Efficacy of GM-CSF-producing Tumor Vaccine after Docetaxel Chemotherapy in Mice Bearing Established Lewis Lung Carcinoma

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Summary: In this report, we evaluated the efficacy of a GM-CSF-producing tumor vaccine given before and after docetaxel in mice bearing established lung tumors. Mice bearing established 3LL tumors were treated with docetaxel and tumor vaccines transduced with either control or GM-CSF adenoviral vectors. Docetaxel (5–20 mg/kg) treatment alone had only a minimal effect on growth of established 3LL tumors in vivo, although docetaxel was cytotoxic to 3LL cells in vitro. When mice bearing established 3LL tumors were pretreated with docetaxel followed by vaccination with irradiated GM-CSF-transduced 3LL tumor cells, significant tumor regression and prolonged survival were observed compared with chemotherapy alone. Delaying docetaxel treatment until after tumor vaccination abrogated the vaccine's anti-tumor effects. Mice that survived treatment were able to resist a lethal rechallenge of 3LL tumor cells. Memory CTL specific for an epitope (MUT-1) derived from 3LL were detected in surviving mice. Docetaxel induced a mild lymphodepletion in mice, both CD4 and CD8 subsets were reduced in LN and spleens. Interestingly, docetaxel also diminished the number of memory $CD8^+$ T cells ($CD122^+$) and possible $CD4^+CD25^+Foxp3^+$ natural T_{reg} cells. Docetaxel treatment did not affect antigen-driven proliferation of naive T cells but significantly promoted survival of activated T cells. Thus, augmentation of vaccine induced antitumor immunity in docetaxel-treated mice primarily due to the enhanced survival of antigen-experienced T cells.

Key Words: GM-CSF, docetaxel, Lewis lung carcinoma, vaccine, T cells

(*J Immunother* 2006;29:367–380)

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This study was supported by grants from Cell Genesys, Inc., American Cancer Society (RSG LIB-105800), and NIH (1R01-CA107243-01).

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INTRODUCTION

Docetaxel is highly active for human cancers. Approximately 30% of advanced NSCLC patients will respond to docetaxel-based treatment. A moderate survival benefit has been reported, but less than 15% of patients survive more than two years.[1,2] Clearly, better therapies are needed for the one million patients diagnosed with lung cancer each year worldwide. Immunotherapy approaches has been pursued as an alternative or adjunctive modality to chemotherapy. Because cancer patients' immune systems may be suppressed by their disease and chemotherapy may further suppress immune function, the utility of cancer vaccines in patients with advanced diseases is very limited. However, recent publications in experimental breast cancer models suggest that a combination of paclitaxel-based chemotherapy and a tumor vaccine could improve survival of tumor-bearing mice. The survival benefit was attributed to stronger antitumor responses developed in mice that received paclitaxel followed by tumor vaccination.[3,4] The strategy of active immunization with a tumor vaccine following chemotherapy could lead to prolonged survival and potentially cure of mice bearing established tumors. Despite many beneficial outcomes were observed in both pre-clinical and clinical studies with the combination of chemotherapy and immunotherapy, the mechanisms underlay these observations are complex and vary depending on the nature of chemotherapeutic agents or tumor models under studies.[5] Cyclophosphamide (CY) is the best studied cytotoxic agent used in combination with tumor vaccines and the immune potentiation effect of CY was attributed to the reduction of the number and function of regulatory T cells[3,6,7] and the recruitment of latent pools of self-reactive high-avidity $CD8^+$ T cells to the antitumor immune response.[8] Taxane, such docetaxel or paclitaxel, is widely used chemotherapy agent for multiple malignancies, including non small cell lung cancers. We reported here that the combination of docetaxel and a GM-CSF secreting tumor vaccine had anti-tumor activity in mice bearing established 3LL Lewis lung tumors. Vaccination after docetaxel treatment produced the optimal efficacy. Because the tumor rejection antigen, mutated connexin 37 and its epitope MUT-1 (FEQNTAQP) have been characterized for the

3LL tumor model,[9] we also examined whether MUT-1-specific memory CTL were generated in mice that received combination therapy. In addition, the contribution of CD4 and CD8 T cells to tumor regression was assessed by T-cell subset depletion experiments. Long-term protective immunity was assessed in mice cured by the combination therapy. The effect of docetaxel on different T-cell subsets, such as naive, memory, and T_{reg} T-cell populations was also determined. Depletion of regulatory T cells could promote the antigen-driven T-cell proliferation by removing their suppressive activity on dendritic cells or activated T cells. Reduced number of preexisting memory CD8 T cells after docetaxel treatment could promote the survival of vaccine induced memory T cells by removing the competition of "T-cell space" or "cytokine sink".[10,11] We have examined the consequence of docetaxel treatment on the populations of $CD4^+$ $CD25^+FoxP3^+$ regulatory T cells and $CD8^+CD122^+$ memory T cells. Recently, we have successfully use a TCR transgenic model system to track T-cell fate in lymphodepleted mice after vaccination.[12] Using this model system, we determined the effect of docetaxel treatment on the proliferation and survival of adoptively transferred melanoma-specific naive TCR transgenic T cells after vaccination with peptide-pulsed dendritic cells. The data presented in this study provides a strong impetus to design new vaccine trials in cancer patients will be treated with docetaxel and insight into the mechanisms how docetaxel might augment vaccine-induced T-cell mediated antitumor immunity.

MATERIALS AND METHODS

Mice and Tumor Cell Lines

Female C57BL/6j (H-2^b) (B6) or congenic B6.SJL-Ptprc mice (B6/CD45.1) (Jackson Laboratory, ME) aged 8–12 weeks were used for all experiments. Pmel-1 mice were originally obtained from Dr Restifo, National Cancer Institute, NIH and breaded in our facility. Most $CD8^+$ T cells of pmel-1 mice express a Vα1/Vβ13-transgenic TCR that recognizes an epitope of pmel-17 corresponding to amino acids 25–33 of gp100 presented by H2-D^b MHC class I molecules. Recognized principles of laboratory animal care (Guide for the Care and Use of Laboratory Animals, National Research Council, 1996) were followed. All animal protocols were approved by the Earle A. Chiles Research Institute Animal Care and Use Committee. Lewis lung cancer cell line (3LL), a gift from Dr. Robert H. Wiltrout, NIH, was derived from a spontaneous lung tumor of C57BL/6 mice. 3LL-TR40 is a docetaxel resistant cell line generated by in vitro selection with 40 nM docetaxel. Tumor cell lines were cultured in complete medium (CM), RPMI 1640 (Bio-Whittaker, Walkersville, MD) supplemented with 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 μg/ml gentamicin sulfate, 50 μM 2-ME (Aldrich, Milwaukee, WI), and 10% FBS (Life Technologies, Grand Island, NY). Tumor cells were splited two to three times per week and maintained in T-150 culture flasks. The MUT-1 octapeptide (FEQNTAQP), a H-$2K^b$–restricted epitope from the mutated connexin protein found in 3LL tumor cells and gp9 (KVPRNQDWL) peptide were custom synthesized by Invitrogen Co. (Carlsbad, CA).

Vaccine Preparation

2×10^6 3LL cells were infected with either control adenoviral vector (Ad-Null) or GM-CSF adenoviral vector (Ad-GM-CSF) virus at MOI of 100 in CM at 37°C for 1 hour (3LL-Null or 3LL-GM). After infection, cells were irradiated (10,000 rads) and frozen in CM with 10% DMSO in a 20×10^6 cells/ml aliquots in liquid N_2. To determine GM-CSF production, cells were thawed and cultured in CM for 24 hours. The GM-CSF concentration in the cell supernatant was determined by ELISA using a mouse GM-CSF kit (B-D Pharmingen, CA).

Immunogenecity of 3LL

On day 0, mice were vaccinated by subcutaneous injection of 2×10^6 of the following irradiated tumor cells: 3LL, 3LL-null or 3LL-GM. Fourteen days later, immunized mice were challenged with either 2×10^4 or 10^5 live 3LL cells by s.c. injection. Tumor growth was measured with digital calipers every two or three days. Tumor area was calculated by multiplying the largest tumor dimension with the smallest dimension.

Sensitivity of 3LL Tumor Cells to Docetaxel In Vitro and In Vivo

Docetaxel (Aventis), was obtained commercially and diluted for injection with PBS to 0.5 mg/ml and frozen as 1 mL aliquots at −20°C. Docetaxel was further diluted in CM to the indicated concentration and added to 3LL cultured in 6-well plates at $0.2–0.5 \times 10^6$ cells per ml. Both adherent and non-adherent cells were harvested after 24 or 48 hours. Cell viability was determined by PI staining and FACS analysis. For in vivo sensitivity, 2×10^4 cultured 3LL cells were injected into the right flank of C57BL/6 mice on day 0. On days 3 and 13, mice were treated with 5, 10, or 25 mg/Kg docetaxel by i.p. administration. Tumor growth was measured as described above.

