

Transfer of *in vitro* expanded T lymphocytes after activation with dendritomas prolonged survival of mice challenged with EL4 tumor cells

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Abstract. Adoptive T cell transfer after *in vitro* expansion represents an attractive cancer immunotherapy. The majority of studies so far have been focusing on the expansion of tumor infiltrated lymphocytes (TIL) and some have shown very encouraging results. Recently, we have developed a unique tumor immune response activator, dendritomas, by fusion of dendritic cells and tumor cells. Animal studies and early clinical trials have shown that dendritomas are able to activate tumor specific immune responses. In this study, we hypothesized that naïve T cells can be primed with dendritomas and expanded *in vitro* to develop an adoptive transfer therapy for patients who do not have solid tumors, such as leukemia. T cells were isolated and purified from lymph nodes of mice. The cells were then incubated with dendritomas made from syngeneic DCs and tumor cells and expanded *in vitro* using Dynabeads mouse CD3/CD28 T cell expander for approximately three weeks. The *in vitro* primed and expanded T cells showed tumor cell specific CTL activity and increased secretion of IFN- γ . Tumor bearing mice receiving the *in vitro* expanded T cells survived significantly longer than control mice. Furthermore, the depletion of regulator T cells enhanced the survival of the mice that received the adoptive transfer therapy.

Introduction

Dendritic cells (DC) are professional antigen presenting cells that play a vital role in stimulating immune responses. DCs not only activate naïve CD4⁺ T helper cells but also stimulate unprimed CD8⁺ T lymphocytes (1-4). Recent studies have also demonstrated that DCs are also involved in the innate

immune responses (5,6). Because of these characteristics, DCs have been widely studied as antigen presenting cells for cancer immunotherapy (7). We have developed a novel technique by which hybrid cells can be easily purified from a fusion mixture of dendritic cells and tumor cells (8). By using this technique, hybrid cells were instantly purified from fusions between DCs and tumor cells and named dendritomas. Dendritomas retained the characteristics of the tumor cell as well as the ability of the dendritic cell to act as an effective APC. Our animal studies have confirmed that dendritomas are better activators than fusion mixtures in stimulating tumor specific anti-tumor immunity (9). *In vitro* studies using human cells also showed that dendritomas made from patients' peripheral blood DCs and autologous primary tumor cells efficiently lysed autologous tumor cells (8). Our phase I studies using dendritomas as a vaccine on melanoma and renal cell carcinoma patients not only demonstrated anti-tumor immune responses but also clinical responses (10, unpublished data).

However, the success of the dendritoma vaccine depends on a functional immune system to kill cancer cells. Unfortunately, the immune system of the leukemia patients is always compromised either by the disease itself or by the chemotherapy treatment or both. Therefore, direct administration of dendritomas into leukemia patients would not be very successful.

T lymphocytes play a key role in maintaining anti-tumor immunity. Consequently, they provide an important opportunity for the immunotherapy of cancer. In adoptive immunotherapy, T cells with anti-tumor activity are transferred into tumor-bearing hosts. Successful therapy depends on the type of T cells transferred and their effector functions, the ability of the cells to reach the tumor location, and the ability of the cells to overcome any tolerance or immunosuppression in the host (11-13).

Animal studies have demonstrated that adoptive immunotherapy with tumor-reactive T lymphocytes has been highly effective against established tumors (14-16). The clinical application of the principles has also been investigated in human trials with encouraging results (17-21). However, despite the occurrence of durable complete tumor responses in clinical studies, it is often confined to a minority of treated

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patients. The inability to generate a large amount of tumor-reactive T lymphocytes *in vitro* from a tumor bearing host has been a major impediment for the development of adoptive immunotherapy of cancer. Recently, a new development has been made to expand T cells either primed or unprimed to a significant scale *in vitro* and these *in vitro* expanded T cells effectively destroy tumors *in vivo* (22). The same study also showed that after expansion, the previously primed T cells demonstrated better anti-tumor activity.

