are among the most widely used cancer chemotherapeutic agents, induce antineoplastic activity by disrupting intracellular microtubular networks. Members of the taxane family, including docetaxel and the closely related compound paclitaxel (Taxol), have been used in a variety of malignancies, such as breast, prostate, and lung cancers, and have been reported to modulate components of the immune system in mice (6). However, it has been shown that chemotherapeutic agents can also have a direct effect on host immune responses (7). In previous *in vivo* studies of the immunoregulatory effect of

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Table 1. Effect of docetaxel on splenic cell populations

| Cells | Treatment | Pretreatment | | Time posttreatment | | | | | | | | | |
|-------------------|-----------|-----------------------|-----------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------|-----------------------------|-----------------------|-----------------------------|
| | | | | Day 1 | | Day 2 | | Day 3 | | Day 4 | | Day 7 | |
| Splenoctyes | PBS | 1.1 × 10 ⁸ | | 1.4 × 10 ⁸ | | 1.1 × 10 ⁸ | | 1.1 × 10 ⁸ | | 9.6 × 10 ⁷ | | 1.1 × 10 ⁸ | |
| | Docetaxel | — | | 8.1 × 10⁷ | | 7.4 × 10⁷ | | 8.6 × 10⁷ | | 1.1 × 10 ⁸ | | 1.1 × 10 ⁸ | |
| CD4 ⁺ | PBS | 18% | 2.0 × 10 ⁷ | 17% | 2.4 × 10 ⁷ | 18% | 2.0 × 10 ⁷ | 17% | 1.9 × 10 ⁷ | 15% | 1.4 × 10 ⁷ | 25% | 2.8 × 10 ⁷ |
| | Docetaxel | — | | 19% | 1.5 × 10⁷ | 19% | 1.4 × 10 ⁷ | 19% | 1.6 × 10 ⁷ | 10% | 1.1 × 10 ⁷ | 19% | 2.1 × 10 ⁷ |
| CD8 ⁺ | PBS | 13% | 1.4 × 10 ⁷ | 11% | 1.5 × 10 ⁷ | 8% | 8.8 × 10 ⁶ | 10% | 1.1 × 10 ⁶ | 9% | 8.6 × 10 ⁶ | 14% | 1.5 × 10 ⁷ |
| | Docetaxel | — | | 13% | 1.0 × 10⁷ | 14% | 1.0 × 10 ⁷ | 12% | 1.0 × 10 ⁷ | 7% | 7.7 × 10 ⁶ | 13% | 1.4 × 10 ⁷ |
| CD19 ⁺ | PBS | 60% | 6.6 × 10 ⁷ | 62% | 8.7 × 10 ⁷ | 64% | 7.0 × 10 ⁷ | 58% | 6.4 × 10 ⁷ | 52% | 5.0 × 10 ⁷ | 61% | 6.7 × 10 ⁷ |
| | Docetaxel | — | | 54% | 4.4 × 10⁷ | 58% | 4.3 × 10⁷ | 52% | 4.5 × 10⁷ | 40% | 4.4 × 10⁷ | 49% | 5.4 × 10⁷ |
| DX5 ⁺ | PBS | 2.8% | 3.1 × 10 ⁶ | 2.7% | 3.7 × 10 ⁶ | 2.4% | 2.6 × 10 ⁶ | 2.5% | 2.8 × 10 ⁶ | 2.0% | 1.9 × 10 ⁶ | 3.4% | 3.7 × 10 ⁶ |
| | Docetaxel | — | | 2.7% | 2.2 × 10⁶ | 2.5% | 1.8 × 10⁶ | 2.2% | 1.9 × 10⁶ | 1.8% | 2.0 × 10 ⁶ | 4.4% | 4.8 × 10 ⁶ |
| Tregs | PBS | 5% | 5.5 × 10 ⁶ | 4.0% | 5.6 × 10 ⁶ | 8.8% | 9.7 × 10 ⁶ | 7.2% | 7.9 × 10 ⁶ | 8.1% | 7.8 × 10 ⁶ | 7.3% | 8.0 × 10 ⁶ |
| | Docetaxel | — | | 6.7% | 5.4 × 10 ⁶ | 11.5% | 8.5 × 10 ⁶ | 9.5% | 8.2 × 10 ⁶ | 9.2% | 1.0 × 10 ⁷ | 8.5% | 9.4 × 10 ⁶ |

NOTE: C57BL/6 mice ($n = 5$ per group) were given 0.5 mg docetaxel i.p. on days -4, -2, and 0. Control mice received PBS only. Mice were sacrificed 1, 2, 3, 4, and 7 d posttreatment. After lysis of RBC, splenocytes were counted and stained for T cells (CD3⁺, CD4⁺, CD8⁺), B cells (CD19⁺), natural killer cells (DX5⁺), and Tregs (CD4⁺CD25⁺FoxP3⁺). Of the CD4⁺ cells that expressed CD25, >98% also stained positive for FoxP3. Cells were analyzed by flow cytometry. Percentages denote the percent of splenocytes. Bold numbers indicate a significant difference ($P < 0.05$) in docetaxel versus PBS treated mice. This experiment was repeated twice.

taxanes in non-tumor-bearing mice, paclitaxel reduced the number of splenocytes, lymphocytes, and WBC for several days (8), and docetaxel dropped neutrophil and lymphocyte cell numbers for at least 2 weeks (9). However, no study has shown the effect of taxanes on individual immune cell subsets or, more importantly, on the function of individual T-cell subsets.

Several preclinical studies have examined the use of vaccines in combination with chemotherapies, including three studies that combined a vaccine with a taxane (docetaxel or paclitaxel) in murine tumor models (9–11). In each of these studies, both vaccine and taxanes had clear antitumor effects as monotherapies and enhanced antitumor effects when used in combination. These results could be explained by (a) the effect of taxane on immune cell subsets or regulatory T cells (Treg) and/or (b) the ability of taxane to reduce tumor burden and thus reduce the level of immune inhibitory elements, such as transforming growth factor- β , secreted by the tumor. In the studies reported here, we investigated both of these possibilities. First, we examined the *in vivo* effects of docetaxel on individual immune cell subsets and on the function of CD4⁺, CD8⁺, and Treg populations in response to antigen-specific vaccination. These studies were conducted in non-tumor-bearing mice to rule out the indirect effects of docetaxel on the immune system that result from a reduction in tumor size. Next, we used a combination of docetaxel and vaccine in tumor-bearing mice in which docetaxel alone has been shown to have no observable effect on tumor growth.

These studies show for the first time that docetaxel modulates T-cell, B-cell, and natural killer cell subsets and enhances CD8⁺ function. These studies were conducted in non-tumor-bearing mice to rule out indirect evidence of docetaxel on tumor. Studies with tumor-bearing mice show for the first time that (a) administering docetaxel with poxviral vaccines results in enhancement of vaccine-specific immune responses and induces antigen-specific T-cell responses to tumor-derived

antigens distinct from the antigen used in the vaccine (antigen cascade).