Combination Therapy

2×10^4 cultured 3LL cells were inoculated into both flanks of C57BL/6 mice on day 0. Vaccinations were given via s.c. injection of thawed cells at both flanks of mice (a total of 2×10^6 cells) on day 6 and day 16. Mice were divided into different groups that received docetaxel treatment (5 mg/kg, i.p.) either before or after vaccination (5–10 mice per group). For pre-vaccination groups, docetaxel was given via i.p. injection on days 4 and 14 post tumor injection. For post-vaccinations, docetaxel were given on day 8, 10, 12, 18, 20, and 22 post tumor injection. Three doses of docetaxel for the post vaccination regimen whereas a single dose of docetaxel treatment of the pre-vaccination was chosen in our experimental protocol simply because three doses of docetaxel started

on day 4 suppressed tumor growth completely without vaccination. Tumor growth was measured as described above.

Long-term Protection Experiments

Survived mice from several independent experiments that received the same treatment were pooled and re-challenged with 2×10^4 live 3LL cells subcutaneously. Naive mice were also injected with the same number of tumor cells on the same day serving as controls. Tumor growth was measured as described above.

In Vivo Cell Depletion Study

MAb to CD4 and CD8 were purified from the culture supernatants of the GK1.5 (anti-CD4, ATCC, TIB 207) and lyt2.2 (anti-CD8, ATCC, TIB 210) hybridomas by ammonium sulfate precipitation and ion-exchange chromatography. In vivo CD4 and CD8 depletion were done by injection i.p with 200 μg of anti-CD4 or 25 μg of anti-CD8 5 days after 3LL tumor inoculation. The antibody injection was repeated every 3 days thereafter during the experiment to maintain the depletion of CD4 and CD8 cells. These doses have been previously shown to deplete more than 90% of corresponding T-cell subset one day after injection. Purified rat Ig (Sigma-Aldrich I-4131, St. Louis, MO) was used as the control antibody (200 μg per injection).

In Vivo CTL Assay

An in vivo CTL assay was used to determine whether MUT-1 peptide-specific memory CTL were generated in mice after vaccination, which has been described previously.[12] Briefly, naive C57BL/6 splenocytes were split into two populations to prepare the control and specific target populations. One population was pulsed with 10 μg/ml MUT-1 peptide, incubated at 37°C for 1 hour, then labeled with a high concentration of CFSE (20 μM) (Molecular Probes, OR) at room temperature for 5 minutes ($CFSE^{Hi}$). As a control target population, naive spleen cells received no peptide pulsing were labeled with 0.5 μM CFSE ($CFSE^{Lo}$). After washing, an equal number of cells from each population were mixed together, and a total of 10^7 cells in 200 μL PBS was injected into the tail vein of naive and surviving mice. Heparinized blood samples were collected from the retro-orbital sinus of mice 3 or 5 days after injection of target cells. After RBC lysis and washing, cells were analyzed by FACS for fluorescence intensity using CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA). Up to 1×10^4 CFSE-positive cells were collected for analysis. To calculate specific lysis, the following formula was used: ratio = (percentage $CFSE^{lo}$/percentage $CFSE^{hi}$). Percentage specific lysis = [1 − (ratio unprimed/ratio primed) × 100].

Flow Cytometry Analysis of T-cell Subsets After Docetaxel Treatment

To determine the effect of docetaxel treatment on different T-cell subsets in naive mice, 2–5 mice received three daily i.p. injection of 5 mg/kg or control PBS. LN, spleens, and blood were collected two days post docetaxel injection and stained with FITC-labeled anti-CD4, CD8, PE-labeled anti-CD122, CD25 antibodies (ebiosciences, CA) after blocking Fc receptors with purified antibody against Fc receptors (2.4G2, ATCC). To enumerate $CD4^+CD25^+FoxP3^+$ regulatory T cell population, cells were first stained with anti-CD4 and CD25 antibodies, then fixed and permeabilized before they were stained with APC-labeled anti-FoxP3 antibody (ebioscience, CA). After washing, cells were run on a FCAScalibor and analyzed with Cellquest software (B-D bioscience, San Jose, CA). A minimal 10,000 events from gated live cells were collected.

Effect of Docetaxel Treatment on the Proliferation and Survival of Antigen-Specific Naive T cells

To gain detailed insight into T-cell immune responses in docetaxel-treated mice, a widely approache that using adoptive transfer of naive TCR transgenic T cells was adapted to track the T-cell proliferation and fate after vaccination in docetaxel-treated mice.[12] Congenic B6/CD45.1 mice were adoptive transferred with 5×10^6 naive spleen cells from pmel-1 transgenic mice[12] and treated with three daily i.p. injections of docetaxel (5 mg/kg), mice were then subsequently vaccinated with 2×10^6 peptide pulsed dendritic cells s.c. two days after the last docetaxel treatment. Naive pmel-1 spleen cells were labeled with 5 μM CFSE (Molecular Probe, Eugene, OR) before the adoptive transfer. Dendritic cells were generated from bone marrow of naive B6 mice with GM-CSF and pulsed with 10 μg/ml peptide drived from gp100 (KVPRNQDWL) for 2 hours before s.c. injection.[12] LN and spleens were harvested on day 7 post DC vaccination. CFSE profile of pmel-1 T cells was analyzed by flow cytometry after gating on pmel-1 T cells with antibodies against CD8 and CD45.2. To investigate whether docetaxel affect the survival of activated pmel-1 T cells, we also examined the number and percentage of pmel-1 in the total $CD8^+$ T-cell population of blood collected from mice a week 1, 2, or 3 post DC vaccination with or without docetaxel treatment. The absolute number of pmel-1 T cells in blood was enumerated by adding a known number of fluorescent microbeads (Bechman Coulter, Fullerton, CA) and calculated based on the ratio of pmel-1 T cells and beads as determined by flow cytometry.

RESULTS

Immunogenicity of 3LL Tumor Cells Before and After Transduction With Recombinant Adenoviral Vectors

Previous reports indicated that 3LL is a poorly immunogenic tumor.[9] Because 3LL tumor cells lines have been in culture for many years, its immunogenicity could change over time. To determine the immunogenicity of 3LL tumor cells before and after transduction with adenoviral vectors, naive C57BL/6 mice were vaccinated

with 2×10^6 irradiated 3LL tumor cells, or irradiated 3LL tumor cells that were transduced with either adenovirus control vector (3LL-Null) or vector containing mouse GM-CSF gene (3LL-GM). Mice were challenged 14 days later with live 2×10^4 or 1×10^5 3LL tumor cells. All five control naive mice that did not receive vaccination developed tumors at both challenge doses, whereas mice vaccinated with irradiated 3LL, 3LL-null and 3LL-GM groups were completely protected from tumor challenge at a dose of 2×10^4. At the higher challenge dose, 1×10^5, 100% mice vaccinated with irradiated 3LL remained tumor-free and 80% mice vaccinated with Ad-Null or Ad-GM remained tumor free (4/5 mice survive) (Table 1). These results indicate that the 3LL tumor cells used in our study were immunogenic. The increased immunogenicity compared with prior report may reflect genetic drifting of inbred mice or changes of 3LL tumor cells after propagation for a long period of time. Nevertheless, irradiated 3LL, 3LL-Null or 3LL-GM induced a strong protective immune response in naive mice.

In Vitro and In Vivo Sensitivity of 3LL to Docetaxel

To determine whether 3LL was sensitive to docetaxel cytotoxicity, 2×10^5 3LL tumor cells were exposed to various concentrations of docetaxel in vitro. Both adherent and non-adherent cells were harvested after 24 or 48 hours incubation. Cell viability was determined by PI staining and flow cytometry analysis. 3LL tumor cells treated with docetaxel in vitro were efficiently killed at nanomolar concentrations (Fig. 1A) with an EC_{50} of approximately 40 nM. Long term exposure to docetaxel could induce resistance in 3LL tumor cells. 3LL-TR40, a docetaxel resistant line, was derived after continuous exposure to 40 nM docetaxel (Fig. 1A). To determine the effect of docetaxel on 3LL tumor growth in vivo, groups of mice were injected with 5, 10 and 25 mg/Kg docetaxel by i.p. administration on days 3 and 13 after s.c. inoculation of 2×10^4 tumor cells. All mice that received no or 5 mg/Kg docetaxel developed tumor, whereas 1 of 7 mice treated with 10 mg/Kg docetaxel and 2 of 8 mice treated with 25 mg/Kg docetaxel did not develop tumors, respectively (Fig. 1B). Statistical analysis revealed no significant difference between these 4 groups. Thus, docetaxel at the concentrations and schedule tested failed to cause significant regression of established tumor.