In this study, we combined the above mentioned two technologies: using dendritoma to prime naïve T cells and the T cell expander (Dynabeads mouse CD3/CD28 T cell Expander, Dynal Biotech) to generate tumor cell specific killer T lymphocytes and tested their anti-tumor effects in mouse models. Dendritomas generated from murine dendritic cell and tumor cell fusion were used to activate lymphocytes *in vitro*. Lymphocytes that were primed specific to tumor cells were then expanded *in vitro* using the Dynabeads coupled with anti-CD3/CD28 antibodies. After *in vitro* confirmation of their activities as indicated by interferon- γ (IFN- γ) production and cytolytic activity (CTL), the anti-tumor activity of the expanded lymphocytes was tested in animal models with transplantable tumors.

Materials and methods

Animals. Female C57BL/6J mice and nude mice, 6-8 weeks of age, were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in our pathogen-free animal facilities.

Tumor cell and DC culture. EL4 tumor cells were cultured in DMEM (Gibco BRL, Grand Island, NY) containing 10% horse-serum (Hyclone, Logan, UT) and 50 μ g/ml of gentamicin (Gibco BRL). Bone marrow DCs were cultured as previously described (9). Briefly, bone marrow cells were flushed from the femur and tibia bones of female C57BL/6J mice (Jackson Laboratories) with RPMI-1640 and passed through a 40- μ m cell strainer. After removing the red blood cells by lysis using ACK lysing solution (0.15 M NH_4Cl , 1 mM KHCO_3 , and 0.1 mM Na_2EDTA , pH 7.3) at room temperature for 5 min, the bone marrow cells were suspended in DC medium containing RPMI-1640 (Gibco BRL), 10% FBS, 50 μ g/ml gentamicin and 20 ng/ml rmGM-CSF (Sigma, St. Louis, MO) and seeded into 100-mm bacterial culture Petri dishes at a concentration of 2×10^6 cells/10 ml/100-mm dish. At day 3, 10 ml of fresh DC medium was added to each dish. At day 6, half of the medium was removed and replaced with fresh DC medium containing 10 ng/ml rmGM-CSF. At day 8, non-adherent cells were transferred to a tissue culture dish and fed with fresh DC medium containing 10 ng/ml rmGM-CSF, 100 ng/ml murine tumor necrosis factor (mTNF- α , Sigma). At day 10, the non-adherent cells (>70% mature DCs) were collected for fusion.

Fluorescent dye staining and fusion. Tumor cells and DCs were stained red and green, respectively, using PKH26-GL and PKH67-GL kits (Sigma) according to the manufacturer's directions. After washing to remove any unbound dyes, the tumor cells were irradiated at a dose of 100 Gy and fused with DCs at a ratio of 1:2 using polyethylene glycol (PEG, Sigma)

according to a previously described protocol (28). After fusion, the cells were suspended in DC medium and incubated overnight at 37°C in 5% CO_2 .

FACS sorting. The overnight culture of fused mixtures (both adherent and non-adherent cells) was collected and resuspended in PBS at a concentration of 1×10^7 cells/ml for sorting. The hybrid cells (dual color) were gated and sorted out using a FACScalibur (Becton Dickinson, San Jose, CA). The sorted cells (dendritomas), displaying both green and red fluorescence, were harvested and resuspended in PBS for *in vitro* activation of T cells.

T cell activation and expansion. T lymphocytes were isolated from mouse lymph nodes using the T cell Negative Isolation Kit from Dynal Biotech. T cells were then activated with dendritomas by co-culturing at a 5:1 ratio in RPMI medium supplemented with 10% FBS, 1% gentamicin, 20 IU/ml of human IL-2, 5 ng/ml of IL-12 and 1000 IU/ml of IL-6 at 5×10^6 cells/well in 6-well plates in 5 ml of medium. One day later T cells were expanded with the mouse CD3/CD28 T Cell Expander Kit from Dynal Biotech at a bead to cell ratio of 1:1 and cultured in RPMI medium supplemented with 10% FBS and 60 IU/ml of human IL-2. Cells were examined every day and counted at least twice each week; fresh medium was added as needed and cells were passed into more wells as determined by cell concentration (normally 0.5- 2×10^6 /ml). One week later, the expanded T cells were restimulated with more fresh beads at a bead to cell ratio of 1:1. The expansions of the restimulated T cells were kept for 2 or 3 more weeks by passing into more wells.