Materials and Methods

Animals. *In vivo* studies were conducted with 6-wk-old to 8-wk-old female C57BL/6 mice or female C57BL/6 mice transgenic for human carcinoembryonic antigen (CEA-Tg; National Cancer Institute). CEA-Tg mice were obtained from a breeding pair provided by Dr. John Thompson (Institute of Immunobiology, University of Freiburg). The generation and characterization of the CEA-Tg mouse has been previously described (12, 13). Mice (mean body weight, 25 g) were housed and maintained under pathogen-free conditions in microisolator cages.

Tumor cells. The murine colon adenocarcinoma cell line MC38 expressing CEA (designated MC38-CEA⁺) has been previously described (14). MC38-CEA⁺ cells were maintained in DMEM-10% FCS supplemented with G418 to maintain transgene expression. Cells were trypsinized and washed in PBS before use. Flasks were incubated at 37°C with 5% CO₂.

Chemotherapeutic agent. Preparations of docetaxel (Taxotere, kindly provided by Sanofi-Aventis) were diluted to 10 mg/mL in 80% ethanol according to the manufacturer's instructions and then further diluted to 5 mg/mL in sterile PBS; 100 μ L/mouse was given i.p. For each experiment, 0.5 mg docetaxel was given i.p. on indicated days.

Phenotypic analysis. C57BL/6 mice were injected i.p. with 0.5 mg docetaxel or PBS on days -4, -2, and 0. Mice were sacrificed 1, 2, 3, 4, or 7 d posttreatment, and total splenocytes were counted after lysis of RBC. To stain for cell surface markers, splenocytes were incubated with anti-CD16/CD32 antibody to block Fc receptors, then labeled with anti-CD3-FITC, anti-CD4-PE, anti-CD8-CyChrome C, anti-DX5-FITC, or anti-CD19-PE antibodies (BD Pharmingen). Splenocytes were also stained for Tregs (CD4⁺CD25⁺FoxP3⁺) using a murine Treg staining kit according to the manufacturer's instructions (Miltenyi Biotec). After acquisition, cells were analyzed on a FACScan cytometer (Becton Dickinson).

Treg functional assay. CD8⁺ cells (5×10^4) from control mice were incubated with 1×10^5 antigen-presenting cells (APC; splenic cells depleted of CD4⁺ cells and irradiated with 30 Gy) and 1 μ g/mL soluble

anti-CD3 cross-linking antibody in the presence or absence of 5×10^4 Tregs from either PBS-treated control mice or docetaxel-treated mice (0.5 mg/mouse on days -4, -2, and 0). Tregs were isolated and assayed on day 1 posttreatment. Assay cultures were harvested and counted after a 72-h incubation. [^3H]Thymidine was added to the culture for the last 12 h of incubation. Control wells containing Tregs, APCs, and anti-CD3 without CD8⁺ cells were used to determine background levels of proliferation in culture. Lack of proliferation of CD4⁺CD25⁺ cells further confirmed their identification as Tregs. CD8⁺ cells and APCs were incubated with concanavalin A as a positive control for proliferation (data not shown). CD4⁺CD25⁺ Tregs were isolated from splenocytes using a Treg isolation kit (Miltenyi Biotec). CD8⁺ cells were isolated using anti-CD8 magnetic cell-sorting beads (Miltenyi Biotec) according to the manufacturer's instructions.

Anti-CD3 cytokine production assay and proliferation. C57BL/6 mice received 0.5 mg of docetaxel on days -4, -2, and 0. Splens from PBS-treated and docetaxel-treated mice were harvested 1, 3, or 7 d posttreatment. Splenocytes were treated with ACK and run over a histopaque gradient. Cells (2×10^5) were then incubated on plates coated with varying concentrations of anti-CD3. After 48 h of incubation at 37°C, supernatants were harvested and cytokine concentrations were determined on a FACScan using cytometric bead array (BD Biosciences). To analyze cytokine production from purified T-cell populations, C57BL/6 mice were given 0.5 mg docetaxel i.p. on days -4, -2, and 0. Mice were sacrificed 1, 3, or 7 d posttreatment, and CD4⁺ and CD8⁺ cells were negatively isolated (Miltenyi Biotec) and purified. CD4⁺ or CD8⁺ cells (2×10^5 per well) were incubated in plates coated with 1 $\mu\text{g}/\text{mL}$ anti-CD3 cross-linking antibody. Cytokine production from cells incubated in uncoated plates was subtracted as background. After 48 h, culture supernatants were harvested and assayed for IFN- γ levels using a cytometric bead array kit. To measure proliferation, assay plates were harvested and counted after 72 h. [^3H]Thymidine was added for the last 18 h of incubation.

Recombinant poxviruses. Recombinant vaccinia (rV) and recombinant fowlpox (rF) viruses containing murine B7-1, ICAM-1, and LFA-3 genes, in combination with either human CEA or LacZ genes (CEA/TRICOM and LacZ/TRICOM), have been previously described (15). The rF virus containing the gene for murine GM-CSF (rF-GM-CSF) has also been previously described (16). Therion Biologics kindly provided all of the orthopoxviruses as part of a Collaborative Research and Development Agreement with National Cancer Institute/NIH.

CD4⁺ lymphoproliferative assay. Antigen-specific CD4⁺ T-cell responses were assayed as previously described (17). Briefly, purified CD4⁺ cells (1.5×10^5 cells per well) were incubated with APCs (5×10^5 irradiated splenocytes) and either β -galactosidase protein (100 $\mu\text{g}/\text{mL}$) or CEA protein (50 $\mu\text{g}/\text{mL}$) for 72 h in flat-bottomed 96-well plates. Proliferation was measured after a 5-d incubation at 37°C by adding [^3H]thymidine (1 $\mu\text{Ci}/\text{well}$) to the wells 18 h before harvesting. Cells were harvested and counted by liquid scintillation spectroscopy (Wallac).

Vaccination regimens. Naive CEA-Tg mice were vaccinated with either whole-tumor cell vaccine (WTCV) or recombinant poxviral vaccines. WTCV-treated mice were injected on day 14 with irradiated (50 Gy) MC38-CEA⁺ cells admixed with 10^7 plaque-forming units (pfu) of rF-GM-CSF. Mice received 100 μL s.c. at three sites (10^6 cells per site; left and right hind limbs and left upper limb), as previously described (18). Mice vaccinated with poxviral vectors received 10^8 pfu rV-CEA/TRICOM admixed with 10^7 pfu rF-GM-CSF on day 0 and boosted with 10^8 pfu rV-CEA/TRICOM admixed with 10^7 pfu rF-GM-CSF on day 14. In the indicated groups, mice were also given 0.5 mg of docetaxel i.p. once every other day over a period of 5 d, resulting in three treatments (days 9, 11, and 13 for day -1 treatment groups; days 21, 23, and 25 for day 7 treatment groups). Splens were harvested 3 wk posttreatment (day 47) and used in a lymphoproliferative assay against CEA.