TABLE 1. Immunogenicity of 3LL Tumor Cell Line

Tumor Vaccine	Challenge Dose	Mice With Tumor Growth/ Total Number of Mice Challenged
None	2×10^4	5/5
	1×10^5	5/5
3LL	2×10^4	0/5
	1×10^5	0/5
3LL-Null	2×10^4	0/5
	1×10^5	0/5
3LL-GM	2×10^4	1/5
	1×10^5	1/5

Cultured 3LL tumor cells were transduced with either adenovirus null (3LL-Null) or GM-CSF (3LL-GM) vectors and cultured overnight before they were irradiated (10,000 rads) and frozen in liquid N_2. Two million transduced tumor cells were injected subcutaneously into C57BL/6 mice on day 0. Irradiated nontransduced 3LL tumor cells were included as vaccine control. On day 14, mice were challenged subcutaneously with 1×10^5 or 2×10^4 live 3LL tumor cells. Tumor growth was monitored from 5 days post-tumor challenge until day 60. Each group consisted of five mice; number of mice that developed tumors over the number of mice per group is indicated.

GM-CSF Production is Critical for the Vaccine Efficacy of Established Tumor After Chemotherapy

Because both low and high doses of docetaxel failed to exhibit significant antitumor activity, the lowest dose (5 mg/kg) was used in the following experiments. We reasoned that the impact of tumor vaccination may be more discernible when chemotherapy alone is ineffective. First we determined whether the combination of 5 mg/kg docetaxel before tumor vaccine would cause tumor regression. Although we did not observe any improvement of protection by vaccination with 3LL tumor cells infected with Ad-GM-CSF over that induced by tumor cells alone or infected with Ad-Null (Table 1), we postulated that GM-CSF production could improve vaccine efficacy in mice with established tumors. Experimental results confirmed this postulation (Fig. 2). Complete regression of pre-existing 3LL tumors was only observed in mice that received docetaxel injection and 3LL-GM vaccination (14 of 46 mice) (Fig. 2B). None of six mice that received 3LL-Null experienced tumor regression. Survival of these mice was not significantly different from either control mice or mice treated with docetaxel alone. Median survival of mice that received docetaxel and 3LL-GM vaccines was 42 days, compared with survival of 26, 29, 31 days for control mice, mice treated with docetaxel alone or mice treated with docetaxel prior to vaccination with 3LL-Null tumor cells. Thus, unlike in the prophylactic model, the GM-CSF production by tumor vaccine was critical for regression of established tumors.

Docetaxel Treatment Prior to Vaccination was Critical for Vaccine-Induced Tumor Regression

To determine when should docetaxel be given to mice related to the time of vaccination, vaccination was performed both before and after docetaxel treatment in same experiments. "Chemo pre" refers to mice that were treated with docetaxel and then vaccinated with irradiated 3LL-GM, whereas "Chemo post" refers to mice vaccinated with 3LL-GM and then treated with docetaxel. The experimental scheme is depicted in Figure 3A. Preliminary results indicated that a single dose of docetaxel before vaccination was effective in the 3LL model, whereas treatment with three daily doses of docetaxel after tumor vaccination was also effective in B16F10 melanoma model (Prell R Mansucript in preparation). Thus, we chose to compare these two different sequential combinations in 3LL model. We only used one dose of docetaxel for "Chemo pre" group because three injections of docetaxel suppressed tumor

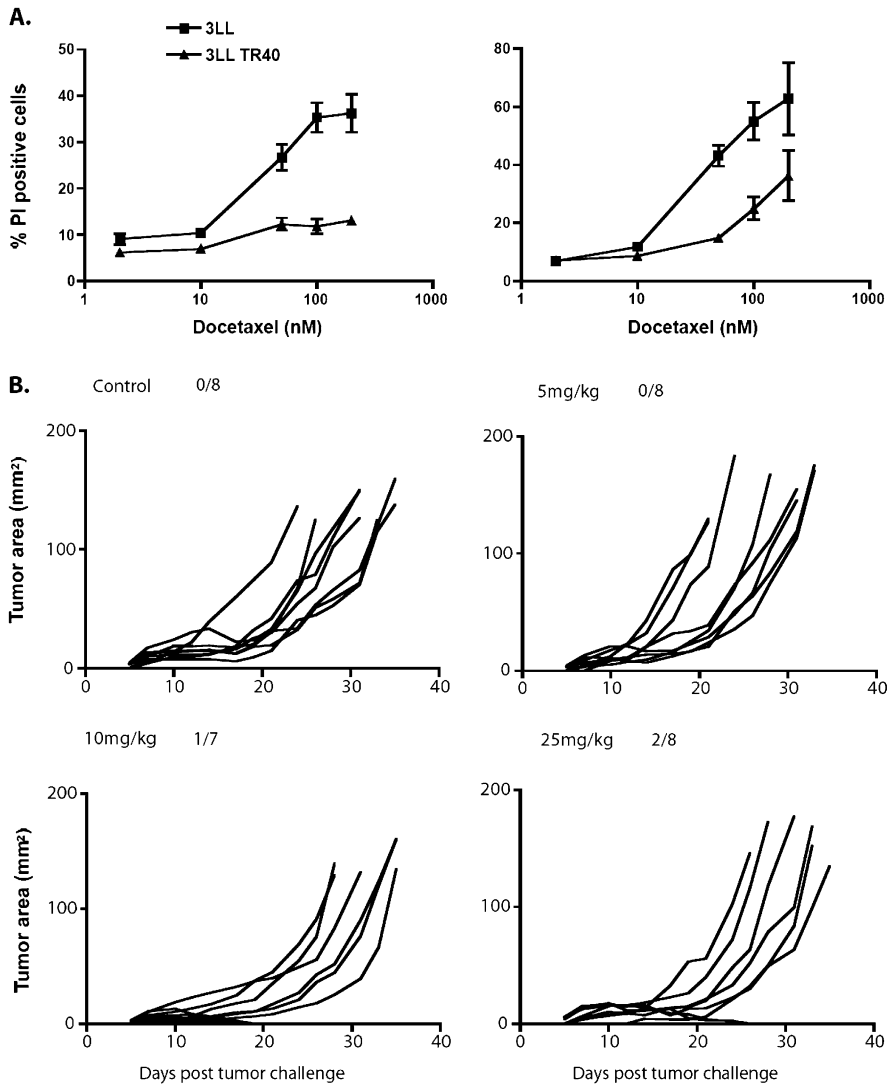

FIGURE 1. Sensitivity of 3LL tumor cells to docetaxel in vitro and in vivo. (A) In vitro sensitivity. 2–5 $\times$ 10^5 3LL and docetaxel resistant line 3LL-TR40 were co-cultured with various concentration of docetaxel in 6-well plates. Both adherent and nonadherent cells were harvested after 24 (left panel) or 48 right panel) hours incubation. Cell viability was determined by PI staining and FACS analysis. (B) In vivo sensitivity. Mice were injected i.p. with 5, 10, or 25 mg/Kg docetaxel on days 3 and 13, following inoculation of 2 $\times$ 10^4 3LL tumor cells on day 0. Tumor area was measured after docetaxel treatment with a digital caliper.

growth completely without vaccination when docetaxel was given on day 4 post tumor inoculation (data not shown). Tumor-bearing mice treated with docetaxel before vaccination showed significant tumor regression (Fig. 3B). Seven of 10 mice initially developed palpable tumor that eventually regressed completely. However, docetaxel treatment after vaccination failed to mediate tumor regression, all 10 mice grew tumors. These results demonstrated that administration of docetaxel before tumor vaccination mediated dramatic tumor regression with complete regression in 70% of mice. Because docetaxel treatment after vaccine was completely ineffective, vaccines should be given shortly after but not before docetaxel treatment.

$CD8^+$ T cells are the Major Effector T cells Whereas $CD4^+$ T cells might be Suppressive

To determine the role of $CD4^+$ and $CD8^+$ T cells in the generation of anti-tumor immune responses, $CD4^+$ and $CD8^+$ T cells were depleted by anti-CD4 or CD8 antibody injections before and after vaccination. Depletion of $CD8^+$ T cells, or both $CD4^+$ and $CD8^+$ T cells completely abolished the efficacy of docetaxel and the vaccine in combination (Fig. 4). All mice with CD8 depletion rapidly developed tumors with a kinetics faster than observed in naive mice. Seven of 10 mice (70%) that received combination therapy and a control antibody developed tumor. Surprisingly, all mice that received combination therapy and anti-CD4 antibody treatment had complete tumor regression, indicating that $CD4^+$ T cells were not required and may function as regulatory T cells that suppress antitumor immune responses in this model (Fig. 4). Taken together, these results suggested that $CD8^+$ T cells were the major effector T cells that mediated tumor regression.