FACS analysis of the expanded T cells. T cells in expansion were collected, stained with various antibodies conjugated with either FITC or PE (BD Biosciences, San Jose, CA), and analyzed with a FACScalibur according to the standard procedures.

IFN- γ assay and CTL assay. For IFN- γ ELISA, T cells were seeded into wells of 24-well plates (1×10^6 cells/well in 2 ml medium). Supernatants of expanded T cell cultures were collected and tested using an IFN- γ ELISA kit (BD Biosciences). A 6-h chromium release assay was performed to determine the cytolytic activity of these expanded T cells. Tumor cells were labeled with ^{51}Cr and incubated with T cells at ratios of 1:100, 1:30, 1:10 and 1:3. Six hours later, the radio activity of the supernatants of the incubations was measured by liquid scintillation counting. The specific lysis was calculated as (experimental CPM - spontaneous CPM)/(maximum CPM - spontaneous CPM) \times 100.

Tumor study. In order to determine the anti-tumor activity of the expanded T cells, female C57BL/6J mice were challenged with EL4 tumor cells by intravenous injection. Three days later, the tumor bearing mice were infused with three doses of the *in vitro* expanded T cells (10×10^6 /mouse/day). In order to boost the T cell proliferation *in vivo*, the mice were administered with human IL-2 by intraperitoneal injection at a dose of 20,000 IU/day/mouse for 5 days. T cells that were also expanded *in vitro*, but not activated with dendritomas,

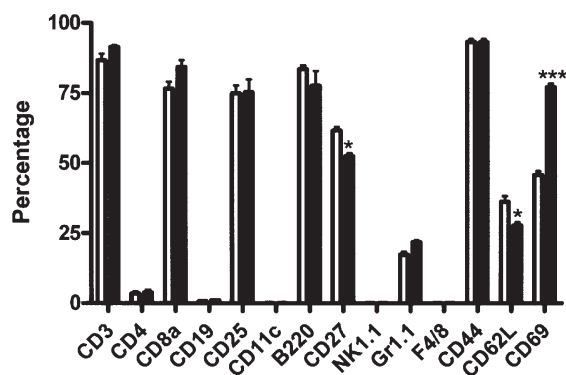


Figure 1. Phenotype analysis of *in vitro* expanded T cells. Lymph node T cells were expanded with the mouse CD3/CD28 T Cell Expander Kit from Dynal Biotech with (solid bars) or without (open bars) dendritoma priming. Two weeks after expansion, the cells were harvested, stained with antibodies as labeled, and analyzed on a FACScalibur.

were used in the study as a control. In some other experiments, anti-CD25 or anti-CTLA4 antibodies were injected before the infusion of the T cells.

Statistical analysis. GraphPad Prism version 4 software was used in this study to generate graphs and to perform the statistical analysis using either one-way ANOVA or two-way ANOVA depending on the designs. The survival curve statistical analysis was also used.

Results

***In vitro* expansion of lymph node T cells.** T lymphocytes were isolated from lymph nodes of naïve C57BL/6J mice using the T cell Negative Isolation Kit from Dynal Biotech. T cells were then activated with dendritomas by co-culturing at a 5:1 ratio in RPMI medium supplemented with 10% FBS, 1% gentamicin, 20 IU/ml of human IL-2, 5 ng/ml of IL-12 and 1000 IU/ml of IL-6 at 5×10^6 cells/well in 6-well plates in 5 ml of medium for 24 h. The T cells were then expanded with the mouse CD3/CD28 T Cell Expander Kit from Dynal Biotech at a bead to cell ratio of 1:1 and cultured in RPMI medium supplemented with 10% FBS and 60 IU/ml of human IL-2. The same unprimed T cells isolated from lymph nodes were also expanded as controls. After three- to four-week expansion, the T cells grew by more than 100-fold. There was no significant growth difference between the dendritoma primed T cells and control T cells (data not shown).

