ORIGINAL ARTICLE

Treatment of pulmonary metastatic tumors in mice using lentiviral vector-engineered stem cells

X Zhang^{1,3}, P Zhao^{1,3}, C Kennedy¹, K Chen², J Wiegand^{1,2}, G Washington¹, L Marrero^{1,2} and Y Cui^{1,2}

¹Gene Therapy Program, Department of Medicine, Louisiana State University Health Sciences Center, New Orleans, LA, USA and ²Department of Genetics, Louisiana State University Health Sciences Center, New Orleans, LA, USA

Active cancer immunotherapy relies on functional tumor-specific effector T lymphocytes for tumor elimination. Dendritic cells (DCs), as most potent antigen-presenting cells, have been popularly employed in clinical and experimental tumor treatments. We have previously demonstrated that lentiviral vector-mediated transgene delivery to DC progenitors, including bone marrow cells and hematopoietic stem cells, followed by transplantation supports systemic generation of great numbers of tumor antigen-presenting DCs. These DCs subsequently stimulate marked and systemic immune activation. Here, we examined whether this level of immune activation is sufficient to overcome tumor-induced tolerogenic environment for treating an established aggressive epithelial tumor. We showed that a combination treatment of granulocyte macrophage-colony stimulating factor and cytosine-phosphate-guanine-containing oligonucleotide stimulated large numbers of tumor antigen-presenting DCs *in situ* from transgene-modified stem cells. Moreover, these *in situ* generated and activated DCs markedly stimulated activation of antigen-specific CD4 and CD8 T cells by augmenting their numbers, as well as function, even in a tumor-bearing tolerogenic environment. This leads to significant improvement in the therapeutic efficacy of established pulmonary metastases. This study suggests that lentiviral vector-modified stem cells as DC progenitors may be used as an effective therapeutic regimen for treating metastatic epithelial tumors. *Cancer Gene Therapy* (2008) **15**, 73–84; doi:10.1038/sj.cgt.7701108; published online 14 December 2007

Keywords: antigen-specific CTL function; antitumor immunity; dendritic cells; hematopoietic stem cells; lentiviral vector; renal cell carcinoma

Introduction

Successful tumor immunotherapy requires effective mobilization of activated T cells to tumor sites for productive tumor eradication. This is especially vital for the treatment of epithelial tumors, as the accessibility of T cells to solid tumors is usually limited and tolerance to tumor antigens develops shortly after they encounter tumors (reviewed by Finn¹ and Pardoll²). Thus, therapeutic strategies to persistently recruit antigen-specific T cells to tumor sites and, more importantly, to maintain their effector function in tumor-induced tolerogenic environment will be crucial.^{3–7}

Dendritic cells (DCs), especially activated DCs, are considered optimal candidates for active cancer immunotherapy and have been widely used as vaccines for

experimental and clinical treatment of various types of hematological and epithelial malignancies (reviewed in references^{8–10}). However, DC vaccines in their current form, with *ex vivo* tumor antigen-loaded DCs^{11–13} or other new strategies in targeting tumor antigen delivery to *in vivo* derived DCs^{14–16} do not facilitate the migration and/or localization of a large number of activated tumor antigen-presenting DCs to lymphoid compartments, which are determining factors for a high level of immune activation and therapeutic efficacy. New improvements to increase the number of injected DCs and employing DC-differentiating cytokines, such as the Flt-3 ligand or granulocyte macrophage-colony stimulating factor (GM-CSF), and DC activation agents, such as ligands of the Toll-like receptor (TLR) or CD40 receptor^{15,17–21} appear to improve DC numbers and/or activation status and, thus, antitumor immunity. Nevertheless, the limited longevity and migratory capacity of *ex vivo* prepared tumor antigen-loaded DCs postinjection restrict the durability of immune activation.^{22–25}

To greatly enhance the number and durability of tumor antigen-presenting DCs in the lymphoid compartment, we developed a new stem cell-based approach with lentiviral vector (LV)-mediated tumor antigen gene delivery.^{17,26,27}

Correspondence: Dr Y Cui, Gene Therapy Program, Department of Medicine, Louisiana State University Health Sciences Center, 533 Bolivar Street, CSRB 601F, New Orleans, LA 70112, USA.
E-mail: ycui@lsuhsc.edu

³These authors contributed equally to this work.