Viral infectivity assay. To measure the ability of poxviruses to infect susceptible cells during exposure to docetaxel, murine tumor cells were infected with 20 MOI of rF-TRICOM. Cells were incubated with 1,100 ng/mL of docetaxel 24 h before infection in the indicated group. In

the other groups, docetaxel was added to the cells 0, 1, or 5 h after addition of the virus. At 24 h after addition of the virus, cells were harvested and stained for transgene expression using an FITC-conjugated B7-1 antibody. Cells were analyzed using CellQuest (BD PharMingen).

IFN- γ production against class I peptide. To determine cytokine production from CD8⁺ T cells in response to tumor-associated antigens, mice were sacrificed after treatment and splenocytes were bulk cultured for 6 d with 10 $\mu\text{g}/\text{mL}$ CEA₅₂₆₋₅₃₃ class I peptide (EAQNTIYL; ref. 19). Lymphocytes were recovered by centrifugation through Ficoll-Hypaque gradient and counted. Recovered lymphocytes (5×10^5 cells per well) were then restimulated with fresh irradiated naive splenocytes as APCs (5×10^6 cells per well) and CEA peptide (10 $\mu\text{g}/\text{mL}$) for 24 h. As a control, HIV-gag₃₉₀₋₃₉₈ peptide (SQVTNPANI) was used for H-2D^b-restricted CEA peptide. Supernatant fluid was collected and analyzed for murine cytokines using the cytometric bead array kit (BD PharMingen). Cytokine production in response to control peptide was subtracted from cytokine production induced by the corresponding tumor-associated antigen peptide, and data were depicted as Δ pg/mL.

Vaccination of CEA⁺ tumor-bearing mice. CEA-Tg mice were injected s.c. with 3×10^5 MC38-CEA⁺ tumor cells on day 0. Mice were primed s.c. on day 4 with 10^8 pfu rV-CEA/TRICOM (plus 10^7 pfu rF-GM-CSF) and boosted s.c. on day 11 with 10^8 pfu rF-CEA/TRICOM (plus 10^7 pfu rF-GM-CSF). In the indicated groups, mice were given 0.5 mg of docetaxel i.p. on days 15, 17, 19, 21, and 23. Tumors were measured twice a week.

Tetramer staining. To evaluate the generation of CEA-specific or gp70-specific CTLs in treated mice, splenocytes were stained with FITC-conjugated anti-CD3e monoclonal antibody, CyChrome-conjugated anti-CD8 monoclonal antibody, PE-conjugated CEA₅₂₆₋₅₃₃/H-2D^b tetramer (CEA tetramer; Beckman Coulter), or PE-conjugated p15E₆₀₄₋₆₁₁/H-2K^b tetramer (gp70 tetramer; Beckman Coulter). As a negative control, splenocytes were stained with PE-conjugated HIV-gag₃₉₀₋₃₉₈/H-2D^b tetramer (for CEA tetramer) or PE-conjugated OVA₂₅₇₋₂₆₄/H-2K^b tetramer (for gp70 tetramer). Immunofluorescence staining was done after Fc receptor blocking with anti-CD16/CD32 monoclonal antibody. Immunofluorescence was compared with the appropriate negative control and analyzed with CellQuest software using a FACSCalibur cytometer (Becton Dickinson). Negative control tetramer samples were <5% positive in CEA tetramer⁺ fraction and gp70 tetramer⁺ fraction after gating of CD3⁺CD8⁺ T cells.

Statistical analysis. P values were derived from Student's *t* test using a two-tailed distribution and calculated at 95% using Statview 4.1 software (Abacus Concepts). Significant differences in flow cytometry data were determined using the Kolmogorov-Smirnov test.

Results

Effect of docetaxel on splenic cell populations and Treg function. The U.S. Food and Drug Administration approved indication for docetaxel as a single agent is 75 to 100 mg/m² (20). Based on this dose, it was calculated that 0.5 mg docetaxel would be equivalent for a 20-g to 25-g mouse. In addition, this dose has been used by others in murine models given i.p. every other day for three to five times (21, 22). Studies were first conducted on the effect of docetaxel on individual immune cell subsets, using non-tumor-bearing mice to rule out the indirect effects of docetaxel on the immune system that result from reduced tumor size. C57BL/6 mice received 0.5 mg docetaxel on days -4, -2, and 0 and were then sacrificed at multiple time points posttreatment. Total splenocytes and individual immune cell subsets as well as percentage of splenocytes were analyzed at each time point (Table 1). There was a statistically significant decrease in total splenocytes on days 1 to 3 posttreatment. A decrease in the number of CD4⁺ and CD8⁺ T-cell subsets was statistically significant on day 1 posttreatment but not on

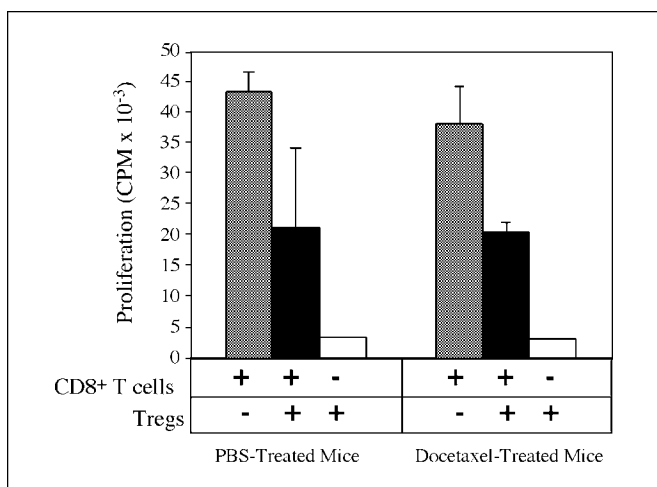


Fig. 1. Lack of effect of docetaxel on Treg function. C57BL/6 mice ($n = 5$ per group) were given 0.5 mg docetaxel i.p. on days -4, -2, and 0. Control mice received PBS only. Mice were sacrificed 1 d posttreatment. CD8⁺ cells from control mice were incubated with APCs (irradiated splenocytes depleted of CD4⁺ cells) and soluble anti-CD3 in the presence (black columns) or absence (gray columns) of Tregs from either control mice or docetaxel-treated mice. Control wells containing Tregs, APCs, and anti-CD3 without CD8⁺ cells (white columns) were used to determine background levels of proliferation in culture.