Long-Term Survivors Resisted a Secondary Tumor Challenge

To determine whether mice cured by combination therapy developed specific antitumor memory responses,

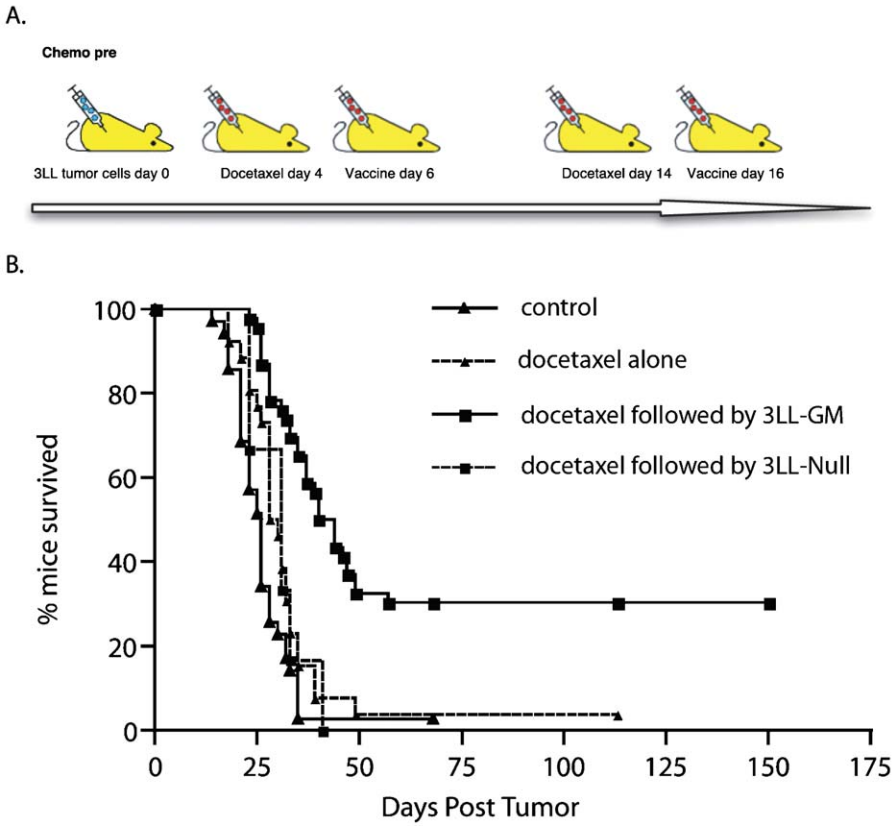

FIGURE 2. Regression of pre-existing 3LL tumors after treatment with docetaxel and 3LLGM vaccine. (A) Schema. (B) Mice were inoculated subcutaneously with 2×10^4 3LL tumor cells at day 0, followed by no treatment (n = 26), or docetaxel alone (5 mg/kg) at day 4 (n = 26), docetaxel at day 4 followed by vaccination with 3LL-Null tumor cells at day 6 (n = 6), or docetaxel at day 4 followed by vaccination with 3LL-GM tumor cells at day 6 (n = 46). A second cycle of docetaxel and vaccination was repeated once at day 14 and day 16 respectively. A significant difference was found between the docetaxel pre plus 3LL-GM group and all other groups ($P < 0.05$).

survived mice that were treated with docetaxel and vaccine with or without CD4-depletion were rechallenged with 2×10^4 live 3LL tumor cells. Tumor-challenged naive mice were served as the control. Mice cured by the initial therapy completely resisted a lethal tumor challenge, indicating that a long-term memory response was developed in these CD4-independent mice and memory persisted as long as 191 days after tumor inoculation (Table 2).

Memory CTL in Long-Term Survivors

To measure memory CTL response in survival mice, an in vivo CTL assay was adapted to measure CTL activity directly in mice after treatment. Naive spleen cells were used as the targets after loading with MUT-1 peptide and labeling with CFSE at a high concentration (5 μM). Naive spleen cells without peptide loading were labeled with low levels of CFSE (0.5 μM) and used as the control targets. The proportional disappearance of peptide-loaded $CFSE^{high}$ cells over $CFSE^{low}$ cells without MUT-1 peptide indicates the level of peptide-specific in vivo cytotoxicity. Labeled cells were injected into mice at day 20 after initial tumor injection and the relative number of $CFSE^{high}$ and $CFSE^{low}$ cells in blood at day 23 were determined by flow cytometry (Fig. 5). Compared with naive mice, 2.78% and 14.69% of MUT-1 pulsed spleens cells were killed in mice bearing 3LL tumor cells that received no or docetaxel treatment respectively. Docetaxel treatment might increase cross-priming of MUT-1 specific CTL in mice bearing 3LL tumors. However, vaccination with 3LL-GM tumor cells greatly increased MUT-1 specific killing whether or not in combination with docetaxel. 52% of MUT-1 loaded cells were eliminated in mice received only 3LL-GM vaccines and 41% killing was observed in mice that received docetaxel plus the 3LL-GM vaccine (Fig. 5). Thus, surviving mice treated with either the 3LL-GM vaccine alone or vaccine in combination with docetaxel developed peptide-specific memory CTL. Pretreatment of mice with docetaxel might increase cross-priming of CTL by growing 3LL tumor cells, docetaxel treatment before vaccination with 3LL-GM vaccination had no significant effect on the peptide-specific CTL responses induced by 3LL-GM vaccines.

Docetaxel Treatment Reduced Total Number of T cells as well as $CD4^+CD25^+Foxp3^+$ Regulatory and $CD8^+CD122^+$ Memory T cell Subsets

To examine whether docetaxel treatment induced lymphopenia in mice, the number of $CD4^+$ and $CD8^+$ T cells were enumerated from both LN and spleens of mice two days after i.p. injection of PBS or docetaxel (5 mg/kg). The number of $CD8^+$ or $CD4^+$ T cells in LN and spleens of mice received docetaxel treatment was significantly reduced compared with mice received PBS injection (Figs. 6A, D). A greater reduction was observed in LN compared with spleens, both numbers of $CD4^+$ and $CD8^+$ T cells in mice treated with docetaxel were less than

