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Dendritic Cells Strongly Boost the Antitumor Activity of Adoptively Transferred T Cells *In vivo*

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Abstract

Dendritic cells (DCs) have been well characterized for their ability to initiate cell-mediated immune responses by stimulating naive T cells. However, the use of DCs to stimulate antigen-activated T cells *in vivo* has not been investigated. In this study, we determined whether DC vaccination could improve the efficacy of activated, adoptively transferred T cells to induce an enhanced antitumor immune response. Mice bearing B16 melanoma tumors expressing the gp100 tumor antigen were treated with cultured, activated T cells transgenic for a T-cell receptor specifically recognizing gp100, with or without concurrent peptide-pulsed DC vaccination. In this model, antigen-specific DC vaccination induced cytokine production, enhanced proliferation, and increased tumor infiltration of adoptively transferred T cells. Furthermore, the combination of DC vaccination and adoptive T-cell transfer led to a more robust antitumor response than the use of each treatment individually. Collectively, these findings illuminate a new potential application for DCs in the *in vivo* stimulation of adoptively transferred T cells and may be a useful approach for the immunotherapy of cancer.

INTRODUCTION

Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) in the induction of primary immune responses (1-3). Because of their central role in controlling cell-mediated immunity, DCs hold much promise as cellular adjuvants in therapeutic cancer vaccines. DC vaccines have been reported to induce strong antitumor immune responses in animal experiments and in selected clinical trials involving cancer patients (4-6). However, although the majority of clinical trials using DC vaccination have succeeded in generating tumor antigen-reactive CTLs in cancer patients, only sporadically have tumor regressions been induced (6-8). Several mechanisms may account for the limited effectiveness of DC vaccine-induced immune responses to tumors. One possibility is that insufficient numbers of CD8⁺ CTLs, the ultimate effector cells thought to mediate the rejection of solid tumors, may be induced in response to DC vaccination alone (8,9). Alternatively, CTLs that are generated in this manner may possess suboptimal antitumor function *in vivo*, possibly because of insufficient activation, inadequate migration to tumor sites, or susceptibility to host-derived regulatory mechanisms (9).

To overcome these limitations, adoptive cell transfer (ACT) therapy, which directly provides large numbers of *in vitro* selected, highly active, tumor-specific T lymphocytes, may be one of the most promising immunotherapeutic approaches for treatment of patients with cancer (10). ACT therapy has been shown to induce T-cell-mediated antitumor immune responses in

patients with lymphoma, metastatic renal cell carcinoma, and melanoma (10-14). However, although clinical responses have been observed, many have been transient (15-17). The rapid disappearance of adoptively transferred cells frequently observed in these patients suggests that insufficient T-cell persistence *in vivo* may be largely responsible for the lack of antitumor efficacy (18,19).

On the basis of the aforementioned clinical observations, we hypothesized that a combination of ACT therapy and DC vaccination may be an optimal method to induce antitumor immune responses in patients by providing sufficient numbers of activated tumor-specific cytolytic cells and potent *in vivo* stimulation. Although DC vaccination has been shown to efficiently initiate immune responses by activating host naive T cells, it is not known whether DC vaccination also can effectively stimulate adoptively transferred, *in vitro*-activated T cells, and whether the combination of DC vaccination and ACT can induce an enhanced antitumor response (20).

To investigate these questions, we have used an animal model consisting of C57BL/6 mice harboring large, established subcutaneous B16 tumors expressing the melanoma tumor antigen gp100. For tumor treatment, we adoptively transferred *ex vivo*-cultured, activated, transgenic T cells (pmel-1 cells) that express a T-cell receptor (TCR) specifically recognizing an H-2D^b-restricted epitope of gp100, in conjunction with gp100 peptide-pulsed DC vaccination and interleukin 2 (IL-2) administration. Although adoptive transfer of pmel-1 T cells alone with or without IL-2 failed to induce tumor regressions, inclusion of vaccination with gp100 peptide-pulsed DCs resulted in cytokine production, proliferation, and increased tumor infiltration of transferred gp100-specific T cells *in vivo* and significantly improved antitumor responses. These results show a potentially useful application of DCs for the stimulation of activated, adoptively transferred T cells *in vivo*.

MATERIALS AND METHODS

Mice and Tumor Cells

Pmel-1 transgenic mice express a TCR specific for an H-2D^b-restricted epitope of the melanoma tumor antigen gp100 (gp100₂₅₋₃₃) on a C57BL/6 background as described previously (21,22). To facilitate tracking of transferred T cells, pmel-1 transgenic mice were bred with homozygous Thy1.1 mice (also C57BL/6 background) to generate Thy1.1⁺ pmel-1 transgenic mice. Virtually all (>95%) of the CD8⁺ T cells in these transgenic mice were V β 13⁺Thy1.1⁺ as confirmed by flow cytometry. C57BL/6 mice and pmel-1 transgenic mice were maintained in a pathogen-free facility at the NIH. All of the protocols conformed to NIH guidelines for the care and use of laboratory animals. B16 melanoma was obtained from the National Cancer Institute tumor repository (Bethesda, MD) and maintained in complete medium consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Biofluids, Rockville, MD), 0.03% L-glutamine, 100 μ g/mL streptomycin, 100 μ g/mL penicillin, and 50 μ g/mL gentamicin sulfate.

Generation of Thy1.1⁺ Pmel-1 Transgenic T Cells

Splenocytes from Thy1.1⁺ pmel-1 transgenic mice were depleted of erythrocytes with ACK lysing buffer (Cambrex, Walkersville, MD) and cultured in complete medium with 30 units/mL recombinant human IL-2 (a gift from Chiron Corp., Emeryville, CA) in the presence of 1 μ mol/L hgp100₂₅₋₃₃ peptide for 7 days (22,23). Such cultured pmel-1 T cells develop an activated phenotype, as determined by the up-regulation of CD25, CD44, and CD69 and down-regulation of CD62L (22,23). More than 95% of the 7-day cultured splenocytes were CD8⁺V β 13⁺ Thy1.1⁺ cells, thus allowing for efficient tracking of gp100-specific T cells after adoptive transfer into Thy1.2⁺ recipients. The purity of all of the pmel-1 T-cell cultures was

confirmed by flow cytometry using monoclonal antibodies (mAbs) against V β 13 and Thy1.1 before adoptive transfer.

Dendritic Cell Preparation

Bone marrow-derived murine DCs were generated as described previously (24,25). Briefly, bone marrow cells from the femur and tibiae of C57BL/6 mice were grown at a starting concentration of 1×10^6 cells/mL in RPMI 1640 and supplemented with 5% fetal bovine serum, L-glutamine (2 mmol/L), penicillin/streptomycin (50 units/mL), 10% nonessential amino acids, and 50 mmol/L 2-mercaptoethanol plus 20 ng/mL of recombinant granulocyte macrophage colony-stimulating factor and 100 ng/mL of IL-4 (Peprotech Inc., Rocky Hill, NJ). Fresh medium supplemented with granulocyte macrophage colony-stimulating factor and IL-4 was added on days 2 and 4, and all of the loosely adherent cells were transferred to Petri dishes on day 6. The following day, nonadherent cells were harvested, washed, and pulsed for 4 hours at 37°C with 10 μ mol/L of the appropriate peptide in Opti-MEM media (Life Technologies, Inc., Rockville, MD). DCs were washed three times with PBS before using for mouse injections. DC purity and maturation were analyzed by flow cytometry before use to ensure staining for markers CD11c, I-A^b, CD80, and CD86 on DCs was positive and staining for CD3, B220, and NK1.1 was negative (24,25).