Received 22 July 2007; revised 9 October 2007; accepted 10 November 2007; published online 14 December 2007

Because bone marrow (BM) cells or hematopoietic stem cells (HSC) are DC progenitors and can be efficiently transduced by LVs,^{17,26,28–30} we believe that LV-modified stem cells would serve as a constant pool of tumor antigen gene carrying DC progenitors posttransplantation. Recently, we demonstrated that upon appropriate cytokine and immune adjuvant treatments, large numbers of activated tumor antigen-expressing DCs are differentiated and situated in multiple lymphoid tissues to stimulate a strong antitumor immunity,^{17,27} which is capable of eliminating established hematological malignancy.^{17,27}

In this study, we examine whether this stem cell-based immunotherapy approach would provide sustained systemic antitumor immunity to recruit sufficient numbers of tumor-specific T cells to established metastatic epithelial tumors and overcome tumor-induced immune tolerance in maintaining their function. More importantly, we want to determine whether this level of T-cell activation and effector function would translate to marked improvement in controlling the size and metastases of solid tumors, as well as significant therapeutic benefit in prolonged survival of mice with established pulmonary metastases.

Materials and methods

Animals and cell lines

BALB/c mice (6- to 8-week old) of either gender were purchased from Charles River (Wilmington, MA). Transgenic (Tg) mice clone 4, with TCR specifically recognizing influenza hemagglutinin (HA) major histocompatibility complex (MHC) I (HA_{518–526}) epitope, were generated in Dr Sherman's laboratory (Scripps Research Institute, La Jolla, CA). Tg mice line 6.5, whose TCR specifically recognize MHC II (HA_{110–120}) epitope, was generated in Dr Von Boehmer's laboratory (Harvard Medical School, Boston, MA). They were bred in the Louisiana State University Health Sciences Center (LSUHSC) animal care facility in accordance with protocols approved by the Animal Care and Use Committee. Human embryonic kidney 293T cells and mouse mastocytoma p815 cell line were obtained from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 50 units per ml penicillin-streptomycin. GM-CSF producing cell line B78H1-GM³¹ was maintained in RPMI 1640 medium (Invitrogen), supplemented with 10% FBS, 50 units per ml penicillin-streptomycin, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1 mM sodium pyruvate and 0.1 mM nonessential amino acids.

Antibodies, HA peptides and CpG-containing oligodeoxynucleotide

Various fluorochrome-conjugated antibodies against murine CD4, CD8, Thy1.1 (CD90.1), CD44 and CD62L were purchased from BD Biosciences (San Diego, CA). MHC I and II restricted HA peptides,⁵¹⁸ IYSTVASSL⁵²⁶ and ¹¹⁰SFERFEIIPKE¹²⁰, were ordered from the LSUHSC peptide core laboratory. PE-conjugated Pro5

HA-specific MHC I pentamer was purchased from ProImmune (Oxford, UK). Stimulatory cytosine-phosphate-guanine (CpG)-containing oligodeoxynucleotide (ODN) 1826 (T*C*C*ATGACGTTCTGAC*G*T*T) was synthesized through IDT (Coralville, IA) with phosphorothioate bond (*) modification for enhanced *in vivo* stability.

Lentiviral production and transduction of murine bone marrow cells

Lentiviruses (LV) EF.GFP and EF.HA were produced through cotransfection of transducing vectors EF.GFP or EF.HA with the packaging construct pCMVdl-8.4 (kindly provided by Dr Naldini, Vita Salute San Raffaele University) and enveloped pMD.G to 293T cells using conventional calcium phosphate precipitation method as previously described.²⁶ Mouse BM cells, harvested from femurs and tibias of BALB/c mice, were transduced with EF.GFP or EF.HA LVs as previously described.²⁷ The transduction of efficiency of these cells was confirmed by flow cytometry analysis of green fluorescent protein (GFP) or HA expression via specific antibody staining. They were then transplanted to lethally irradiated (850 cGy) syngeneic BALB/c mice at 4×10^6 BM per mouse with an additional 1×10^7 fresh splenocytes to reduce radiation toxicity via lateral-orbital vein injection.