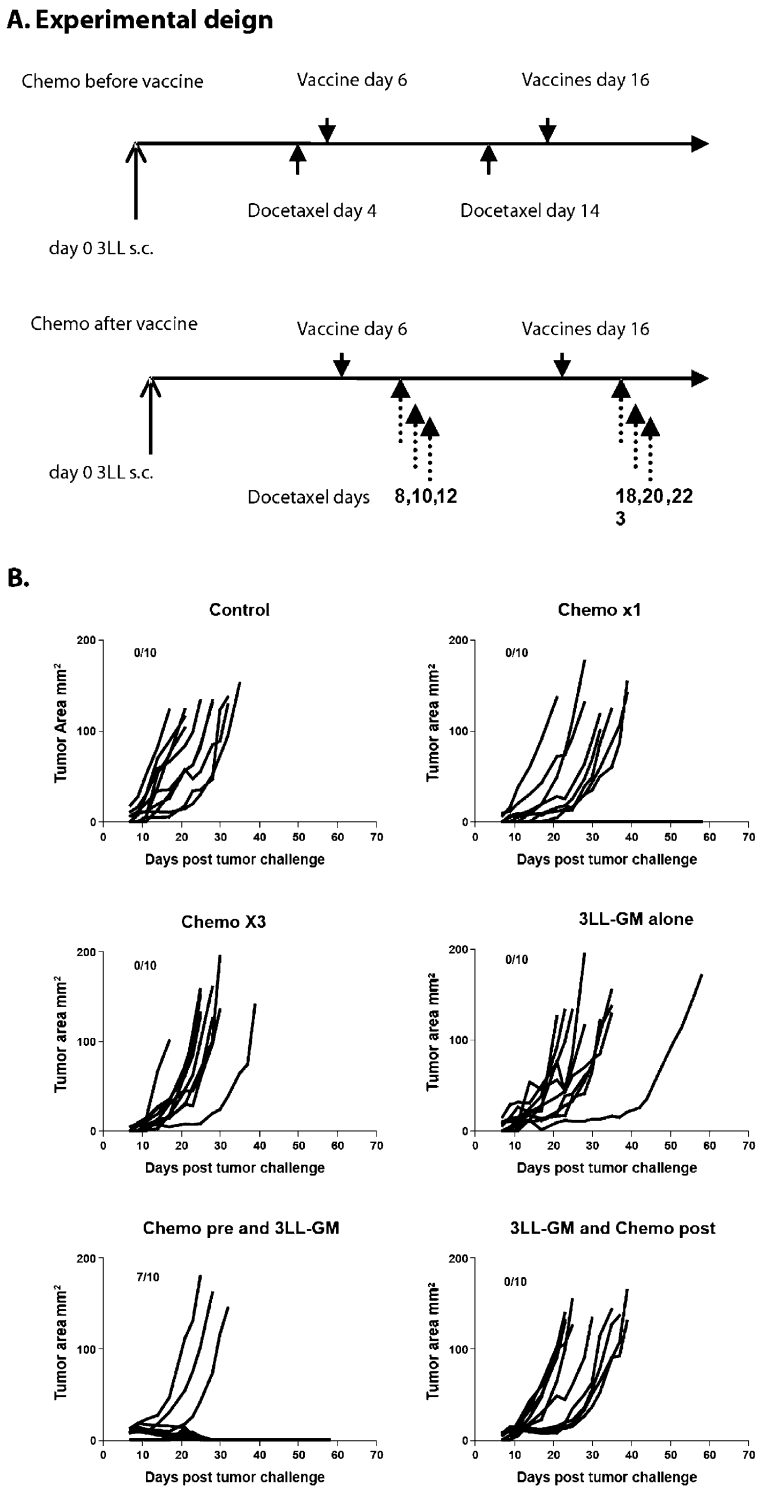

FIGURE 3. Optional combination of chemotherapy and immunotherapy. Experiment Scheme (A): The days for vaccine injection are listed above the horizontal arrows, whereas the docetaxel injections are listed below. The solid upward arrows represent docetaxel regimen in pre vaccine setting, whereas the dotted arrows indicated the post vaccine chemotherapy regimen. Sixty mice were inoculated subcutaneously with 2×10^4 3LL tumor cells at day 0. At day 4, mice were randomized and divided into six different groups (n = 10 each). One group received no treatment (Control). A second group of mice received 5 mg/Kg docetaxel at days 4 and 14 and tumor vaccine at days 6 and 16 (Chemo × 1). Third group of mice received 5 mg/kg docetaxel at days 8, 10, 12, 18, 20, 22 (chemo 3x). A fourth group of mice received 3LL-GM tumor vaccines at days 6 and 16 (3LL-GM alone). A fifth group received docetaxel as in the third group plus 3LL-GM at days 6 and 16 (Chemo pre plus 3LL-GM). The last group of mice received 3LL-GM tumor vaccines at days 6 and 16 plus docetaxel at days 8, 10, 12, 18, 20, 22 (3LL-GM and Chemo post). The numbers on top left indicated the number of mice that did not develop tumor or experienced complete regression over total number of mice.

half of that in PBS-treated mice, whereas the reduction of both T-cell subsets was less than two fold in spleens (Figs. 6A, D). We also examined the effect of docetaxel on the number of preexisting memory $CD8^+$ T cells of naive mice, a similar degree of reduction of $CD8^+CD122^+$ T cells were observed in either LN or spleens two days after docetaxel treatment as seen with total population of $CD8^+$ T cells (Fig. 6B). This result indicates that docetaxel may exhibit a similar cytotoxicity against naive or memory $CD8^+$ T cells. This notion was further supported by the observation that no difference in the frequency of $CD122^+$ $CD8^+$ T cells were found in blood

TABLE 2. CD4-independent Antitumor Immunity in Mice Treated With Chemotherapy and Vaccine

Treatment	CD4 Depletion	Tumor-free Mice/ Total Number of Mice Challenged
None	None	0/5
Chemo and Vaccine	None	6/6
Chemo and Vaccine	Yes	10/10

Naive mice were inoculated subcutaneously with 2×10^4 3LL tumor cells at day 0. At day 4 and 14, mice were treated with 5 mg/kg docetaxel followed by 3LL-GM vaccine at day 6 and 16. The CD4 depletion was done at day 4, 7, 14, and 17. At day 49, mice were rechallenged 2×10^4 3LL tumor cells. As control, age-matched naïve mice were also challenged with the same number of tumor cells. Protected mice were survived at least 60 days after second tumor challenge.

obtained from mice treated with either PBS or docetaxel (Fig. 6C).

Studies on the mechanisms of the CY and vaccine combination demonstrated that reduction of the number and functions of $CD4^+CD25^+$ regulatory subset of T cells played a critical role for its immune potentiating effect.[6–8] Whether docetaxel treatment could also result in deletion of regulatory T cells was investigated next. A general reduction of $CD4^+$ T cells was observed in LN and spleens of mice treated with docetaxel compared with mice injected with PBS (Fig. 6E). As seen with $CD8^+$ T cells, reduction of LN $CD4^+$ T cells was greater than that of splenic $CD4^+$ T cells. Beside its expression on regulatory T cells, CD25 is also expressed on recently activated T cells. To specifically identify the regulatory T-cell population, a more specific marker for regulatory T cells, Foxp3,[13] was included in the staining protocols in addition to CD25. Cells were first stained with antibodies to CD4 and CD25 and then permeabilized before they were stained with anti-Foxp3 antibody intracellularly (Fig. 6). As shown in Figure 5D and 5E, a reduction of $CD4^+CD25^+Foxp3^+$ T cells was also found in both LN, spleens, and blood from mice treated with docetaxel.

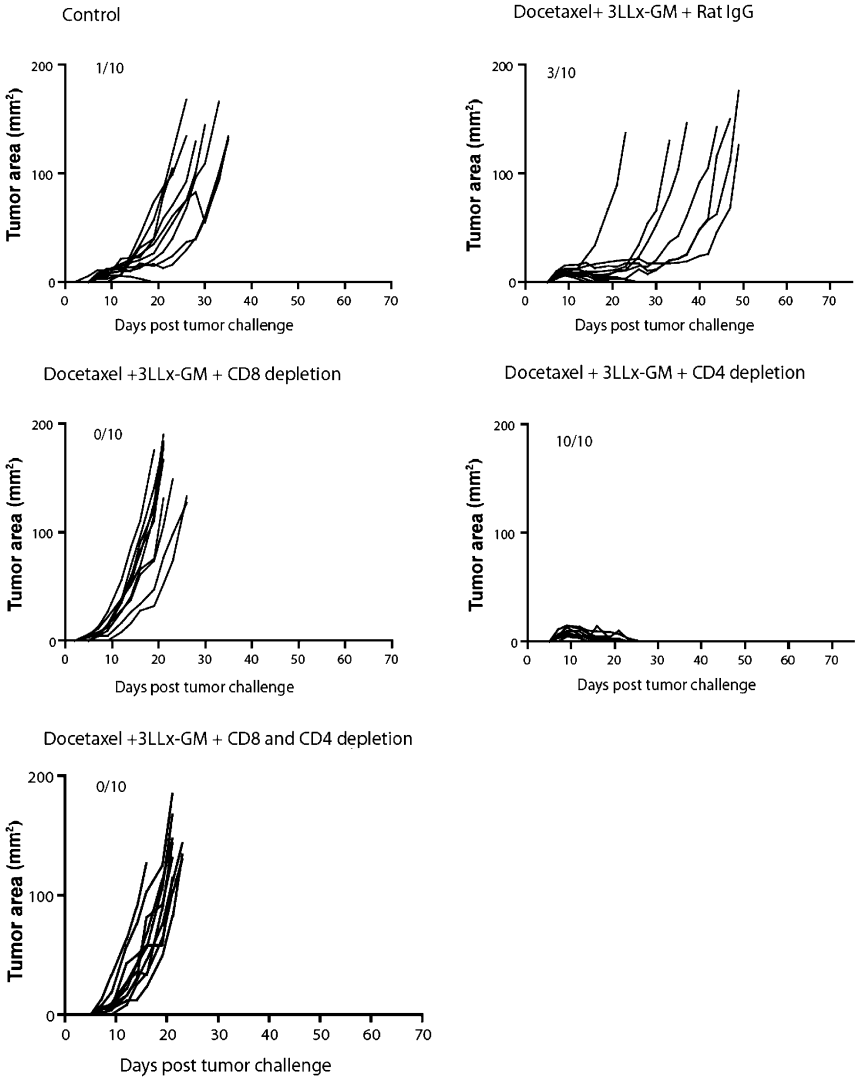

FIGURE 4. Detection of MUT-1 peptide-specific CTL in mice after combination therapy with in vivo CTL assays. 10×10^6 naive splenocytes pulsed with 10 μg/ml MUT-1 peptide and then labeled with 20 mM CFSE (marked as High) were mixed with 10×10^6 naive splenocytes labeled with 5 mM CFSE (marked as Low). The mixed population were injected i.v. followed by 20 days of tumor inoculation. Peripheral lymphocytes were harvested after 3 days. At least 30,000 live cell events were collected by forward and side scatter gating from either CFSE high or CFSE Low population and data were analyzed with CellQuest software. Percentage of lysis were calculated as the formula: % lysis = [1 − (naive ratio/survival ratio)] × 100. Experiments were repeated twice with similar results.