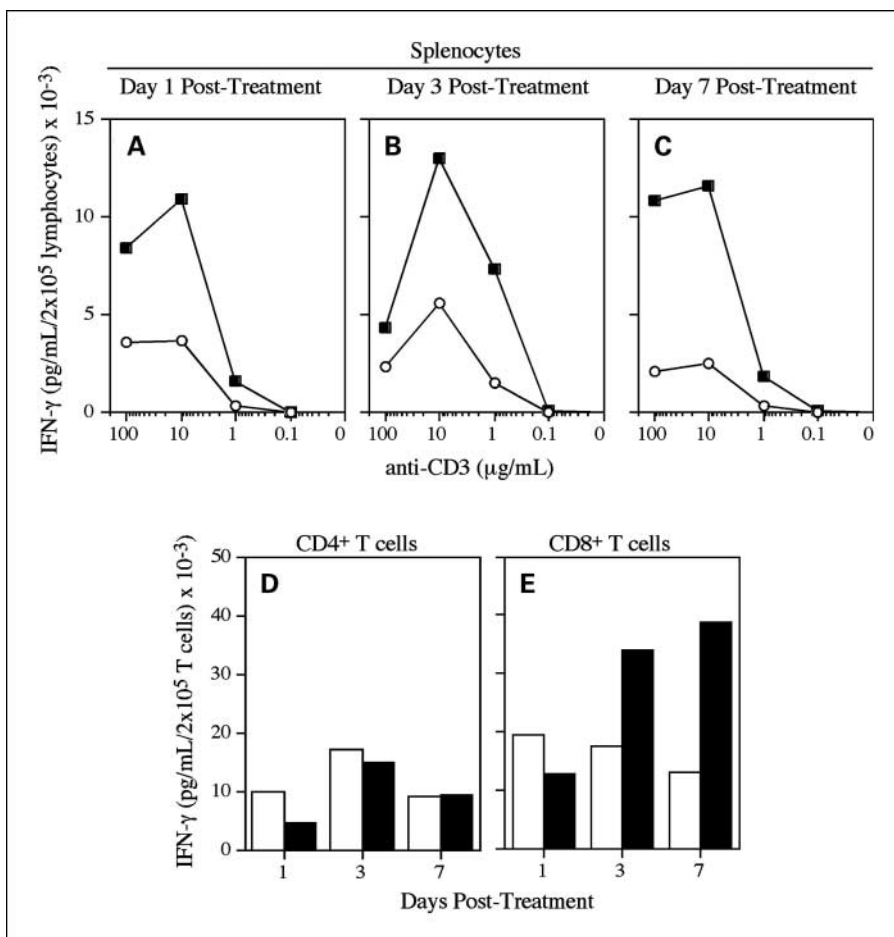
subsequent days. Both the percentage and total number of CD19⁺ B cells decreased on days 1 to 7 posttreatment. The number of natural killer cells, as monitored by antibody to DX5, also decreased on days 1 to 3. There was no change in the number

of Tregs (CD4⁺ cells expressing surface CD25, >98% of which also stained positive for FoxP3) at any of the time points indicated (Table 1). The cell population analysis presented in Table 1 was conducted on pooled mice ($n = 5$) and has been repeated twice with similar results. The depicted data is from a representative experiment, whereas the statistical analysis examining population changes is representative of two experiments.

It has been reported that a potential side effect of docetaxel treatment is transient myelosuppression and anemia, occurring 4 to 7 days after administration and returning to reference ranges within 21 days (20). To determine if this was the case here, C57BL/6 mice received 0.5 mg docetaxel on days -4, -2, and 0 and were then sacrificed 14 days posttreatment and complete differential blood counts taken. Control mice received PBS. On day 14 posttreatment, there were no significant differences observed in any cell population between the docetaxel-treated or control mice. In addition, all values fell within the reference ranges.

Previous studies have shown a decrease in Treg functionality after treatment with the chemotherapeutic agent cyclophosphamide. We therefore examined the functionality of Tregs in mice after treatment with docetaxel. CD4⁺CD25⁺ cells from untreated and docetaxel-treated mice were purified by microbead separation and used in an *in vitro* Treg assay. After separation, cells were stained for FoxP3 to confirm their identity as Tregs; >98% were FoxP3⁺. On day 1 posttreatment, the proliferation of CD8⁺ cells in the presence of Tregs from untreated mice was similar to the proliferation of CD8⁺ cells in the presence of Tregs from docetaxel-treated mice (Fig. 1),

Fig. 2. Effect of docetaxel on splenic CD4⁺ and CD8⁺ T-cell cytokine production. C57BL/6 mice ($n = 5$ per group) were given 0.5 mg docetaxel i.p. on days -4, -2, and 0. Control mice received PBS only. Mice were sacrificed 1, 3, and 7 d posttreatment. A-E, splenocytes from untreated mice (open circles and open columns) or docetaxel-treated mice (closed squares and closed columns) were incubated on plates coated with dilutions of anti-CD3 antibody. A-C, supernatants were harvested after 48 h and analyzed for IFN- γ production. D and E, CD4⁺ and CD8⁺ T cells were purified and incubated on plates coated with 1 μ g/mL of anti-CD3 antibody. Supernatants were harvested after 48 h and assayed for IFN- γ levels. Proliferation of CD4⁺ and CD8⁺ T cells was similar.