Phenotype of the expanded T cells. T cells expanded for three to four weeks *in vitro* were analyzed for their surface marker expression by FACS. The cells were stained with different antibodies and analyzed on a FACScalibur. Surprisingly, the majority of the expanded cells were CD3⁺ and CD8⁺ T cells (>80%); while only about 2-3% were CD4⁺ T cells. There were almost no B cells or NK cells (<1%) in the culture either. Compared to unprimed T cells, the dendritoma priming prior to expansion significantly increased the number of CD69⁺ cells ($p < 0.001$). On the other hand, the numbers of CD27⁺ cells ($p < 0.05$), and CD62L⁺ cells ($p < 0.05$) were significantly decreased (Fig. 1).

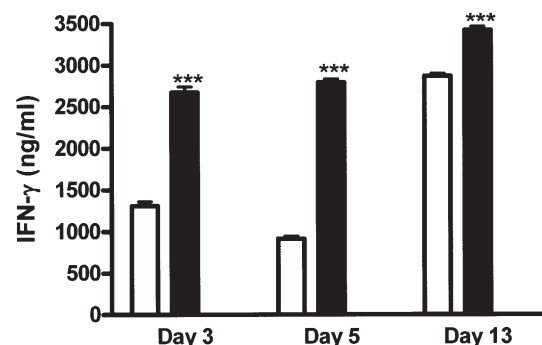


Figure 2. IFN- γ analysis of *in vitro* expanded T cells. Supernatants of T cells in expansion were taken at different time-points and assayed for IFN- γ using the IFN- γ ELISA kit (BD Biosciences). Open bars represent samples from unprimed, but *in vitro* expanded T cells; solid bars represent samples from dendritoma primed and *in vitro* expanded T cells.

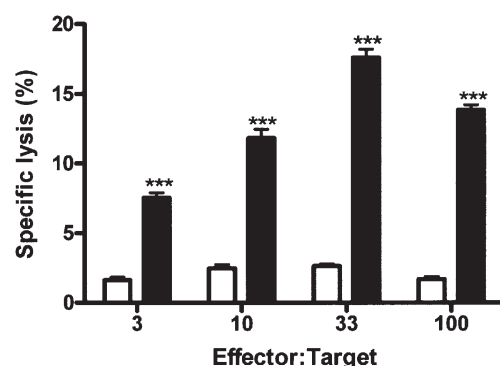


Figure 3. CTL activity of *in vitro* expanded T cells. T cells expanded *in vitro* for two weeks were harvested and used as effector cells to lyse ^{51}Cr -labeled EL4 cells at different ratios. Open bars represent samples from unprimed, but *in vitro* expanded T cells; solid bars represent samples from dendritoma primed and *in vitro* expanded T cells.

IFN- γ expression. The expression of IFN- γ by the expanded T cells was detected by ELISA. At all three checking points (days 3, 5, 13), the levels of IFN- γ expression by T cells that were primed with dendritomas and expanded were significantly higher than those of the unprimed but expanded T cells ($p < 0.001$, Fig. 2).

CTL activity. In order to determine whether the expanded T cells are able to kill the tumor cells *in vitro*, a standard 6-h chromium 51 release CTL assay was performed using EL4 tumor cells as targets. The results demonstrated that dendritoma primed and *in vitro* expanded T cells were able to specifically lyse tumor cells, while the unprimed T cells had much lower CTL activities (Fig. 3).

Animal studies. The infusion of the unprimed *in vitro* expanded T cells increased the survival of the EL4 tumor bearing mice slightly, but was not statistically significant ($p > 0.05$), while the infusion of the dendritoma primed *in vitro* expanded T cells significantly increased the survival of the tumor bearing mice ($p < 0.05$, Fig. 4A). The injection of IL-2 after T cell infusion enhanced the survival of the tumor bearing mice. Fifty percent of the mice survived >50 days after receiving the combined