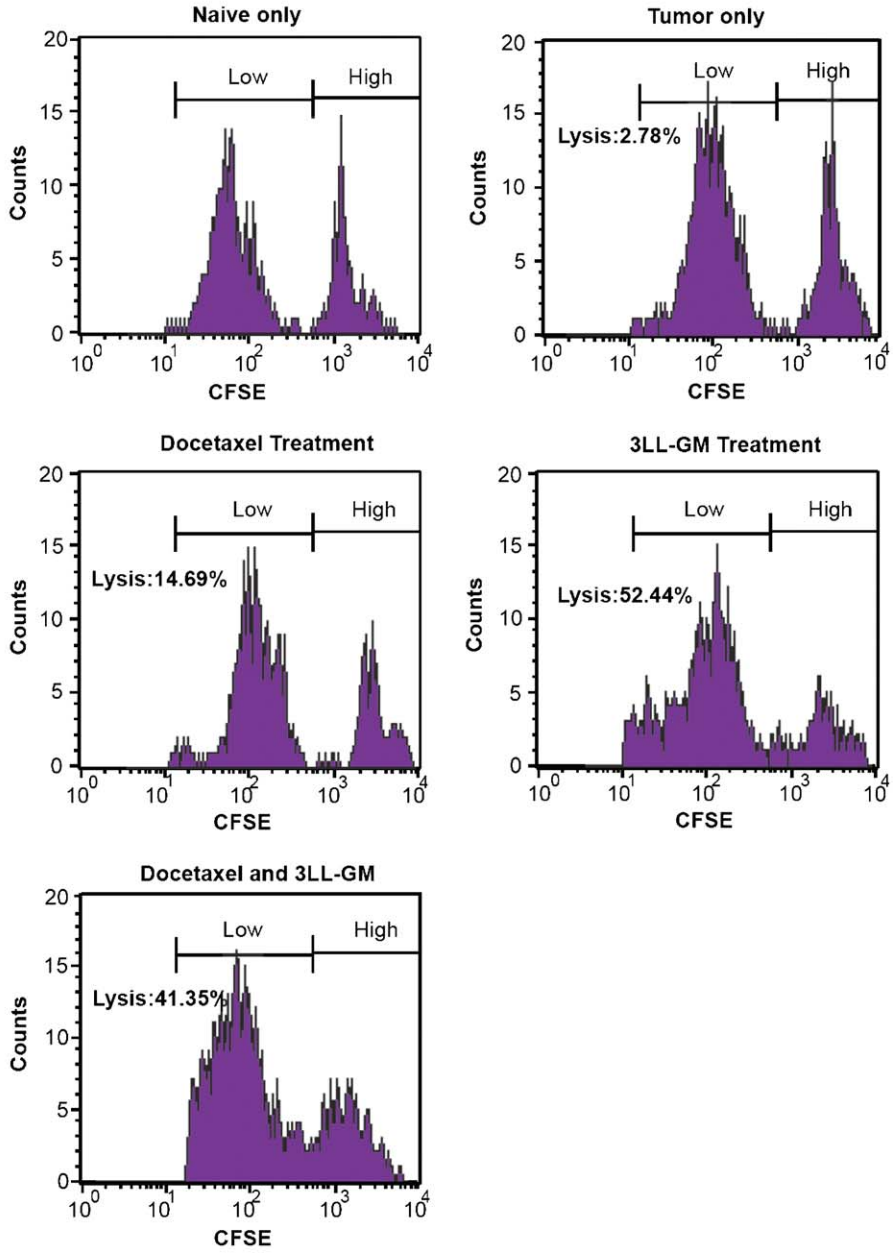

FIGURE 5. CD8 T cells were critical for tumor protection in combination therapy. Mice from combination therapy groups were treated with control rat IgG, anti-CD4, anti-CD8 and both anti-CD4 and anti-CD8 every 3 days after tumor inoculation on day 0. Tumor area were measured by digital calipers. Data in this figure were pooled from two independent experiments. The numbers in parentheses indicated the number of mice did not develop tumor or experienced complete regression over the total number of mice.

Interestingly, the percentage of blood $CD4^+CD25^+$ $Foxp3^+$ T cells was slightly, but significantly, lower than that found in docetaxel-treated mice compared with PBS-treated mice. Although we did not have direct evidence yet, it remains an interesting possibility that docetaxel could selectively deplete $CD4^+CD25^+Foxp3^+$ T cells.

Taken together, these results showed that docetaxel induced a mild lymphopenia in mice, the degree of lymphodepletion was similar to that was observed in mice treated with low-dose of CY (100 mg/kg).[6] The lymphodepletion occurred in either naive $CD4^+$ and $CD8^+$ T cells or memory $CD8^+$ $CD122^+$ memory and $Foxp3^+$ $CD4^+CD25^+$ regulatory T cells.

Docetaxel Treatment Did Not Affect T-cell Proliferation But Promoted Survival of Primed T cells

Recently we have demonstrated that lymphodepletion before vaccination with peptide pulsed dendritic cells greatly increased the expansion and survival of adoptive transferred naive melanoma-specific $CD8^+$ T cells.[12] The increased expansion and survival of adoptive transfer pmel-1 T cells depended on IL-7, a cytokine critical for