Peptides

In all of the experiments, H-2D^b-restricted human gp100 (hgp100₂₅₋₃₃, KVPRNQDWL), an altered peptide ligand of mouse gp100 (mgp100₂₅₋₃₃, EGSRNQDWL), was used as the immunogen as described previously (22,23). The H-2D^b-restricted epitope of the influenza nucleoprotein (NP₃₆₆₋₃₇₄, ASNENMETM) was used as an irrelevant control peptide. All of the peptides were made by standard 9-fluorenylmethoxycarbonyl chemistry and purified by reverse-phase high-performance liquid chromatography, and purity of >99% was confirmed by mass spectrometry.

Adoptive Transfer, Vaccination, and Tumor Treatment

Eight- to 10-week-old C57BL/6 mice were inoculated subcutaneously with 5×10^5 B16 melanoma cells. On day 7 or 8 after tumor injection, 4 to 6×10^6 *in vitro*-activated Thy1.1⁺ pmel-1 T cells were adoptively transferred into tumor-bearing mice ($n = 5$ per group), followed immediately by intravenous vaccination with 1 to 4×10^6 DCs pulsed with either hgp100₂₅₋₃₃ or NP₃₆₆₋₃₇₄ peptide. In some experiments, mice were administered one or two additional DC vaccinations at 6-day intervals. Recombinant human IL-2 was administered intraperitoneally directly following each vaccination (6×10^5 units twice daily for 3 days after the first vaccination, and 3×10^5 units twice daily for 3 days after the second and third vaccinations). B16 tumor growth was monitored by measuring the perpendicular diameters of tumors. Mice were sacrificed when tumors exceeded 20 mm in diameter. All of the experiments were carried out in a blinded, randomized fashion and performed at least twice with similar results.

Flow Cytometry

The percentage of Thy1.1⁺ pmel-1 T cells in total peripheral blood lymphocytes (PBLs) was analyzed as described previously (22,23). Briefly, mice were tail-bled on the indicated days following vaccination. Erythrocytes were depleted with ACK lysing buffer, and the remaining PBLs were stained with mAbs against V β 13 (clone, MR12-3) and Thy1.1 (clone, OX-7) and analyzed by flow cytometry using a lymphocyte gate. Total numbers of lymphocytes were enumerated by either automated complete differential count or hemocytometry with trypan blue exclusion. The absolute number of Thy1.1⁺ pmel-1 T cells in tissues was calculated by multiplying the percentage of Thy1.1⁺ pmel-1 cells in lymphocytes (as determined by flow

cytometry) by the total numbers of lymphocytes present in tissues. To monitor the proliferation of adoptively transferred T cells *in vivo*, Thy1.1⁺ pmel-1 T cells were labeled with 5 $\mu\text{mol/L}$ carboxyfluorescein succinimidylester (CFSE) (Molecular Probes, Eugene, OR) for 10 minutes at 37°C and washed, and 6×10^6 labeled cells were adoptively transferred into B16 tumor-bearing mice. A total of 2×10^6 peptide-pulsed DCs were injected intravenously after T-cell transfer, followed by IL-2 administration as described previously. On the indicated days, lymphocytes from four mice per group were isolated from peripheral lymph nodes, mesenteric lymph nodes, spleen, peripheral blood, and tumors as described previously, and Thy1.1⁺ V β 13⁺ lymphocytes were analyzed by flow cytometry (26,27). To assay intracellular interferon γ (IFN- γ) release, lymphocytes were isolated from different tissues as described previously and cultured with Golgiplug (BD Biosciences, San Jose, CA) for 4 hours at 37°C in the absence or presence of 1 $\mu\text{mol/L}$ mgp100₂₅₋₃₃ peptide (26,27). Cells were washed, stained with mAbs against Thy1.1 and V β 13, and permeabilized with Cytotfix/Cytoperm (BD Biosciences) according to the manufacturer's recommendations. After incubating with an anti-IFN- γ mAb, cells were washed and analyzed by flow cytometry. To analyze expression of cell surface markers CD44 or CD62L on pmel-1 T cells, cells were stained with mAbs against Thy1.1, V β 13, CD44, and CD62L (BD PharMingen, San Diego, CA). Samples were analyzed using a FACSCalibur flow cytometer and CELLquest software (both from BD Biosciences).

Statistical Analysis

The statistical analyses to compare tumor size and T-cell numbers between treatment and control groups were determined using the Mann-Whitney nonparametric *U* test. The statistical analyses to compare mouse survival between treatment and control groups were determined with a Kaplan-Meier test. Unless noted, data are presented as the mean \pm SE of data from four to five mice.

RESULTS

Antigen-Specific DC Vaccination Induces Cytokine Production and Proliferation of Adoptively Transferred T Cells *In vivo*

We first sought to determine whether antigen-specific DC vaccination could lead to stimulation and proliferation of activated, adoptively transferred T cells *in vivo*. To use a mouse model that paralleled ACT therapy in human cancer patients, we chose to address this issue in a large tumor-burden setting. Thus, mice were first inoculated with gp100-expressing B16 melanoma for 7 days before given *ex vivo*-activated gp100-specific pmel-1 T cells by adoptive transfer, followed by IL-2 administration and concurrent intravenous vaccination with DCs pulsed with either hgp100 or NP peptide (hereafter referred to as DC/hgp100 and DC/NP, respectively). *In vivo* function of pmel-1 T cells in the blood, lymph nodes, and spleen was analyzed 3 days later by measuring IFN- γ production in the absence of further *in vitro* stimulation. As shown in Fig. 1, only DCs bearing the relevant hgp100 peptide were capable of inducing IFN- γ production in the adoptively transferred T cells as determined by intracellular staining and flow cytometry. These cytokine-producing cells localized largely to the spleen but were not observed in the blood or lymph nodes. Upon *in vitro* restimulation with mgp100 peptide, IFN- γ -producing pmel-1 T cells were found in all of the tested tissues from the DC/hgp100- and DC/NP-treated mice (data not shown), showing that the transferred pmel-1 T cells maintain their functionality when stimulated with cognate antigen.

In vivo proliferation of the transferred pmel-1 T cells also was analyzed following DC vaccination. For these experiments, pmel-1 T cells were labeled with CFSE before adoptive transfer into tumor-bearing mice. At 5 days following vaccination, mice were sacrificed, and the extent of pmel-1 T-cell proliferation in the lymph nodes, spleen, and blood was determined

by flow cytometry. The results showed that DC/hgp100 led to significantly higher T-cell proliferation *in vivo* compared with DC/NP, particularly in the spleen and blood (Fig. 2A).