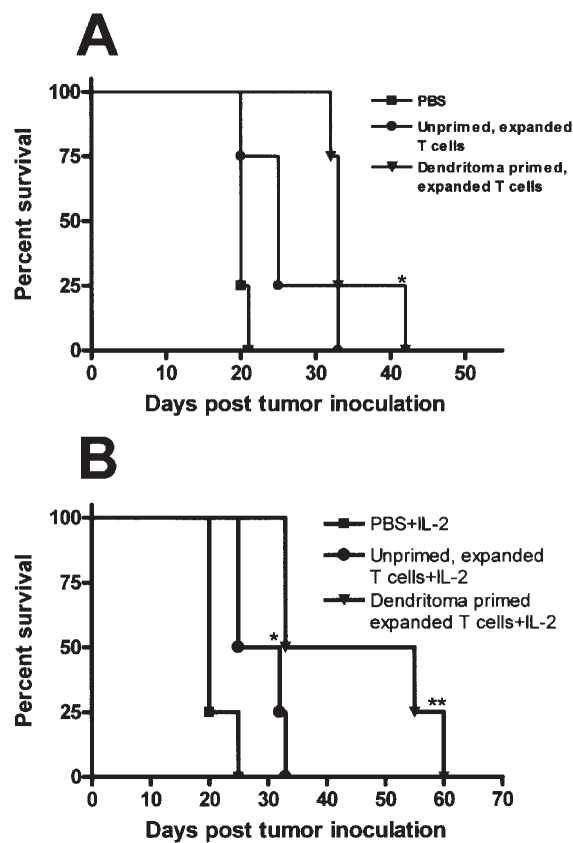


Figure 4. Infusion of dendritoma primed and *in vitro* expanded T cells improved the survival of EL4 tumor bearing mice. Female C57BL/6J mice were i.v. injected with 5×10^5 EL4 tumor cells. Three days later, the mice were i.v. injected with PBS, 10×10^6 *in vitro* expanded T cells, or dendritoma primed and *in vitro* expanded T cells, respectively, once a day for three consecutive days without (A) or with (B) IL-2 therapy. The survival of these mice was then monitored.

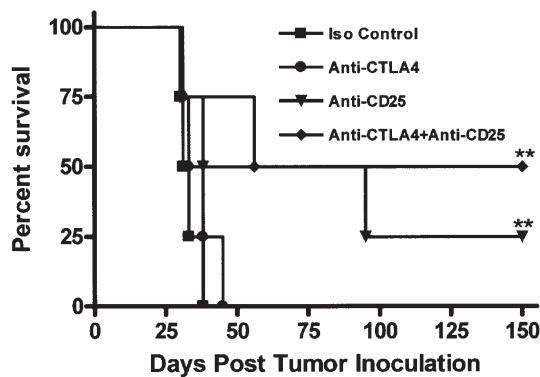


Figure 5. Survival study of T cell infusion combined with anti-CTLA4 and/or anti-CD25 antibodies. Female C57BL/6J mice were i.v. injected with 5×10^5 EL4 tumor cells. Two days later, the mice were i.p. injected with PBS, $100 \mu\text{g}$ anti-CTLA4 antibody/mouse, $100 \mu\text{g}$ anti-CD25 antibody/mouse, or $100 \mu\text{g}$ anti-CTLA4 antibody/ $100 \mu\text{g}$ anti-CD25 antibody/mouse, respectively. On the next day, the mice were i.v. injected with 5×10^6 dendritoma primed and *in vitro* expanded T cells once a day for three consecutive days. The survival of these mice was then monitored.

treatment of dendritoma primed *in vitro* expanded T cells and IL-2, while the control mice that received IL-2 alone lived <27 days (Fig. 4B).

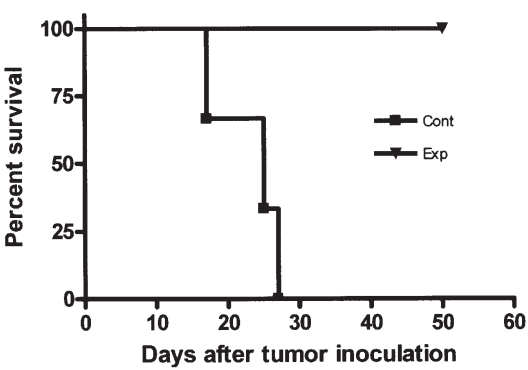


Figure 6. An antitumor effect was initiated by the injection of anti-CTLA4/CD25 antibodies and the dendritoma primed *in vitro* expanded T cells successfully rejected further tumor challenge. The mice that received anti-CTLA4/CD25 antibodies and dendritoma primed *in vitro* expanded T cells were tumor-free for five months from the previous experiment in Fig. 5 and some regular C57BL/6J mice were i.v. re-injected with 5×10^5 EL4 tumor cells. The survival of these mice was monitored.