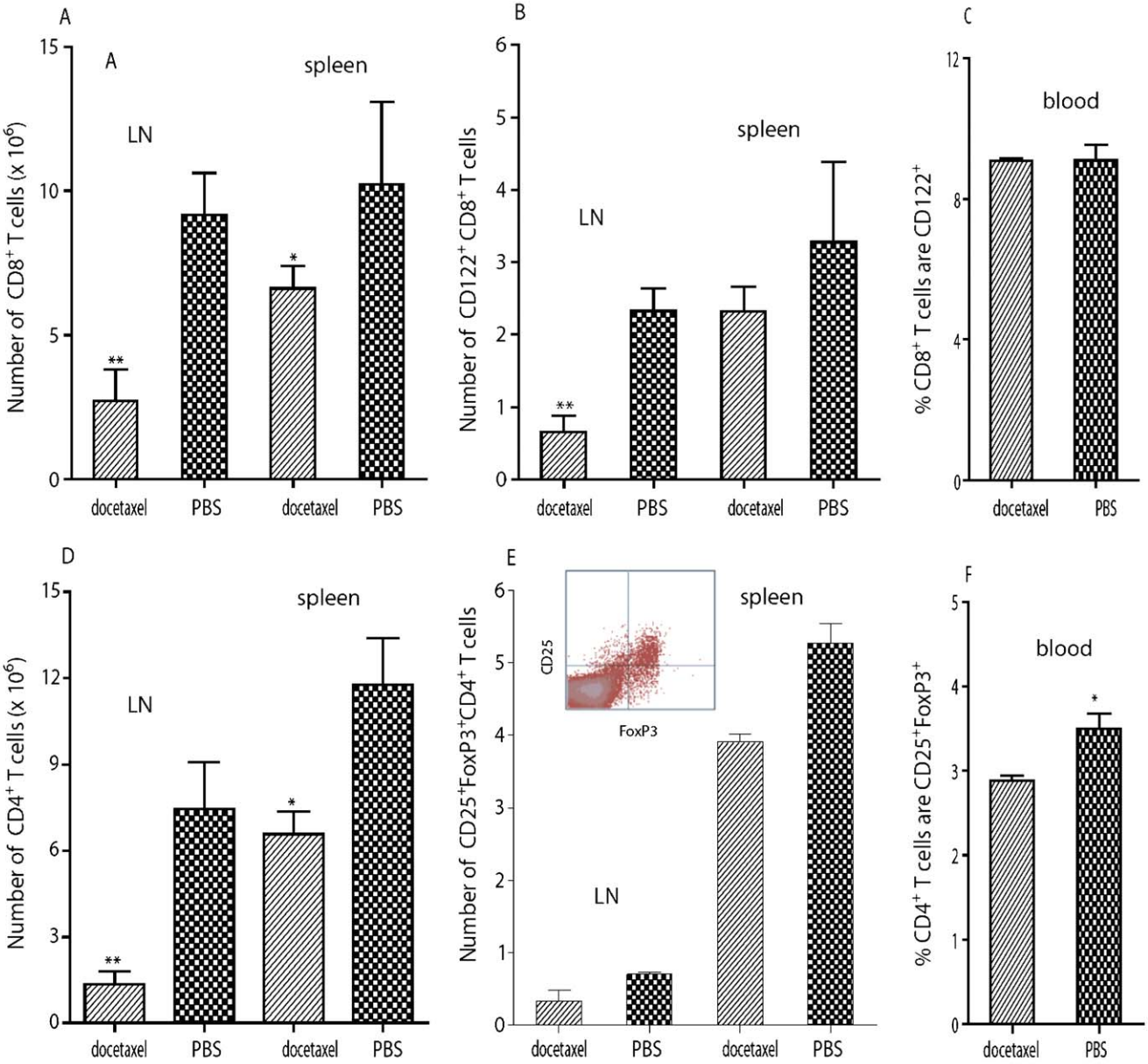

FIGURE 6. Reduction of total $CD4^+$ and $CD8^+$ T cells, or $CD4^+CD25^+FoxP3^+$ and $CD8^+CD122^+$ T cells after docetaxel treatment. Naive B6 mice were treated with i.p. injection of 5 mg/kg of docetaxel or PBS. The number of total $CD8^+$ T cells (A), $CD122^+$ $CD8^+$ T cells (B), total $CD4^+$ T cells (D), $CD4^+CD25^+FoxP3^+$ T cells (E) in 6 LN (2 inguinal and 4 axillary lymph nodes of each mouse) and spleen were determined by flow cytometry and counting of total number of cells. The percentage of $CD8^+$ T cells expressing CD122 (C) and $CD4^+$ T cells expressing surface CD25 and intracellular FoxP3 in bloods from docetaxel treated mice was also determined by flow cytometry (insert). The data represented one of two experiments with similar results. Each bar represents the mean and standard deviation of values obtained from four different mice. $*P < 0.05$ and $**P < 0.01$. P value was determined by paired t test (two tailed).

survival of both naive and memory T cells. Depletion of regulatory T cells could promote the antigen-driven T-cell proliferation by removing their suppressive effect on dendritic cells or activated T cells. Reduced number of preexisting memory CD8 T cells after docetaxel treatment could promote the survival of vaccine induced memory T cells by removing the competition "T-cell space" or "cytokine sink".[10,11] To investigate whether docetaxel treatment before vaccination would affect the T-cell proliferation and survival, mice were adoptively transferred with CFSE-labeled naive T cells (pmel-1) specific for melanoma antigen, gp100, and subsequently treated

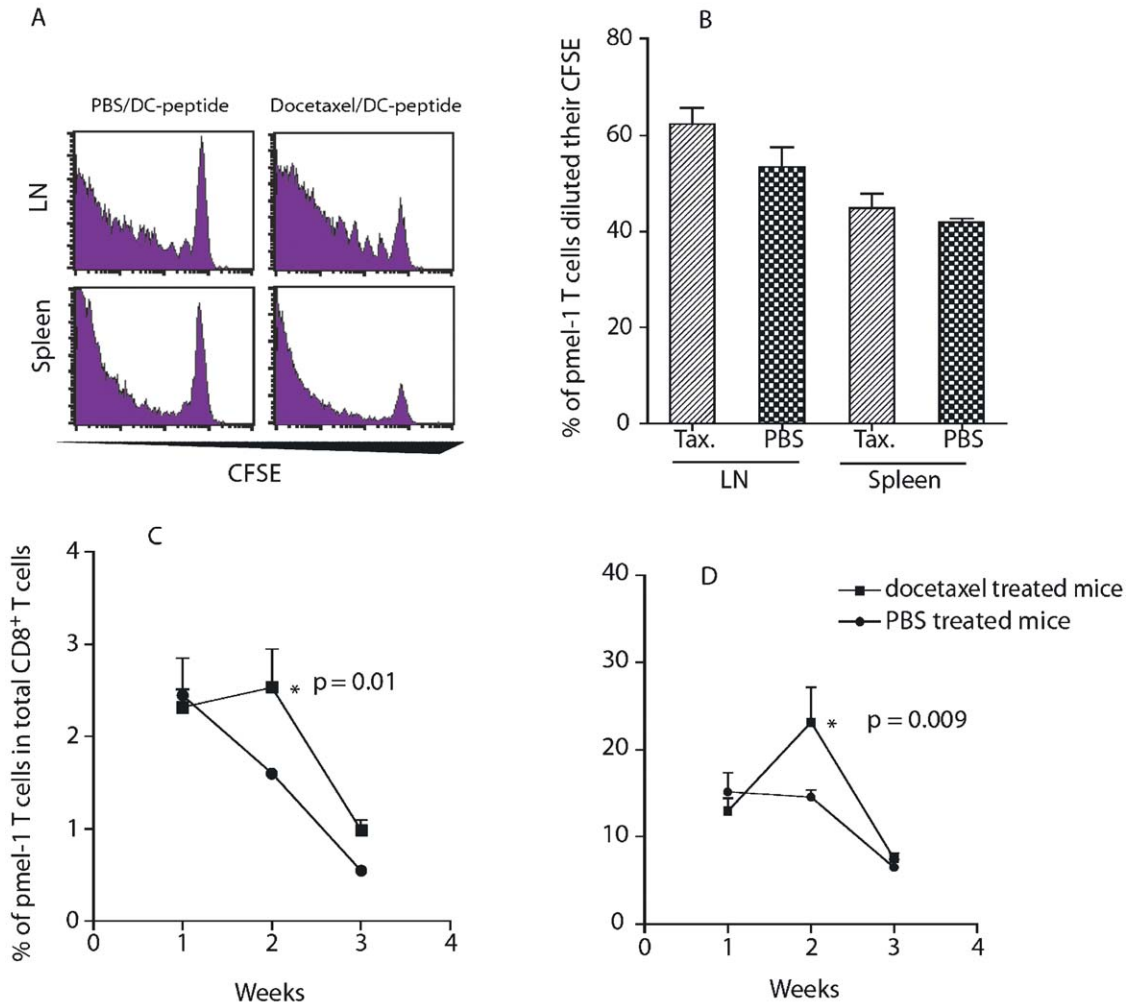

FIGURE 7. Docetaxel treatment did not affect proliferation of naive pmel-1 T cells induced by peptide-pulsed dendritic cells but promoted their survival. Congenic B6CD45.1 naive mice were adoptive transferred with CFSE-labeled pmel-1 spleen cells (CD45.2) and then treated with 5 mg/Kg doecetaxel or PBS daily for three days. Mice were then vaccinated with peptide pulsed dendritic cells by s.c. injection of 2×10^6 DC. LN and spleen cells were then stained with antibodies against CD8 and CD45.2 and analyzed by flow cytometry. (A) CFSE dilution of gated donor $CD8^+CD45.2^+$ T cells (pmel-1) from LN and spleen 7 days post DC vaccination; (B) percentage of pmel-1 T cells diluted their CFSE over total number of peml-1 T cells. Data represent the mean and standard deviation obtained from 3 docetaxel-treated and 2 PBS-treated mice. No statistically difference between docetaxel or PBS treatment. Congenic B6/CD45.1 naive mice (n = 5) were treated with 5 mg/Kg docetaxel or PBS and adoptive transferred with 5 million naive pmel-1 spleen cells and vaccinated with peptide pulsed DC two days post docetaxel treatment. Both the percentage of pmel-1 T cells in the total $CD8^+$ T cell population (C) and the absolute count (D) of pmel-1 T cells ($CD8^+$ and $CD45.2^+$) were determined by flow cytometry at week 1, 2 and 3 post vaccination. Each data points represents the mean and standard deviation calculated from the values obtained from 5 mice in PBS or docetaxel treated mice. Significant difference is indicated by *.

with docetaxel and then vaccinated with dendritic cells pulsed with gp100 peptide. The division of CFSE-labeled pmel-1 T cells as indicated by the dilution of CFSE fluorescence at day 7 post DC vaccination did not differ in both LN and spleens from mice treated with either PBS or docetaxel (Figs. 7A and B). Thus, docetaxel did not affect vaccine induced T-cell proliferation.