T-cell proliferation was further quantitated by determining the total numbers of pmel-1 cells present in the different tissues. As shown in Fig. 2B, DC/hgp100 vaccination led to substantially higher numbers of pmel-1 T cells in blood and spleen compared with DC/NP vaccination. Overall, DC/hgp100 vaccination resulted in a 7- to 12-fold increase in the total number of pmel-1 T cells compared with DC/NP vaccination, as measured 5 days after adoptive transfer. Antigen-specific DC vaccination clearly induced *in vivo* expansion of adoptively transferred pmel-1 T cells, given that the absolute number of pmel-1 T cells recovered on day 5 was significantly more than the 6×10^6 cells originally transferred (Fig. 2B). These data show that, in addition to the well-documented ability of DCs to stimulate naive T cells, antigen-pulsed DCs also can effectively stimulate and expand *in vitro* cultured, adoptively transferred, antigen-specific T cells *in vivo*.

Combination of Antigen-Specific DC Vaccination and ACT Therapy Results in Enhanced Antitumor Activity

Our observation that antigen-specific DC vaccination led to cytokine production and proliferation of transferred pmel-1 T cells *in vivo* prompted us to examine the therapeutic antitumor potential of combined DC vaccination and ACT therapy. Seven-day B16 tumor-bearing mice were subjected to adoptive transfer of pmel-1 T cells, followed by concurrent intravenous DC vaccination and IL-2 administration as described previously. Compared with untreated mice, DC/hgp100 vaccination alone had no significant antitumor effect, whereas treatment with pmel-1 T cells alone resulted in only a modest inhibition of tumor growth (Fig. 3A). In contrast, the combination of DC/hgp100 vaccination and pmel-1 T cells resulted in a significant delay in tumor growth compared with the treatment regimens of pmel-1 cells alone or pmel-1 T cells with irrelevant peptide-pulsed DCs (Fig. 3A; $P = 0.006$). The combination regimen of DC/hgp100 with pmel-1 T cells also resulted in a significant prolongation of survival compared with the other treatment groups, as depicted in Fig. 3B ($P = 0.003$). Notably, however, this regimen only provided a temporary inhibition of tumor growth, and all of the mice eventually succumbed to the B16 melanoma.

Because a single vaccination with specific antigen-pulsed DCs resulted in a significant delay in tumor growth and extension of mouse survival, we next explored whether sequential DC vaccinations could enhance the antitumor effects as reported previously in an active immunotherapeutic setting (28). For these experiments, mice were adoptively transferred with pmel-1 T cells as described previously, followed by one, two, or three vaccinations at 6-day intervals with either DC/hgp100 or DC/NP plus IL-2 administration. As depicted in Fig. 4, increasing numbers of vaccinations with DC/hgp100 correlated with increasingly delayed B16 tumor growth *in vivo* and also extended survival of mice. Three vaccinations with DC/hgp100 resulted in a significant inhibition of tumor growth and prolonged mouse survival compared with a single DC/hgp100 vaccination (Fig. 4A, $P = 0.014$; Fig. 4B, $P = 0.003$). Importantly, only those mice receiving three vaccinations of DC/hgp100 showed 100% survival for the entire observation period extending up to 6 weeks after treatment. In contrast, sequential vaccinations with DC/NP did not result in significant inhibition of tumor growth, showing that the improved antitumor effects induced by multiple DC vaccinations were antigen specific. Flow cytometric analysis showed that the numbers of pmel-1 T cells in the peripheral blood also correlated with the number of DC/hgp100 vaccinations administered (Fig. 4C). In mice receiving three sequential DC/hgp100 vaccinations, pmel-1 T cells accounted for ~13% of all of the PBLs at day 24 after cell transfer, approximately eightfold higher than mice receiving a single DC/hgp100 vaccination ($P = 0.021$). These results suggest that prolonging the

persistence of antigen-specific pmel-1 T cells *in vivo* may contribute to the enhanced antitumor response.

Collectively, these results show that antigen-specific peptide-pulsed DC vaccination in conjunction with ACT therapy can induce considerably stronger antitumor effects than either treatment individually. Furthermore, these experiments show that the use of multiple peptide-pulsed DC vaccinations following ACT results in increased T-cell persistence and enhanced antitumor responses *in vivo*. Importantly, vaccination with either hgp100 peptide alone or hgp100 peptide-pulsed splenocytes failed to enhance the antitumor activity of adoptively transferred pmel-1 cells (data not shown), implying that the use of DCs was an important component of this treatment.

Previous Lymphodepletion Further Improves the Efficacy of Combined Immunotherapy

To verify that the antitumor responses described previously were caused by the adoptively transferred pmel-1 T cells rather than by endogenous host immune cells, tumor-bearing mice were subjected to 500 rad of whole body irradiation 1 day before transfer of pmel-1 T cells and DC vaccination. This sublethal dose of irradiation results in lymphodepletion of mice and only slightly impacts B16 tumor growth. Interestingly, sublethal irradiation before ACT led to a much more significant improvement in antitumor responses in the combined immunotherapeutic setting (Fig. 5; $P = 0.009$). Similarly, we found that the combination of DC/hgp100 vaccination and pmel-1 T-cell transfer induced more effective antitumor activity in Rag-1^{-/-} knockout mice than in wild-type C57BL/6 mice (data not shown). These data suggest that the observed antitumor responses were caused by the adoptively transferred pmel-1 T cells rather than by the endogenous host immune cells. These results also indicate that the antitumor efficacy of ACT is significantly enhanced in mice devoid of endogenous T and B lymphocytes.

The most effective ACT regimen in this mouse tumor model occurred in the lymphodepletion setting with three sequential DC/hgp100 vaccinations (Fig. 6A). Under these conditions, complete tumor regressions were observed in a majority of mice within this treatment group. Furthermore, the remaining mice had tumors that were considerably smaller at 30 days post-treatment compared with tumors found in mice receiving only a single DC/hgp100 vaccination ($P = 0.013$). Increased persistence of transferred T cells also was observed in the lymphodepletion setting, with pmel-1 T cells accounting for ~75% of all of the PBLs at day 9 and ~25% of all of the PBLs at day 20 in mice receiving three sequential DC/hgp100 vaccinations (Fig. 6B), a significant improvement over that observed with only a single DC/hgp100 vaccination ($P = 0.021$).

Although the mechanisms leading to enhanced antitumor responses in the irradiation setting remain to be determined, similar findings by our group using a Rag1^{-/-} tumor model (data not shown) suggest that lymphodepletion may play a major role in determining the outcome of ACT therapy. Because the expansion of pmel-1 T cells *in vivo* was similar with or without previous irradiation (data not shown), homeo-static expansion of tumor-specific pmel-1 T cells is not likely to explain the enhanced antitumor effects of irradiation-induced lympho-depletion. Additional experiments will be required to more precisely determine the nature of this observation.