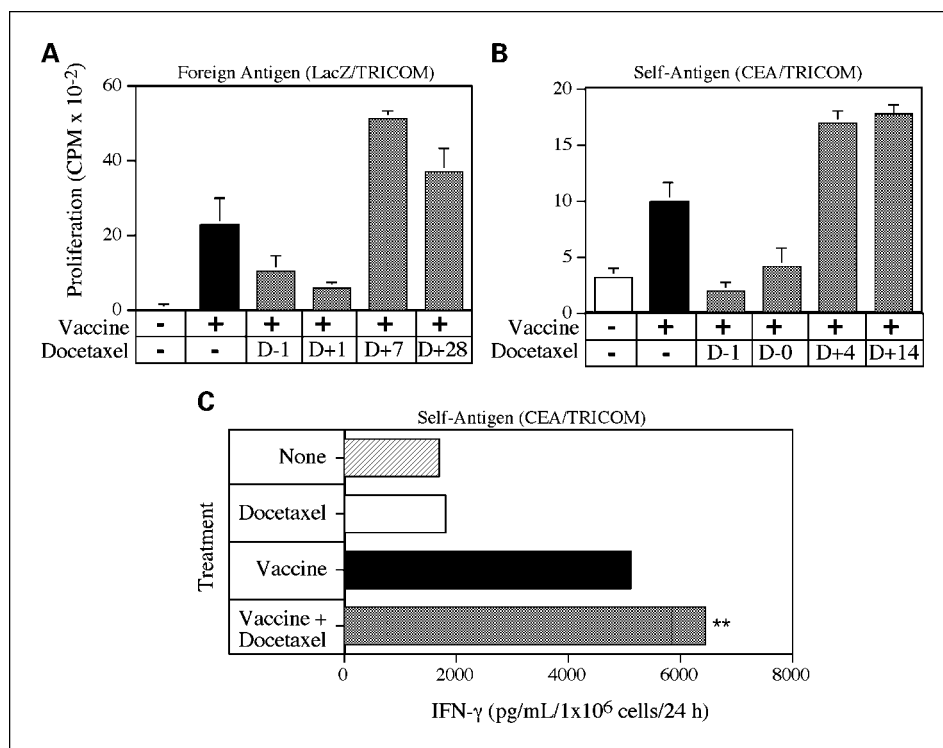


Fig. 3. Antigen-specific T-cell responses combining vaccination plus docetaxel. **A**, CD4⁺ T-cell responses to foreign antigen. C57BL/6 mice ($n = 3$ per group) were primed on day 0 with *rV*-LacZ/TRICOM s.c. and boosted on day 14 with *rF*-LacZ/TRICOM s.c. Both vaccinations were given with *rF*-GM-CSF. Docetaxel (0.5 mg) was given i.p. 1 d before (D-1; days 9, 11, 13), 1 d after (D+1; days 15, 17, 19), 7 d after (D+7; days 21, 23, 25), or 28 d after (D+28; days 42, 44, 46) the boost. Three weeks postdocetaxel administration, purified CD4⁺ splenic T cells were tested for reactivity to β -galactosidase protein (100 μ g/mL) in an *in vitro* lymphoproliferation assay. **B**, CD4⁺ T-cell responses to a self-antigen. CEA-Tg mice ($n = 3$ per group) were primed on day 0 with *rV*-CEA/TRICOM s.c. and boosted on day 14 with *rF*-CEA/TRICOM s.c. Both vaccinations were given with *rF*-GM-CSF. Docetaxel (0.5 mg) was given i.p. 1 d before (D-1; days 9, 11, 13), the day of (D-0; days 14, 16, 18), 4 d after (D+4; days 18, 20, 22), or 14 d after (D+14; days 28, 30, 32) the boost. Four weeks after the boost, purified CD4⁺ splenic T cells were tested for reactivity to CEA protein (50 μ g/mL) in an *in vitro* lymphoproliferation assay. **C**, CD8⁺ T-cell responses to a self-antigen. CEA-Tg mice ($n = 5$ per group) were primed with *rV*-CEA/TRICOM on day 0, followed by boosts with *rF*-CEA/TRICOM on days 7 and 19. *rF*-GM-CSF was given with each vaccination. Docetaxel (0.5 mg) was given i.p. on days 11, 13, and 15. Mice were sacrificed 3 wk after the last vaccine (day 45), and splenocytes were bulk cultured for 6 d with CAP-M8 peptide. Lymphocytes were recovered and restimulated for 24 h with CEA CAP-M8 peptide or negative control HIV-gag peptide. Specific IFN- γ secretion (HIV peptide background subtracted) is depicted. **, $P < 0.05$ versus vaccine.

demonstrating that docetaxel does not inhibit the suppressor function of Tregs. The lack of proliferation in the wells containing only CD4⁺CD25⁺ cells, APCs, and soluble anti-CD3 antibody further confirms their identity as Tregs.

Effect of docetaxel on splenic CD4⁺ and CD8⁺ T-cell function. Assays were conducted to determine the effects of docetaxel on the function of total splenocytes and CD4⁺ and CD8⁺ subsets. CD3⁺ splenocytes obtained from docetaxel-treated mice on days 1, 3, and 7 posttreatment showed a clearly enhanced IFN- γ production when exposed *in vitro* to varying concentrations of anti-CD3 antibody (Fig. 2A-C); however, no difference in proliferation was observed between the treatment groups at any of the indicated time points. Further analysis revealed that, posttreatment, docetaxel had no effect on CD4⁺ T cells but had a substantial effect on the function of the CD8⁺ T-cell subset (Fig. 2D and E). On days 1, 3, and 7 posttreatment, CD4⁺ and CD8⁺ T cells were isolated and purified cells were incubated with 1 μ g/mL of anti-CD3 cross-linking antibody. This led to an increase in IFN- γ production by CD8⁺ T cells on days 3 and 7 posttreatment. No differences in proliferation of CD4⁺ and CD8⁺ T cells were observed between docetaxel-treated and untreated mice. These data suggest that docetaxel specifically enhances IFN- γ production by CD8⁺ T cells and that this enhancement is not due to increased proliferation of these cells.

Antigen-specific T-cell responses to foreign and self-antigens after vaccination in combination with docetaxel. To determine if docetaxel had an effect on vaccine-mediated antigen-specific T-cell responses, C57BL/6 mice were vaccinated with recombinant vectors containing the LacZ gene that encodes the antigen β -galactosidase protein. Mice were primed with *rV*-LacZ/TRICOM s.c. on day 0 and then boosted with *rF*-LacZ/TRICOM s.c. on day 14. Docetaxel was given i.p. before, during, or after vaccine. Docetaxel given before or during vaccination clearly inhibited vaccine-mediated CD4⁺ T-cell responses compared with vaccine alone. However, docetaxel given after the booster vaccination clearly enhanced vaccine-mediated CD4⁺ T-cell responses, as indicated by increased CD4⁺ T-cell proliferation to β -galactosidase protein (Fig. 3A).

To study the combination of docetaxel and vaccine in the setting of a self-antigen, CEA-based vaccine was given to C57BL/6 mice transgenic for human CEA-Tg. CEA-Tg mice received a priming vaccination with *rV*-CEA/TRICOM s.c. on day 0, followed by a booster vaccination with *rF*-CEA/TRICOM s.c. on day 14. Docetaxel was given before or after the booster vaccine, and in terms of dose scheduling, vaccine-mediated CD4⁺ responses (Fig. 3B) were markedly similar to those seen with the LacZ-based vaccine (Fig. 3A). CEA-specific CD8⁺ T-cell responses were also evaluated by measuring IFN- γ production against a CEA class I-restricted peptide. Mice were sacrificed

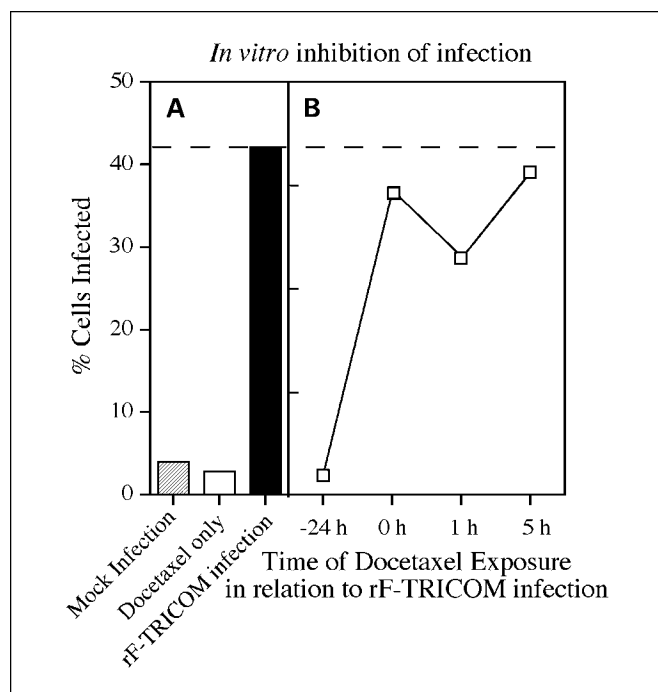


Fig. 4. Docetaxel given before recombinant viral vaccine can inhibit infection or transgene expression of susceptible cells. **A**, murine tumor cells (MC38-CEA⁺) were infected with rF-TRICOM (black bar). **B**, cells were incubated with 1,100 ng/mL (equivalent to clinical dose of 30 mg/m²) of docetaxel either 24 h before infection, concurrent with infection (0 h), or 1 or 5 h after infection (open squares). After 24 h, cells were analyzed for transgene expression (B7-1).