The treatment of anti-CTLA4 antibody before the infusion of dendritoma primed *in vitro* expanded T cells did not enhance the survival of the tumor bearing mice compared to the isotype antibody treatment. However, the treatment of anti-CD25 antibody before the T cell infusion significantly enhanced the survival of the tumor bearing mice ($p < 0.01$). When both antibodies were used, 50% of the mice lived longer than 150 days (Fig. 5). Furthermore, 100% of the mice that survived the initial tumor challenge were resistant to further tumor challenge (Fig. 6).

Discussion

Adoptive T cell transfer represents a very promising immunotherapy. Many studies have shown the effectiveness of the therapy (11-13). However, tumors that develop in the presence of a competent immune system evolve complex immune evasion strategies to avoid and subvert T cell-mediated killing (23). Therefore, T cells isolated from this environment can be expanded and may show some CTL activity *in vitro*, but may not have therapeutic effect when introduced back to the patient. Approaches have been taken to overcome this problem, such as the genetic modification of antigen-specific T cells to allow them to perform better *in vivo* and conditioning the host to improve *in vivo* expansion and function of transferred cells. In this study, we primed T cells isolated from naïve mice with dendritomas, an effective-tumor specific T cell activator (9), and then expanded them *in vitro* in order to develop a new approach for adoptive T cell transfer by using either naïve donor T cells or patient T cells for leukemia patients.

An early study showed that the anti-CD3/CD28 bead expansion system favors the expansion of CD4⁺ T cells isolated from lymph nodes and the longer the cells were expanded, the more dominant the CD4⁺ T cells were (22). In contrast, our study showed that CD8⁺ T cells dominated the cell expansion (>80%), while CD4⁺ T cells were only 3-4% in the culture (Fig. 1). This difference may be due to the fact that the mice we used to isolate T cells were naïve mice, while the mice in the previous study were tumor bearing mice. The

dendritoma priming significantly increased the number of CD69⁺ T cells ($p < 0.001$), an indicator of T cell activation. On the other hand, the dendritoma priming significantly decreased the numbers of CD27⁺ cells and CD62L⁺ cells. Gattinoni *et al* reported that naïve CD8⁺ T cells express high levels of CD62L and CD27, while the effector T cells express no CD62L and low levels of CD27 (24). Together, this suggests that the dendritoma priming may facilitate the differentiation of a CD8⁺ T cell to an effector cell. The results of IFN- γ assay and CTL assay (Figs. 2 and 3) also demonstrated that the dendritoma priming activated the CD8⁺ T cells.

IL-2 has been widely used in adoptive T cell transfer because of its ability to boost cell proliferation after infusion. In this study, the injection of IL-2 after T cell transfer significantly increased the survival of the mice compared to either T cell transfer alone or unprimed T cell transfer plus IL-2 (Fig. 4). Previous studies have shown that the blockade of the CTLA4 signal transduction in T cells (25,26) or the depletion of CD4⁺CD25⁺ regulatory T cells (27) enhances the anti-tumor activity of transferred T cells. In this study, the treatment of anti-CTLA4 antibody alone did not enhance the anti-tumor activity of infused T cells. On the other hand, the injection of anti-CD25 antibody before T cell infusion significantly enhanced the survival. Furthermore, when combined with anti-CTLA4 antibody, 50% of the mice were tumor-free five months after tumor injection while the control mice died within a month (Fig. 5). All of these tumor-free mice survived a second tumor challenge (Fig. 6), indicating that memory T cells were developed in these mice.

In conclusion, naïve lymph node T cells can be successfully primed with dendritomas and expanded *in vitro*. The majority of expanded T cells in this system are CD8⁺ T cells which show anti-tumor activity both *in vitro* and *in vivo*. The depletion of CD4⁺CD25⁺ regulatory T cells not only enhanced the anti-tumor activity of the *in vitro* primed and expanded T cells after infusion but also facilitated the development of memory T cells in mice.

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