Antigen-Specific DC Vaccination Results in Increased Pmel-1 T-Cell Infiltration into Tumors

To investigate the possible mechanisms by which the combined immunotherapy regimen induced tumor regression, we next analyzed lymphocytes infiltrating the B16 tumors. Tumors were excised from mice 5 days following ACT and DC vaccination, and the percentage of pmel-1 T cells in the tumor-infiltrating lymphocyte population was analyzed by flow

cytometry. As shown in Fig. 7A, mice receiving DC/hgp100 vaccination had a much higher percentage of pmel-1 T cells in their tumors compared with mice receiving DC/NP (32% versus 2.1%). Although the numbers of total lymphocytes per milligram of tumor were only slightly higher in mice receiving DC/hgp100 compared with DC/NP vaccination, ~20-fold more total gp100-specific pmel-1 T cells per milligram of tumor tissue were found in DC/hgp100-vaccinated mice as compared with DC/NP-vaccinated mice (Fig. 7B).

To further delineate the mechanisms by which antigen-specific DC vaccination resulted in improved tumor infiltration, we analyzed pmel-1 T-cell surface expression of CD62L and CD44, both of which have been implicated in cell migration to the site of antigen deposition (29,30). By day 5 after cell transfer, pmel-1 T cells in blood and spleen showed significantly down-regulated CD62L (Fig. 7C) and up-regulated CD44 (Fig. 7D) expression when activated by DC/hgp100 vaccination compared with DC/NP vaccination. These changes are consistent with a reduced level of T-cell trafficking through lymphoid tissues and thus increased chance of migration to sites of antigen deposition in the periphery, such as tumor sites. In support of this, the CD62L^{low} cells clearly showed an enhanced ability to migrate to tumor sites, regardless of whether they were derived from DC/NP- or DC/hgp100-immunized mice (Fig. 7C). Collectively, these data suggest that it is the increased quantity and improved tumor-homing quality of pmel-1 T cells induced by antigen-specific DC vaccination that is responsible for the enhanced tumor infiltration and augmented antitumor responses observed.

DISCUSSION

The identification of CTLs as key effector cells that mediate the rejection of solid tumors has facilitated the development of a variety of T-cell-based cancer therapies (31). ACT-based immunotherapy, in which large numbers of *in vitro* cultured, highly activated, tumor-specific T cells are infused into patients, represents one of the most promising approaches to cancer treatment. Although ACT has had some success in the clinic, in the majority of reported cases it has resulted in limited and transient antitumor responses. Potential reasons for this lack of clinical efficacy include a lack of persistence of adoptively transferred T cells *in vivo*, which can result from the inability of these cells to survive or proliferate because of insufficient antigen stimulation *in vivo*, activation-induced cell death, or susceptibility to immune regulatory cells (10). Alternatively, transferred cytotoxic T cells that persist may be prevented from infiltrating or functioning optimally at the tumor site because of immunosuppressive mechanisms frequently observed in tumors (32-34). Designing rational strategies to overcome these limitations may augment the ACT therapeutic effectiveness.

A number of vaccination approaches have been used to stimulate antitumor host T cells *in vivo*, including the use of antigenic peptides or recombinant viral vectors to deliver tumor antigens *in vivo* (35,36). Although peptide immunizations frequently have been shown to result in increased numbers of circulating antigen-specific T cells (especially following long series of vaccinations over many months), rarely have they led to objective regressions of large, established tumors (37,38). Immunization with viral vectors have shown some promise in terms of inducing antitumor responses, potentially because of the activation of the innate immune system and induction of inflammation (39).

We have shown previously that immunization of mice with recombinant gp100-encoding fowlpox virus also can result in enhanced T-cell persistence and antitumor activity in our adoptive transfer model (22). However, these approaches are somewhat clinically limited by safety issues, in addition to the potential development of host immunity to the viral vectors themselves or immune inhibition known to be induced by many viral proteins (40). By contrast, the administration of autologous DCs as vaccines to activate endogenous tumor-specific T cells has been widely shown to be safe for clinical applications (41). Although repeated

immunizations with viral vectors have been shown previously to be limited by the development of neutralizing antibody (42), in this study we show that repeated DC immunization does not appear to have such limitations, with enhanced efficacy following multiple immunizations.

Several clinical trials are currently in progress using antigen-loaded DCs to immunize cancer patients. Although many of the trials have resulted in encouraging tumor antigen-specific T-cell reactivity, reports of complete regressions of established tumors in humans using DC vaccination alone remain largely anecdotal.

This study was undertaken to determine whether DCs could boost the antitumor activity of adoptively transferred T cells *in vivo*. Our results show that tumor antigen DC/hgp100 vaccination can induce cytokine production, proliferation, and increased tumor migration of previously activated, adoptively transferred gp100-specific T cells *in vivo*. Furthermore, the combination of peptide-pulsed DC vaccination and ACT therapy led to a more robust antitumor response than the use of either therapy alone, resulting in the substantial regression of large subcutaneous gp100-expressing B16 tumors and prolonged survival of tumor-bearing mice. DCs were clearly a key to improving the antitumor activity of ACT because peptide-pulsed DC immunization resulted in a greatly enhanced tumor treatment compared with vaccination with peptide alone or peptide-pulsed splenocytes.³ Experiments currently are underway to determine which particular attributes of DCs (*e.g.*, the levels of MHC expression, costimulatory molecules, and cytokine secretion) contribute to this function and whether DCs are unique in this capacity.

Efficient immunotherapy requires that cytotoxic T cells not only persist *in vivo* but also migrate to and function optimally at the tumor site. In this study, we showed that activation of adoptively transferred T cells by antigen-specific DC vaccination led to significant proliferation, persistence, and selective migration of transferred T cells to tumor, as evidenced by significant increases in absolute number of transferred cells in blood, spleen, and tumor tissue. The distinct down-regulation of CD62L and up-regulation of CD44 in pmel-1 T cells induced by gp100 peptide-pulsed DC may play a role in increasing T-cell homing to tumor. CD62L is thought to be primarily responsible for the entry of lymphocytes into lymph nodes. It has been suggested that CD62L^{low} cells would migrate more efficiently to the site of antigen deposition because of a reduced ability to recirculate through lymph nodes (30,43). CD44 can mediate T-cell rolling, which ultimately leads to endothelial cell adhesion and extravasation into peripheral tissues and potentially tumor sites (29,44).

Although intravenous immunization with peptide-pulsed DCs has been shown previously to induce significant antigen-specific T-cell responses and can control lung metastases, rarely has it been shown to lead to regression of subcutaneous tumors (45). Studies have suggested that intravenously injected DCs localize to the spleen, leading to effector and memory T cells in the spleen, but absent from major lymph node compartments (45,46). Furthermore, these data suggested that T cells initially activated in the spleen are possibly incapable of infiltrating subcutaneous tumors (45). Consistent with this, we found that most of the adoptively transferred pmel-1 T cells were further activated in the spleen but not in lymph nodes. Surprisingly, we found that intravenous DC immunization controlled the outgrowth of subcutaneous B16 tumor much more effectively than administering DCs via footpad.¹ This discrepancy may stem from the fact that our results, unlike the other studies, were observed in an ACT setting using T cells previously activated *in vitro*. In our model, we injected antigen-specific T cells and peptide-pulsed DCs via the same intravenous route, increasing the likelihood of interaction between the two cell types. Because our results showed large numbers of tumor antigen-specific T cells infiltrating B16 tumors after stimulation with peptide-pulsed

³Unpublished observations.