In vivo differentiation of transgene-expressing DCs to stimulate antigen-specific T-cell activation

In 1–2 weeks after transplantation of LV-modified BM, mice were treated with 1×10^7 B78H1-GM cells, as previously described, to promote *in vivo* differentiation of antigen-expressing DCs from LV-modified BM.²⁷ Some of the mice were also treated with CpG-containing ODN at 100 µg per mouse per day on days 4 and 6 post-GM-CSF treatment. To examine antigen-specific T-cell activation, 3×10^6 HA-specific CD4⁺ or CD8⁺ T cells per mouse from line 6.5 or clone 4 Tg mice was adoptively transferred to the above-treated mice. For monitoring *in vivo* proliferation of these T cells, they were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen) (5 µM for 5 min at room temperature). At various time points, spleen, peripheral lymph nodes (LN) and tumor-draining hilar nodes were harvested. The percentage of HA-specific T cells and their CFSE level were examined via specific antibody recognizing Tg TCR or pentamer followed by fluorescence-activated cell sorting (FACS) analysis. Background staining of T cells from mice that did not receive adoptive transfer of HA-specific T cells was usually less than 0.1%. For functional analyses, these HA-specific CD4 or CD8 T cells were further enriched from lymphoid tissues via magnetic antibody cell sorting beads-based positive selection (Miltenyi Biotec, Auburn, CA). Proliferation capability of HA-specific CD4 T cells was determined via ³H-thymidine incorporation or CFSE dilution in a 3-day culture in the presence or absence of HA MHC II peptide (10 µg ml^{−1}). The cytotoxic T lymphocytes (CTL) function of activated CD8 T cells was evaluated via a new FACS-based CTL assay developed in our laboratory.³²

Murine Renca-HA pulmonary metastases and immunotherapy treatment

Renca-HA 19 stably expressing HA antigen of the influenza virus A/PR/8/34 was cultured in supplemented RPMI medium. Pulmonary metastases were established via i.v. inoculation of 1×10^6 Renca-HA cells to syngeneic BALB/c mice. Tumor burdens were evaluated by the number and size of tumor nodules in the lungs at various time points post-inoculation.

For the therapeutic treatment of these tumor-bearing mice, LV-modified BMs were transplanted (as described above) 1 week after tumor inoculation. Seven days post-bone marrow transplantation (BMT), these mice were then treated with GM-CSF and/or ODN as described above. In parallel, Renca-HA-bearing mice, unmanipulated or transplanted with LV-modified BM and treated with phosphate-buffered saline (PBS) were used as controls. To monitor HA-specific immune responses following our therapeutic treatment regimens in tumor environment, $0.5\text{--}3 \times 10^6$ CFSE-labeled HA-specific CD4 or CD8 T cells were transferred to those Renca-HA-bearing mice. The percentage and immunological status of these HA-specific T cells in the spleen, tumor-draining hilar nodes and peripheral blood were analyzed via FACS. The healthiness of all the tumor-bearing mice was monitored daily. When any signs of unhealthiness were present, they were euthanized. Once the tumor burden was confirmed by autopsy, their death was recorded as tumor-related mortality.