Next, to investigate whether reduced competition from naive or memory T cells after docetaxel treatment could promote the survival of activated pmel-1 T cells, we repeated the same experiment described above except the adoptive transfer of pmel-1 T cells was done two day post docetaxel treatment. It was done because docetaxel did reduce the number of transferred pmel-1 T cells although it did not changed the proliferative responses of pmel-1 T cells if docetaxel was given after adoptive transfer of pmel-1 T cells. Both the percentage and absolute count of pmel-1 T cells at week 1, 2, and 3 post vaccination in blood were determined from mice that received either PBS or docetaxel (n = 5). As it would

be predicated from the result of CFSE-dilution experiments, the percentage and number of pmel-1 T cells were similar at week 1 in either PBS or docetaxel-treated mice. However, a significantly higher percentage and number of pmel-1 T cells were found in mice treated with docetaxel compared with PBS (Figs. 7C, D) two weeks post vaccination. At week 3, the percentage of pmel-1 T cells was still higher in docetaxel-treated mice, however, the number in both groups of mice was not significantly different. This may due to a slow recovery of endogenous $CD8^+$ T cells at week 3 in docetaxel-treated mice.

DISCUSSION

This study demonstrated that a GM-CSF-producing lung cancer vaccine could be combined with docetaxel chemotherapy to achieve long-term survival and cure of mice with established 3LL tumors. The augmented antitumor immunity at least in part due to the docetaxel-induced lymphodepletion, which not only diminished the number of regulatory T cells, but also reduced the number of both naive and memory T cells. Unlike sublethal irradiation, but similar to low-dose chemotherapy with CY, docetaxel only induced mild lymphodepletion. Because an increased survival of antigen primed T cells rather than increased T-cell activation was observed in docetaxel-treated mice, the immune potentiation effect of docetaxel in our model probably due to the deceased competition of naive T cells and memory T cells rather than depletion of regulatory T cells. In studies with low-dose CY, a reduction of number or function of regulatory T cells was the primary mechanisms for immune potentiation effect of CY.[3,6,7] However, the effect of CY on memory T cells has not been examined in these studies, decreased competition of naive and memory T cells in CY-treated mice could also contribute to beneficial effect of low-dose CY treatment. Mice treated with combination therapy also exhibited a peptide-specific CTL memory response and resisted a secondary tumor challenge. These results provide a strong impetus for new clinical trials that combine the benefits of tumor debulking by chemotherapy and stronger antitumor immune responses induced by tumor vaccines after mild lymphodepletion. GM-CSF gene modified NSCLC tumor vaccines have been demonstrated in clinical trials to be safe and have caused a small number of patients to achieve complete responses.[14,15] Docetaxel is one of the most widely used and effective drugs for NSCLC. Our results strongly support a combination of these two distinct modalities in future clinical trials. Because docetaxel is usually given in a 3-week cycle, vaccination shortly after each docetaxel treatment is well suited and spaced to allow the development of immune responses.

The ability to combine traditional lung cancer treatment such as chemotherapy and radiation with vaccines is critical for the success of lung cancer immunotherapy trials. Lung cancer patients often have too high a disease burden to be cured or controlled with vaccination alone, as was also seen in the tumor models used in this study. Vaccination can be quite effective in protecting naive mice from tumor challenge or in delaying tumor growth in mice bearing minimal residual diseases, but is less effective when given to mice with established tumors. Tumor debulking with chemotherapy or radiation has the added advantage of inducing lymphopenia in the host, a condition that is sometimes associated with the sensitization of antigen-specific T cells and exposing T cells to potential novel tumor antigens unveiled by tumor cell death.[23]

As early as the 1970s, preclinical studies suggested that cyclophosphamide inhibited host immune suppressor cells and augmented antitumor immune responses when the drug was given prior to immunization.[16] However, there have been few clinical studies combining chemotherapy and active immunotherapy with tumor vaccines. A favorable outcome for melanoma patients was reported when patients were treated with cyclophosphamide plus an autologous melanoma vaccine; clinical responses correlated with increased DTH response.[17] Clinical responses were achieved in another study in which patients were treated with cyclophosphamide plus an allogeneic melanoma lysate vaccine and adjuvant (DETOX) and correlated with elevated melanoma-specific CTL precursors.[18] Chemotherapeutic drugs may cause various degrees of immune suppression and lymphopenia; sometimes a paradoxical potentiation of immune effects was observed, depending on the type, dose, regimen and temporal relationship to antigen exposure.[19] When chemotherapy was administrated after tumor vaccination, Nigam et al showed that, of 11 agents tested, only doxorubicin had immunostimulatory activity.[20] The detrimental effects of other chemotherapeutic drugs on antitumor immune responses clearly related to the post-vaccine timing of chemotherapy, which led to cytotoxicity to immune cells. Consistent with this notion, Machiels et al demonstrated that pre-vaccine, but not post-vaccine, treatment with cyclophosphamide and paclitaxel at doses just above the level that cause lymphopenia could break tolerance and augment antitumor immune responses in mice treated with GM-CSF-secreting breast cancer vaccine.[3] They also showed that doxorubicin given either before or after vaccination failed to enhance antigen-specific T-cell responses and appeared to augment vaccine efficacy through different mechanisms. The antitumor effect of intratumor injection of dendritic cells was shown to be dramatically enhanced by systemic chemotherapy before vaccinations.[21,22] Similarly, Yu et al documented that paclitaxel before intratumoral injection of dendritic cells given at a dose that causes tumor apoptosis and minimal immune suppression could induce significant antitumor effect without compromise of antitumor immune responses.[4] In agreement with these studies, our data strongly support the notion that immune potentiation of chemotherapy could be achieved only with pre-vaccine chemotherapy. Pre-vaccine docetaxel could cure a high proportion of tumor-bearing mice, whereas post-vaccine docetaxel total failed. In serial experiments

using TCR transgenic T cells to determine the effect of gemcitabine on T cell priming by tumor cells, Nowak AK et al demonstrated that gemcitabine was not detrimental to antitumor immunity and induction of tumor apoptosis could lead to effective cross-priming of tumor antigen specific $CD8^+$ T cells in tumor bearing mice.[23,24] Our results also demonstrated that docetaxel did not affect antigen-driven proliferation of naive T cells and the efficacy of docetaxel and tumor vaccination depended on induction of antitumor $CD8^+$ T cells. Interesting, $CD4^+$ T cells were not required and depletion of $CD4^+$ T cells improved the efficacy of combination therapy. This is consistent with our earlier reports that priming of therapeutic $CD8^+$ effector T cells by GM-CSF producing tumor vaccines was independent of CD4 help.[25,26] Enhanced efficacy after depletion of $CD4^+$ cells may relate to the depletion of $CD4^+CD25^+$ regulatory T cells and results in a stronger antitumor immunity (5, 6, 8, 27). In our model, docetaxel did induced a broad reduction of different T cell subsets, a slight but significant reduction of $CD4^+CD25^+Foxp3^+$ T cells was observed in LN, spleen and blood of docetaxel-treated mice. Another interesting mechanism for immune-potentiating effects of pre-vaccine chemotherapy is related to the induction of lymphopenia and lymphopenia-driven proliferation of antigen-specific T cells.[10,19,28] Many studies including ours provide ample evidence to support the hypothesis that a lymphopenic environment is more conducive to the proliferation and survival of tumor-specific T cells induced by tumor or DC based vaccines[12,28–30] and adoptive transferred tumor-reactive T cells after in vitro activation and expansion.[11,25,26] Although depletion of regulatory T cells is more popular view on how lymphodepletion could augment the activity of self/tumor-reactive $CD8^+$ T cells, the removal of endogenous naive, memory or NK cells that act as sinks for cytokines could also played a very or maybe more important role in promoting T-cell proliferation and expansion in lymphodepleted hosts. The fact we did observe enhanced survival but not proliferation of pmel-1 in docetaxel-treated mice suggested that docetaxel treatment promote antitumor immunity by removing "cytokine sink" in our model. As indicated in CD4 depletion experiments, a more complete depletion of $CD4^+CD25^+Poxp3^+$ T cells could further augment vaccine efficacy. More experiments are needed to address this possibility.

Rosenberg et al, has reported that combination of lymphopenia induction (lymphodeletion) and adoptive transfer of ex-vivo expanded tumor infiltrating lymphocytes could result clinical response in 6 of 13 melanoma patients.[31–33] Cancer patients are often treated with monthly docetaxel and lymphopenia often lasts 1–3 months after treatment,[34] this would provide an excellent window for vaccination to take advantage of lymphodepletion. Effects of lymphopenia-driven proliferation on antitumor immune responses must be investigated in future clinical trials combining tumor vaccines with docetaxel chemotherapy.

ACKNOWLEDGMENTS

This study was supported by grants from Cell Genesys, Inc., American Cancer Society (RSG LIB-105800 to H.-M. Hu) and NIH (1R01-CA107243-01 to H.-M. Hu).

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