DCs, it suggests that adoptively transferred T cells do possess the capability to infiltrate subcutaneous tumor following antigen-specific stimulation *in vivo*.

It has been suggested that repeated sequential DC vaccinations can lead to clonal exhaustion of responding T cells and enhanced activation-induced cell death (46,47). Consistent with this, we also found that weekly vaccination with peptide-pulsed DCs alone, in the absence of IL-2, led to decreased antitumor activity in our model.¹ However, concurrent administration of IL-2 with each DC vaccination led to higher levels of transferred T cells in blood and significantly enhanced antitumor responses compared with IL-2 treatment alone. These results strongly suggest that DC vaccination and IL-2 act synergistically to augment the function of the adoptively transferred T cells. Although IL-2 has been shown previously to increase the potency of DC-based immunizations in an active immunization setting, our results show that the same principles also may apply to the setting of adoptive immunotherapy (48). Our finding that increased numbers of DC vaccinations led to enhanced antitumor immunity also is consistent with data from other groups showing that CD8⁺ T-cell responses to booster DC vaccinations are faster and stronger than the responses to the first DC vaccination (49). These principles are likely to be crucial for the rational design of immunization schedules for future ACT trials in human cancer patients.

Lymphodepletion regimens given before ACT have been shown recently to enhance the persistence and the antitumor activity of adoptively transferred T cells (50,51). It has been hypothesized that this increased antitumor efficacy may result from increased homeostatic proliferation of T cells and/or removal of host-derived regulatory immune cells. Our results confirm that lymphodepletion by irradiation before infusion of pmel-1 T cells increased the antitumor response, a conclusion strengthened by similar experiments using nonirradiated Rag-1^{-/-} knockout mice lacking endogenous B and T lymphocytes. However, careful quantitation of pmel-1 T cells revealed that *in vivo* proliferation following ACT was nearly identical in mice with or without previous irradiation. Thus, homeostatic proliferation may not be the principal means by which lymphodepletion contributes to the effectiveness of ACT. Delineation of the precise mechanisms that lead to enhanced antitumor responses in this model will require additional study.

ACT-based immunotherapy for human cancer patients holds much promise, but substantial limitations in clinical efficacy still remain to be overcome. Our experimental model, involving treatment of a large, poorly immunogenic B16 tumor with ACT consisting of low-to-moderate affinity, *ex vivo*-activated pmel-1 T cells, was designed to mimic human adoptive transfer studies as closely as possible. Although our use of TCR transgenic T cells with monoclonal specificity does not model the behavior of adoptively transferred polyclonal T-cell populations, this system still likely provides a valuable means to uncover general principles of DC vaccination that may be important to shape future immunization approaches. Our finding that antigen-specific DC vaccination can induce substantial *in vivo* proliferation, tumor migration, and increased antitumor activity of previously activated T cells may have significant clinical applications. Thus, the use of DCs to enhance adoptively transferred T cells may represent a promising new approach for cancer treatment.

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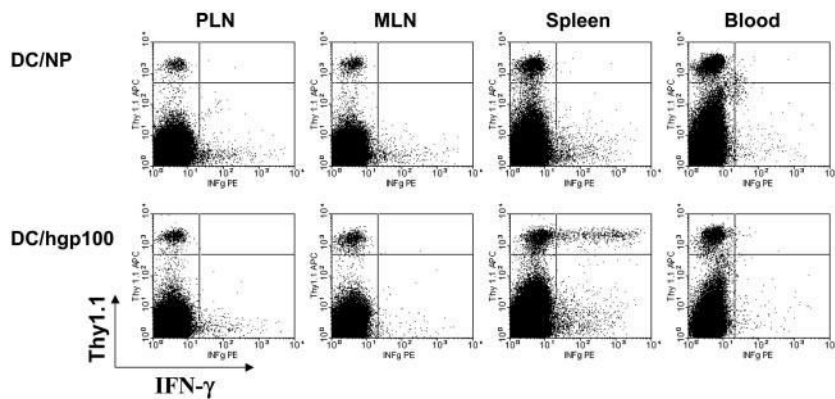


Fig. 1. Cytokine production by pmel-1 T cells following antigen-specific DC vaccination *in vivo*. Mice were immunized intravenously with DCs pulsed with either the melanoma peptide antigen hgp100₂₅₋₃₃ or the irrelevant influenza nucleoprotein-derived peptide NP₃₆₆₋₃₇₄ plus IL-2 administration immediately following adoptive transfer of cultured pmel-1 T lymphocytes. Three days later, pooled lymphocytes from treated mice (four mice/group) were isolated from the indicated lymphoid organs, and pmel-1 T cells were evaluated for intracellular production of IFN- γ in the absence of additional *in vitro* stimulation. *PLN*, peripheral lymph nodes; *MLN*, mesenteric lymph nodes.

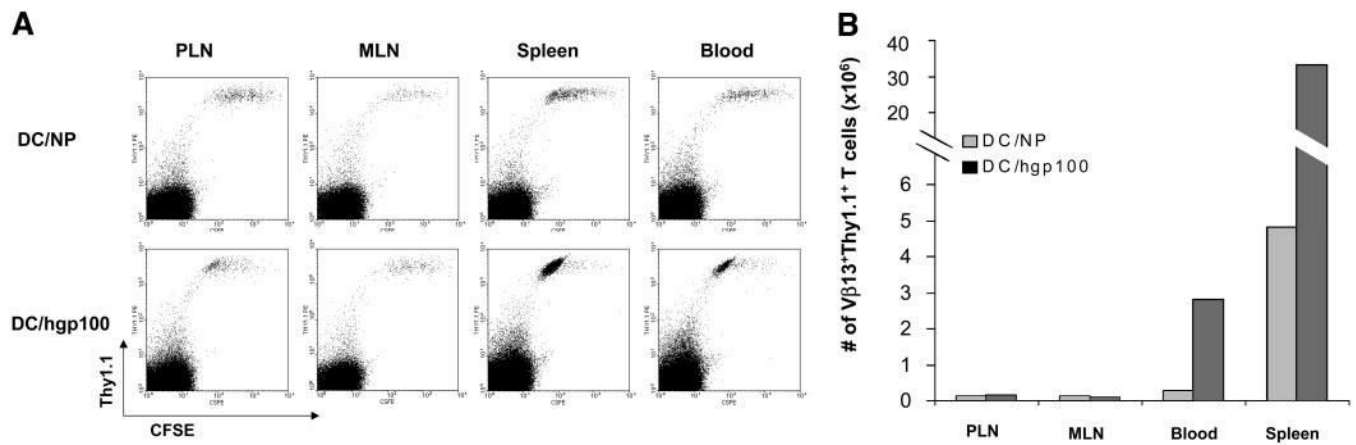


Fig. 2. Enhanced proliferation of pmel-1 T cells following antigen-specific DC vaccination *in vivo*. *In vitro* stimulated pmel-1 T cells were labeled with CFSE, washed, and adoptively transferred into B16 tumor-bearing recipient mice, followed immediately by immunization with DCs pulsed with either hgp100₂₅₋₃₃ or NP₃₆₆₋₃₇₄ peptides plus IL-2 administration. After 5 days, pooled lymphocytes from treated mice (four mice/group) were recovered from the indicated lymphoid organs and (A) analyzed for proliferation by flow cytometry or (B) quantitated for absolute number of pmel-1 cells present within each lymphoid compartment. *PLN*, peripheral lymph nodes; *MLN*, mesenteric lymph nodes.