3 weeks after vaccine administration, and cytokine production was measured as described in Materials and Methods. Docetaxel given 4 days after the booster vaccine increased production IFN- γ in response to a CEA-specific peptide compared with mice receiving vaccine alone (Fig. 3C).

Effect of docetaxel on poxviral infection. A previous study (10) on the use of vaccine and taxanes used a WTCV rather than a poxviral vaccine and paclitaxel instead of docetaxel. In that study (10), administration of paclitaxel 1 day before vaccine resulted in enhanced antitumor activity, whereas paclitaxel given 7 days postvaccine had no antitumor effect and even had a detrimental effect on tumor-specific immune response. To investigate these different outcomes, we conducted controlled studies that combined docetaxel with two different types of vaccines: live viral vaccine (rV-CEA/TRICOM and rF-CEA/TRICOM) and a WTCV (irradiated MC38 colon carcinoma cells expressing CEA, given with GM-CSF, as described in Materials and Methods). Docetaxel, like paclitaxel, enhanced CD4⁺ T-cell responses when given 1 day before administration of a WTCV. However, unlike paclitaxel, docetaxel also enhanced CD4⁺ T-cell responses when given 7 days after a WTCV, suggesting that the two taxanes can function differently. Prell et al. reported similar results with docetaxel and a WTCV (9). Interestingly, in the studies reported here using a viral-based vaccine, docetaxel diminished T-cell responses when given 1 day before vaccine, but enhanced T-cell responses when given postvaccine.

To determine why docetaxel has a pronounced detrimental effect when given before a viral-based vaccine but not when given before a WTCV, we explored the possibility that the drug could inhibit viral infection or transgene expression, thus

inhibiting the ability of the virus to induce an immune response. When fresh murine splenocytes were incubated with 1.1 μ g/mL docetaxel—the *in vitro* equivalent of the 30 mg/m² used in many clinical protocols—24 h before administration of rF-TRICOM, expression of the transgene was reduced by 45% to 63%, a significant inhibition of the ability of virus to infect cells or express the transgene (Fig. 4). However, when cells were first incubated with rF-TRICOM and then treated with docetaxel, there was no inhibition of infection or transgene expression (Fig. 4). Results were similar when human cells were substituted for murine splenocytes and rV-TRICOM was used instead of rF-TRICOM (data not shown). These studies thus have implications for the dose scheduling of docetaxel in combination with live viral vaccines.

Increased antitumor activity of docetaxel plus vaccine. Studies were conducted with CEA-Tg mice transplanted with MC38-CEA⁺ carcinoma cells to determine if the optimal dose-scheduling regimen of docetaxel enhances the antitumor effect of vaccine. The vaccine was given in a prime-and-boost regimen using rV-CEA/TRICOM as prime and rF-CEA/TRICOM as boost. Mice were injected s.c. with 3×10^5 MC38-CEA⁺ tumor cells on day 0 and received either no treatment, docetaxel only, vaccine only, or vaccine plus docetaxel ($n = 15$). The latter two groups of mice began vaccine treatment on day 4 posttumor transplant. Mice in the combination group received docetaxel 4 days after the last booster vaccine. Unlike previous studies using taxanes, docetaxel alone had no effect on the growth of this tumor (Fig. 5). Vaccine alone reduced tumor burden in 30% of mice, but the antitumor effect did not reach statistical significance (Fig. 5). The combination of vaccine and docetaxel, however, had a statistically significant antitumor effect compared with no treatment ($P = 0.003$), vaccine alone ($P = 0.036$), or docetaxel alone ($P = 0.030$; Fig. 5 and Table 2). In addition, tumor progression was delayed in >80% of mice receiving a combination of docetaxel and vaccine.

Induction of antigen-cascade responses by vaccine plus docetaxel. Cohorts of tumor-bearing mice from the same groups described above (see Fig. 5) were sacrificed on day 30 posttumor transplant and splenocytes were analyzed for

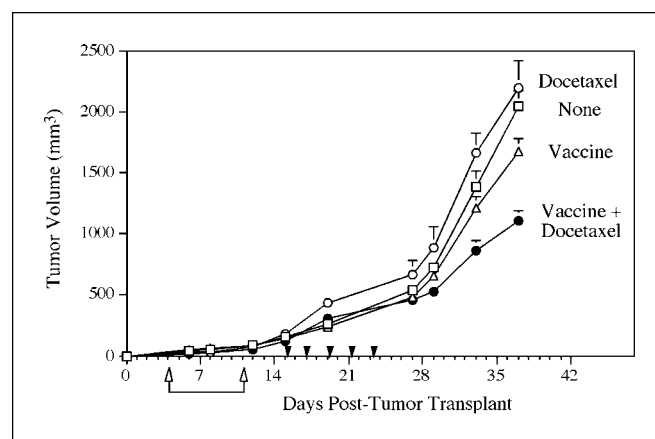


Fig. 5. Increased antitumor activity of vaccine plus docetaxel. **A**, CEA-Tg mice ($n = 15$ per group) were implanted s.c. with MC38-CEA⁺ tumors on day 0. Mice were primed with rV-CEA/TRICOM on day 4 and boosted with rF-CEA/TRICOM on day 11 (open arrows). Both vaccinations were given with rF-GM-CSF. Indicated groups were given 0.5 mg of docetaxel i.p. on days 15, 17, 19, 21, and 23 (closed arrows). Tumor volume was monitored twice a week.

Table 2. Increased antitumor activity of vaccine plus docetaxel

| Treatment | n | Tumor volume (day 37; mm ³) | P = (vs:) | | | |
|---------------------|----|---|--------------|--------------|--------------|---------------------|
| | | | None | Docetaxel | Vaccine | Vaccine + docetaxel |
| None | 13 | 2038 ± 232 | — | 0.76 | 0.12 | 0.003 |
| Docetaxel | 14 | 2199 ± 409 | 0.76 | — | 0.197 | 0.030 |
| Vaccine | 13 | 1575 ± 107 | 0.12 | 0.197 | — | 0.036 |
| Vaccine + docetaxel | 15 | 1206 ± 85 | 0.003 | 0.030 | 0.036 | — |

NOTE: CEA-Tg mice were implanted s.c. with MC38-CEA⁺ tumors on day 0. Mice were either not treated (none), treated with docetaxel alone, vaccine alone, or first vaccinated and then treated with docetaxel (Fig. 5). Shown is average group tumor volumes at day 37. Bold numbers indicate statistical significance ($P < 0.05$).