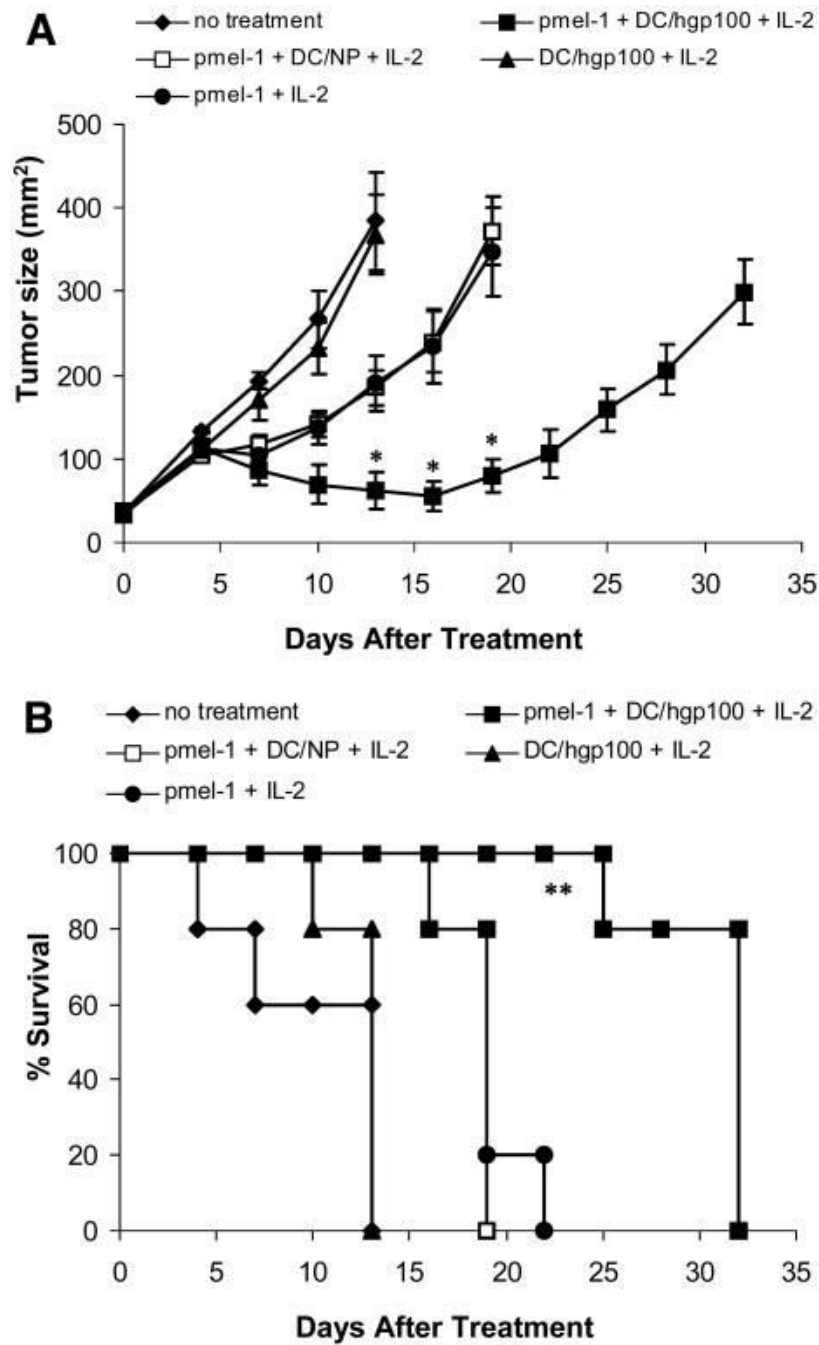


Fig. 3. Enhanced antitumor activity by combining DC vaccination with adoptive transfer of pmel-1 T cells and IL-2 injection. C57BL/6 mice were subcutaneously inoculated with 5×10^5 B16 tumor cells. Seven days later, tumor-bearing mice were intravenously injected with 6×10^6 cultured pmel-1 T cells, followed immediately by intravenous vaccination with DCs pulsed with either the hgp100₂₅₋₃₃ or NP₃₆₆₋₃₇₄ peptides. IL-2 was administered twice daily for a total of six doses. (A) Tumor growth and (B) mouse survival rate were monitored for up to 5 weeks following treatment. * $P = 0.006$; ** $P = 0.003$ for DC/hgp100 *versus* DC/NP. Results shown are representative of three independent experiments.