CEA-specific immune responses. Proliferation of CEA-specific T cells (Fig. 6A) and CEA tetramer⁺ CD8⁺ T cells (Fig. 6B) was clearly enhanced in mice receiving vaccine plus docetaxel versus docetaxel alone or vaccine alone. It is interesting to note that in tumor-bearing mice docetaxel alone had no effect on CD4⁺ T-cell function (Fig. 6A), whereas it had an enhancing effect on CD8⁺ CEA-specific (tetramer) cells. These results are in concordance with the results seen in Fig. 2D and E wherein non-tumor-bearing mice CD8⁺ but not CD4⁺ T-cell function was enhanced by docetaxel.

We have previously shown (23, 24) that CEA vaccine-mediated destruction of tumor cells in this murine model leads to cross-priming and a subsequent antigen cascade, which results in the generation of gp70-specific CD8⁺ T cells postvaccination. gp70 is an endogenous murine leukemia virus antigen expressed in MC38-CEA⁺ tumors (23, 24). In this study, treatment with docetaxel alone also generated more gp70-specific tetramer⁺ T cells than no treatment (Fig. 6C), possibly as a result of some tumor cell destruction and cross-presentation to APCs. However, there was a clear enhancement of gp70-specific tetramer⁺ CD8⁺ T cells in mice receiving vaccine plus docetaxel compared with either agent alone (Fig. 6C).

Discussion

There is growing interest in the potential therapeutic benefits of combining cancer vaccines with traditional therapies, such as surgery, radiation, and/or chemotherapy. Many cancer vaccine clinical trials have been conducted in patients who have failed prior chemotherapy or, in some cases, multiple therapies. von Mehren et al. reported that numerous prior therapies, or initiating vaccine soon after ending chemotherapy, inhibited T cell-mediated response to vaccine (25). Until recently, the general belief was that, if used in combination with a cancer vaccine, chemotherapy would invariably have a negative effect on vaccine-mediated immune responses. However, more recent studies (1, 26, 27) have indicated that chemotherapy can have a positive, negative, or neutral effect on vaccine-mediated immune responses, depending on the tumor stage and the types of immunotherapeutic and chemotherapeutic agents used.

Docetaxel and paclitaxel are both taxanes, a class of chemotherapy drugs whose broad application, mechanism of action, and relatively low toxicity show great promise. Previous studies combining vaccine and chemotherapy have focused on agents, such as doxorubicin and cyclophosphamide, and various vaccine reagents, such as whole-tumor cell vaccines or DNA vaccines. However, there have been few studies on the most

effective sequencing of taxane administration in combination with vaccines (9, 18) and no reports on combining docetaxel and recombinant viral vector vaccines. In a previous study, Machiels et al., using paclitaxel in combination with a GM-CSF-secreting WTCV in a murine tumor model, reported that taxane enhanced vaccine-mediated immune responses when given 1 day before vaccine, but not when given 1 week after vaccine (18). In the studies reported here, we examined the dosing sequence outlined by Machiels et al. and two other schedules to determine the optimal sequence of docetaxel administration when combined with recombinant viral vector vaccines. Vaccine-induced immune responses mediated by a live vector in the studies reported here were enhanced when docetaxel was given after vaccine and diminished when the drug was given before or concurrently with vaccine (Fig. 3). Although the dose sequencing in our studies was different from that of previously reported studies (18), our findings confirm that chemotherapy given at optimal times enhances immune responses to vaccine.

Although Machiels et al. did not report CD4⁺ T-cell responses, they concluded that paclitaxel can increase the pool of IFN- γ -secreting, antigen-specific, T-helper type 1 cells only when given before vaccination (18). In our *in vitro* studies, docetaxel given soon after vaccine inhibited vaccine-mediated immune responses. The simplest explanation for this discrepancy is that Machiels et al. used a different taxane than we did, in combination with a WTCV instead of recombinant viral vectors. Our results suggest that docetaxel may diminish the infectivity or transgene expression of a live viral vector (Fig. 4). Another possible reason for difference could be the dose and schedule used. Taken together, these data suggest that, in a combined chemotherapy/immunotherapy regimen, the type of vaccine used may have a significant effect on the effectiveness of the cancer drug combination therapy.

Prell et al. (9) found that administration of a taxane (in this case docetaxel) either before or after a WTCV enhanced the antitumor efficacy of the vaccine and did not inhibit antigen-specific T-cell responses, resulting in prolonged survival. These data offer further evidence that different taxanes function differently *in vivo* and that findings from studies of paclitaxel are therefore not necessarily applicable to docetaxel.

There are also differences in the models used, moreover, between the study by Prell et al. (9) and the study reported here. For instance, Prell et al. (a) did not evaluate tumor reduction, (b) analyzed the effect of docetaxel on immune response after adoptive transfer of antigen-specific cells and not *de novo* development of T cells, and (c) used a WTCV and not a recombinant viral vector vaccine.