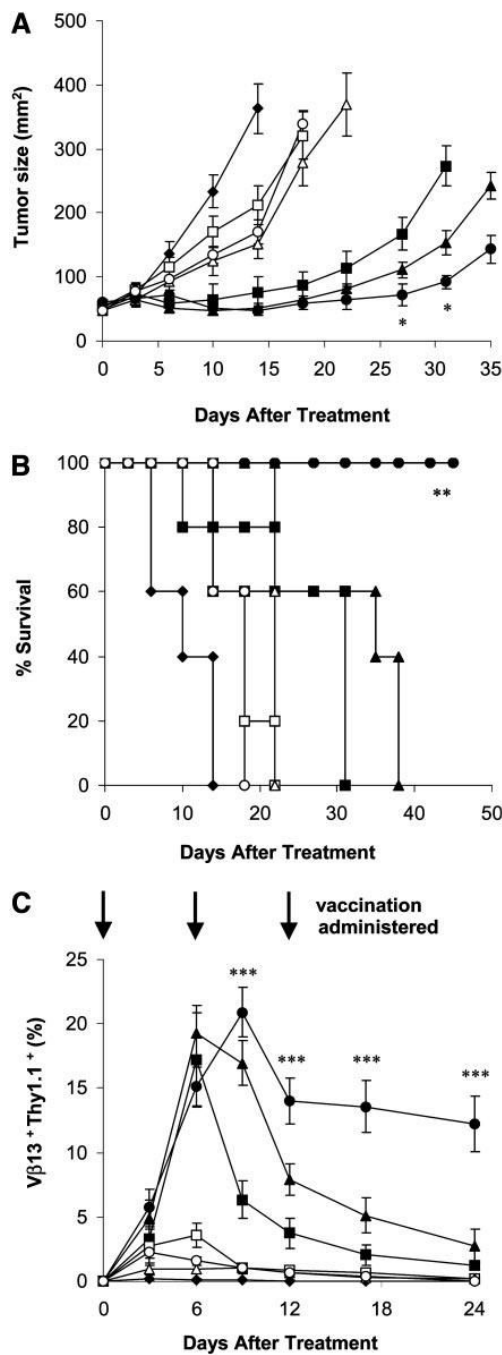


Fig. 4. Sequential DC immunization increases antitumor response, mouse survival, and pmel-1 T-cell persistence. Eight-day B16 tumor-bearing mice were intravenously injected with 4×10^6 pmel-1 T cells, followed by vaccination with DCs pulsed with either the hgp100₂₅₋₃₃ or NP₃₆₆₋₃₇₄ peptides. Indicated groups of mice received booster DC immunizations at 6-day intervals with peptide-pulsed DCs for one, two, or three total vaccinations. IL-2 was administered intraperitoneally immediately following each vaccination. (A) Tumor growth and (B) survival rate of mice were monitored to compare vaccination regimens. (C) Peripheral blood was analyzed by flow cytometry for percentages of pmel-1 cells (Vβ13⁺Thy1.1⁺) in total PBLs at the indicated time points. * $P = 0.014$; ** $P = 0.003$; *** $P = 0.021$ for one versus three

sequential DC/hgp100 vaccinations. Data are representative of two independent experiments with a total of five mice per group. (◆) No treatment; pmel-1 + (■) DC/hgp100 + IL-2 (1), (□) DC/NP + IL-2 (1), (▲) DC/hgp100 + IL-2 (2), (△) DC/NP + IL-2 (2), (●) DC/hgp100 + IL-2 (3), (○) DC/NP + IL-2 (3).

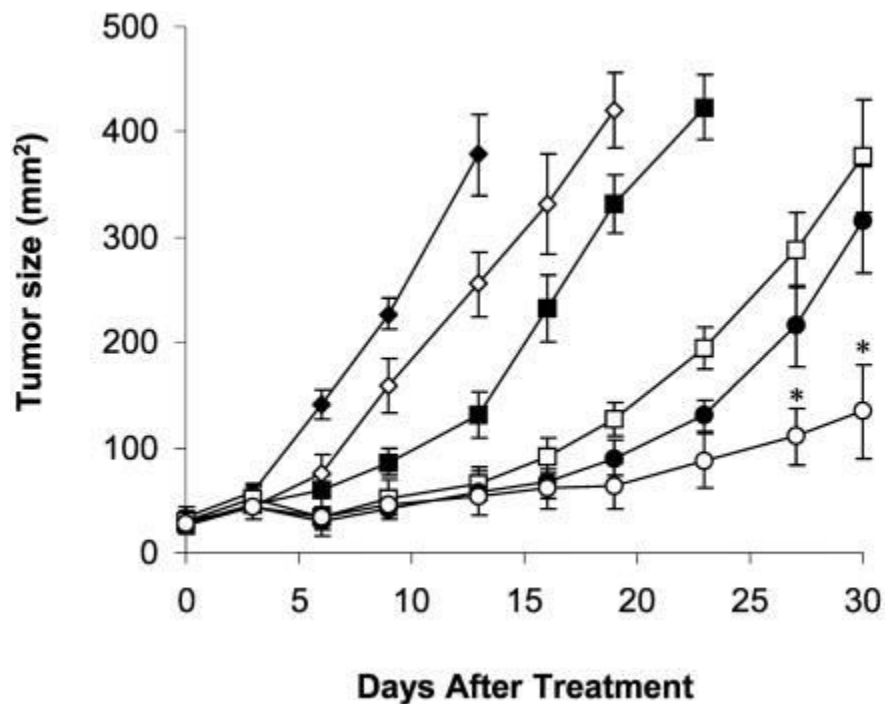


Fig. 5.

Irradiation before adoptive pmel-1 T cells transfer and DC immunization enhances antitumor response. Seven-day subcutaneous B16 tumor-bearing mice were irradiated with 500 rad, followed 1 day later by adoptive transfer of pmel-1 T cells and immunization with DCs bearing either of the hgp100₂₅₋₃₃ or NP₃₆₆₋₃₇₄ peptides plus IL-2 administration. Tumor growth was monitored and compared with that of nonirradiated mice. * $P = 0.009$ for irradiated *versus* nonirradiated hosts receiving DC/hgp100 immunization. Data are representative of two independent experiments with a total of five mice per group. Non-irradiation: (◆) no treatment, (■) DC/NP + IL-2, (●) DC/hgp100 + IL-2. Irradiation: (◇) no treatment, (□) DC/NP + IL-2, (○) DC/hgp100 + IL-2.