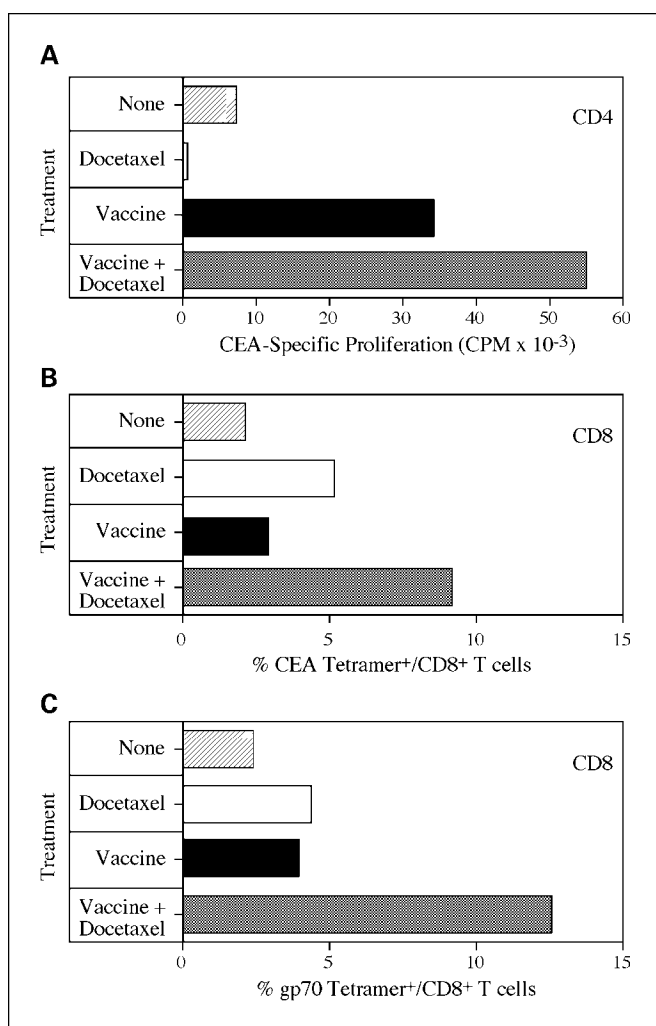


Fig. 6. Antigen-specific responses and antigen cascade after vaccine plus docetaxel. CEA-Tg mice ($n = 3$ per group) were implanted s.c. with MC38-CEA⁺ tumors on day 0. Mice were primed with rV-CEA/TRICOM on day 4 and boosted with rF-CEA/TRICOM on day 11. Both vaccinations were given with rF-GM-CSF. In the indicated groups, mice were given 0.5 mg of docetaxel i.p. on days 15, 17, 19, 21, and 23. **A**, CD4⁺ T-cell responses to CEA. One week after the last dose of docetaxel (day 30), purified CD4⁺ splenic T cells were tested for reactivity to CEA protein (50 μ g/mL) in an *in vitro* lymphoproliferation assay. **B**, CD8⁺ T-cell responses to CEA. One week after the last dose of docetaxel (day 30), splenic T cells were stained for CEA-specific CD8⁺ cells. Specific tetramer binding (control HIV tetramer background subtracted) is depicted. **C**, CD8⁺ T-cell responses to gp70. One week after the last dose of docetaxel (day 30), splenic T cells were stained for gp70 tetramer⁺/CD8⁺ cells. Specific tetramer binding (control HIV tetramer background subtracted) is depicted.

A recent clinical study (28) by Arlen et al. reported prolonged time to progression in crossover patients who received docetaxel after vaccine (6.1 months) compared with patients who received docetaxel and vaccine concurrently (3.2 months). In addition, progression-free survival increased in the crossover group compared with historic controls from the same institution and same patient population who received docetaxel alone (3.7 months; ref. 28). The potential clinical benefit seen in these patients may have been the result of altered gene expression that makes tumors more amenable to immune killing, enhanced immune response (as observed in the non-tumor-bearing mice reported here), or both.

The combination of docetaxel and vaccine in the studies reported here not only enhanced CD4⁺ T-cell responses in

tumor-bearing mice (Fig. 6A) but also led to a broadening of immune responses, as indicated by CD8⁺ T-cell responses to antigens within the tumor but not encoded within the vaccine (Fig. 6C); the combination of docetaxel and vaccine produced a greater antigen cascade than either modality alone. Whether immune response to the antigen cascade is the cause or result of the statistically significant reduction in tumor burden observed in these mice, docetaxel seems to play a role in stimulating or enhancing these immune responses.

Several murine studies of the immunomodulatory effects of taxanes have focused on paclitaxel, which can reportedly mimic the biological effects of lipopolysaccharide by inducing expression of cytokine genes, such as tumor necrosis factor- α , IFN- γ , NO, and IL-1 in macrophages (29–31). In one study, pairing paclitaxel with a priming signal, such as IFN- γ , stimulated macrophages to lyse tumor cells (32). It is generally assumed that docetaxel would produce similar results. In preclinical studies, docetaxel has been shown to mobilize hematopoietic stem cells (33) and to stimulate an influx of T lymphocytes and natural killer cells into the tumor (34). A clinical study in patients with metastatic breast cancer showed that, in humans, paclitaxel and docetaxel can increase IFN- γ , IL-6, and GM-CSF, as well as MLR, natural killer, and lymphokine-activated killer cell activity (35).

As the studies described above suggest, it can be difficult to distinguish between an effect of taxane on immune cells versus its effect on tumor cells and to further distinguish these primary effects from secondary effects brought about by decreased levels of immune inhibitory molecules expressed by shrinking tumor cells. We therefore examined the *in vivo* effects of docetaxel on immune cell populations in splenocytes from non-tumor-bearing mice (Table 1). There was a sharp decline in CD4⁺ and CD8⁺ T cells 1 day posttreatment, but their numbers increased at later time points. CD19⁺ cell levels were down at all time points. Treg numbers remained steady. However, previous studies have shown that chemotherapeutic agents, such as cyclophosphamide, can decrease Treg functionality while leaving their numbers unaffected (36). The observed differences could be chemotherapy specific or related to dose and schedule of the chemotherapeutic administrations. We therefore evaluated Treg function and found no change posttreatment with docetaxel (Fig. 1). We then extended our functionality studies to CD3⁺ T cells. Splenocytes from non-tumor-bearing docetaxel-treated mice produced enhanced levels of IFN- γ in response to CD3 cross-linking compared with splenocytes from untreated mice (Fig. 2). Additional studies determined that IFN- γ production was from CD8⁺ T cells and not from CD4⁺ T cells (Fig. 2D and E). In these studies, proliferation was similar in docetaxel-treated and untreated mice. This result is interesting in light of the fact that in the timing studies, CD4⁺ T cells responded better to the antigen used in the vaccine (Fig. 3A and B); however, T-cell harvest occurred much later in these lymphoproliferation experiments. Our studies show that docetaxel increases IFN- γ secretion in CD8⁺ T cells in response to CD3 cross-linking, although how it does this is unclear at this time.

Docetaxel is used in the therapy of breast, lung, prostate, and stomach cancer and is currently being evaluated clinically for the treatment of esophageal, ovarian, melanoma, and head and neck cancers (37–40). Docetaxel is often used in combination with other chemotherapeutic agents or with radiation, but

there are few reports of its use with cancer vaccines. The results of this study show that, when used with a recombinant viral vector at optimal dose scheduling, docetaxel can enhance both antitumor activity and vaccine-mediated responses to foreign and self-antigens. In the preclinical model used here, docetaxel alone showed no antitumor activity. However, the combination of docetaxel and vaccine increased antigen-specific T-cell responses to both the antigen in the vaccine and tumor-derived cascade antigens. Docetaxel, unlike cyclophosphamide, did not inhibit Treg function. Thus, patients who show no objective clinical response to docetaxel alone may still benefit from the ability of the drug to enhance immune responses and thus potentiate patient benefit from vaccine therapy.

These studies show for the first time that in non-tumor-bearing mice (a) docetaxel modulates CD4⁺, CD8⁺, CD19⁺, natural killer cells, and Treg populations; (b) unlike cyclophosphamide, docetaxel does not inhibit the function of Tregs; and (c) docetaxel enhances CD8⁺ function. These

studies were conducted in non-tumor-bearing mice to rule out indirect evidence of docetaxel on tumor. Studies with tumor-bearing mice show for the first time that (a) administering docetaxel after recombinant poxviral vaccines results in optimal enhancement of vaccine-specific immune responses; (b) the combination of docetaxel and recombinant poxviral vectors is more effective than either agent alone at reducing tumor burden; and (c) the combination of docetaxel and vaccine induces antigen-specific T-cell responses to tumor-derived antigens distinct from the antigen used in the vaccine (antigen cascade). These findings thus have important implications for the combined use of vaccines and chemotherapeutic agents in the clinic.

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Clinical Cancer Research

Combination of Docetaxel and Recombinant Vaccine Enhances T-Cell Responses and Antitumor Activity: Effects of Docetaxel on Immune Enhancement

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