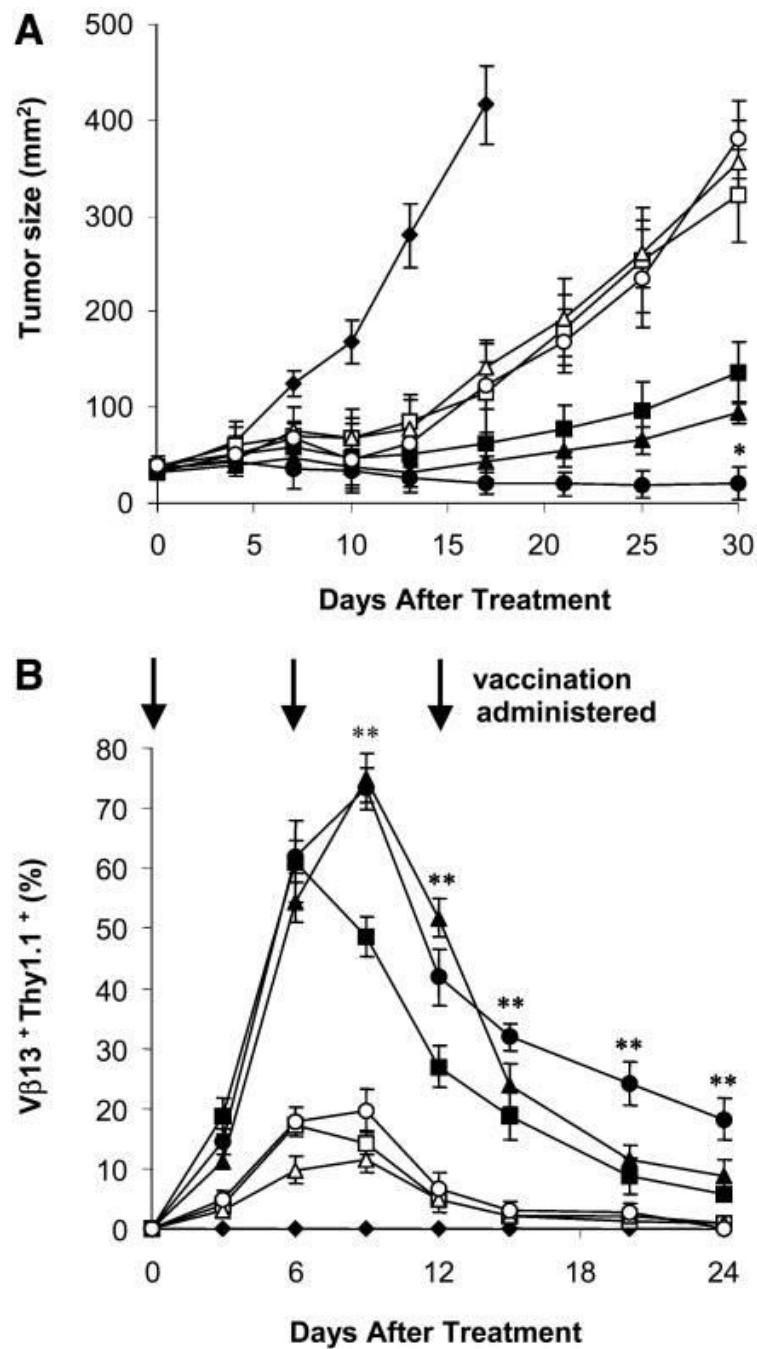


Fig. 6. Sequential DC vaccination plus irradiation in combination with adoptive transfer of pmel-1 T cells provides an optimal antitumor response. Seven-day subcutaneous B16 tumor-bearing mice were irradiated with 500 rad, followed 1 day later by adoptive transfer of pmel-1 T cells and immunization with DCs bearing either of the hgp100₂₅₋₃₃ or NP₃₆₆₋₃₇₄ peptides plus IL-2 administration. Thereafter, indicated mice received one or two booster DC vaccinations at 6-day intervals. (A) Tumor growth of mice was monitored following treatment. (B) Peripheral blood was analyzed by flow cytometry for percentages of pmel-1 cells (Vβ13⁺Thy1.1⁺) in total PBLs at the indicated time points. **P* = 0.013; ***P* = 0.021 for one versus three sequential DC/hgp100 vaccinations. Data are representative of two independent experiments with a total of

five mice per group. (◆) No treatment; pmel-1 + (■) DC/hgp100 + IL-2 (1), (□) DC/NP + IL-2 (1), (▲) DC/hgp100 + IL-2 (2), (△) DC/NP + IL-2 (2), (●) DC/hgp100 + IL-2 (3), (○) DC/NP + IL-2 (3).

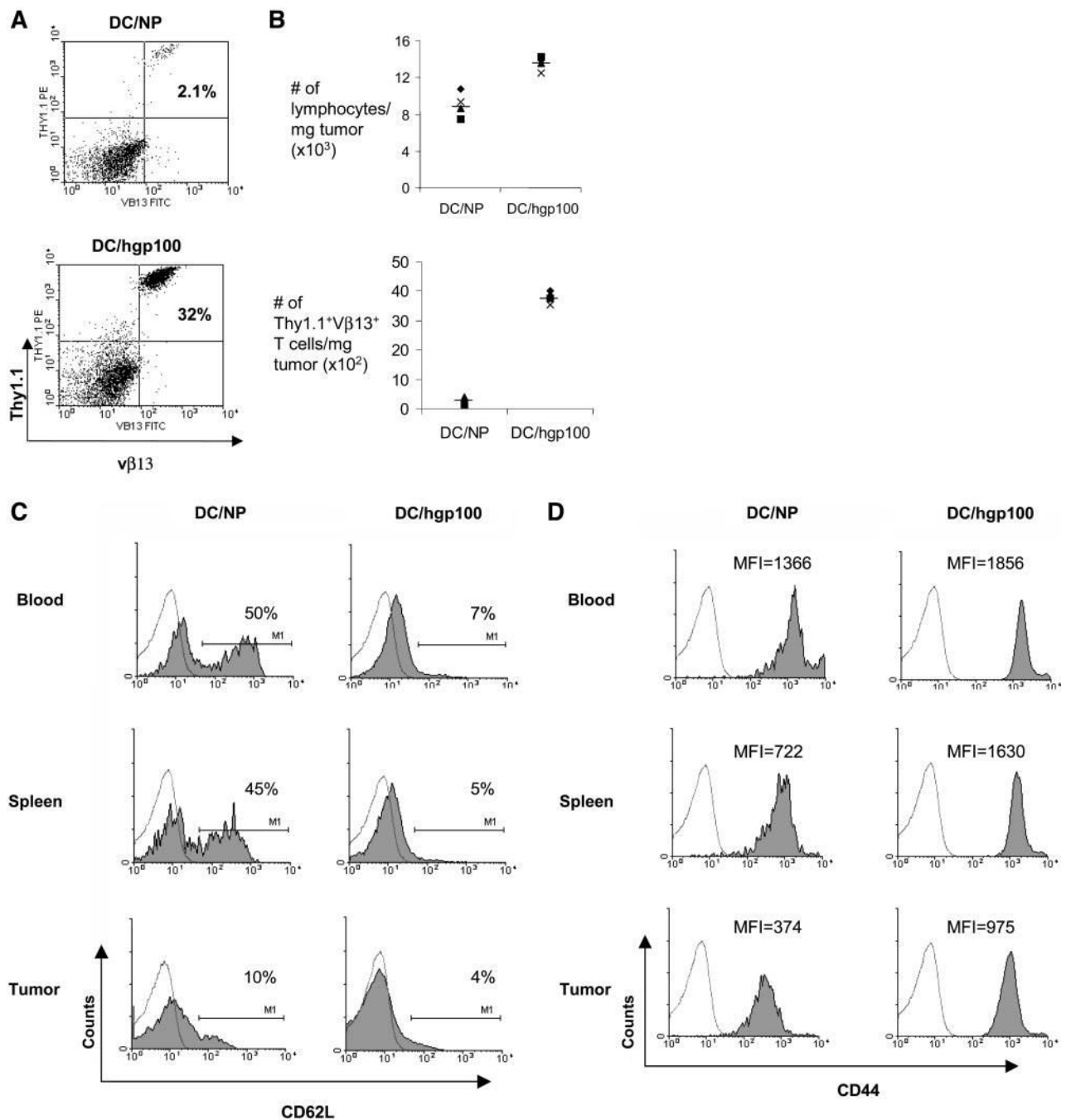


Fig. 7. Antigen-specific DC vaccination enhances the pmel-1 T-cell infiltration into tumors. Seven-day B16 tumor-bearing mice were infused intravenously with pmel-1 T cells, followed by immunization with DCs pulsed with either the hgp100₂₅₋₃₃ or NP₃₆₆₋₃₇₄ peptide plus IL-2 administration. Five days later, blood, spleen, and tumors were harvested, and lymphocytes were isolated and analyzed by flow cytometry. (A) percentage of total tumor-infiltrating lymphocytes that are pmel-1 (V β 13⁺Thy1.1⁺) T cells. (B) number of total lymphocytes and number of pmel-1 cells per milligram of tumor. For (B) each data point represents one individual mouse of four per group. (C and D) expression of activation/homing markers CD62L and CD44 on adoptively transferred pmel-1 T cells. Cells were stained with mAbs against

Thy1.1 and $V\beta 13$ to identify adoptively transferred pmel-1 cells and then stained with CD62L and CD44 mAbs, respectively. Histograms are gated on pmel-1 cells ($V\beta 13^+Thy1.1^+$). Specific staining is depicted as a filled histogram with isotype control as an open histogram.