Histology and immunohistochemistry evaluation of tumor burden and infiltrating lymphocytes

At various time points during treatment, some Renca-HA-bearing mice were euthanized and their lungs were inflated with 30% of Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan) in PBS. They were then flash-frozen in liquid N₂-chilled isopentane and sectioned at 10 μ m in thickness. The number and size of the tumor nodules were determined in hematoxylin-eosin (H&E)-stained sections. Tumor-infiltrating T lymphocytes were stained with rat anti-mouse CD3, followed by goat anti-rat immunoglobulin G conjugated with horseradish peroxidase, and diaminobenzidine (DAB) staining (Chromogenic Kit from Dako, Carpinteria, CA). These sections were counterstained with hematoxylin. The dark brown tumor-infiltrating T cells were visualized using a Nikon Eclipse E600 microscope (Nikon Instruments Inc., Melville, NY) and imaged with a digital camera. To quantitatively determine the tumor burden and tumor-infiltrating T cells, photomicrographs of entire representative lung sections were obtained using a Nikon Eclipse E600 upright brightfield microscope (Nikon Instruments Inc.). The images were loaded to Spot Advanced imaging software (Diagnostic Instruments Inc., Sterling Heights, MI), where the borders of tumor nodules were traced (Figure 5d). The ratio of total tumor nodule area to total lung area was calculated from these images using the Spot Advanced imaging program. Quantification of CD3+ cell area was performed on a total of 10 nodules per section by thresholding DAB+ signals and masking

subsampling with Slidebook software (Intelligent Imaging Innovations, Denver, CO; Figure 5d). The total CD3 area/tumor nodule area was then calculated.

Statistical analyses

The differences in number of HA-specific T cells and effector function of different treatment groups were analyzed via Student's *t*-test using SigmaPlot (Systat Software Inc., Point Richmond, CA) or Microsoft Excel (Redmond, WA). Improvement in survival of tumor-bearing mice between each treatment group was determined by one-way analysis of variance (ANOVA) using Microsoft Excel. Statistical significance was set at $P < 0.05$.

Results

Aggressive renal cell carcinoma pulmonary metastases induce immune tolerance in BALB/c mice and result in rapid tumor-related death

The HA antigen has been popularly used in tumor immunology studies as a model tumor antigen. Introduction of HA to mouse tumors, such as renal cell carcinomas (Renca-HA), does not change their *in vitro* growth or *in vivo* establishment in immune-competent mice.^{19,33} As shown in Figure 1a, 1 week after i.v. inoculation of 1×10^6 Renca-HA cells in BALB/c mice, microscopic lung metastases were established, which became macroscopically visible nodules by the second week post-tumor inoculation. By 3 weeks post-inoculation, tumor masses occupied 50–70% of the lung space and further expanded to occupy ~90% of lung tissue by 4 weeks (Figure 1a). These rapid and massive metastases resulted in tumor-related death of almost all mice between weeks 5 and 6.

To determine whether the failure of host immune system in controlling Renca-HA was the result of immune ignorance or tumor-induced immune tolerance, we adoptively transferred 3×10^6 CFSE-labeled CD4 and CD8 HA-specific T cells to tumor-bearing BALB/c mice 7 days after Renca-HA inoculation. Interestingly, 3 days post-HA specific T-cell transfer, a small percentage of the transferred CD4⁺ T cells in the spleen, peripheral LN and tumor-draining hilar nodes divided a few rounds, as indicated by the decrease in CFSE fluorescent intensity (Figure 1b), whereas CFSE intensity of T cells in nontumor-bearing control mice was unchanged (Figure 1b). This suggests that the HA antigen on tumor surface was processed and presented by antigen-presenting cells (APCs) to T cells in the host although the number of divisions and total percentage of HA-specific T cells in these tumor-bearing mice was far less than those in mice immunized with vaccinia virus carrying the HA antigen (Figure 1b; Vacc-HA). To determine whether these T cells develop tolerance to the HA antigen upon tumor encountering, HA-specific T cells were harvested from the spleens of Renca-HA-bearing mice 7 days posttransfer, relabeled with CFSE and stimulated with HA antigen in a 3-day culture using T cells from nontumor-bearing and Vacc-HA-immunized mice as controls (Figure 1c).

Figure 1 Intravenous inoculation of Renca-HA to immune-competent BALB/c mice resulted in aggressive pulmonary metastases and tumor-induced immune T-cell tolerance. A total of 1×10^6 Renca-HA cells were injected i.v. to BALB/c mice. **(a)** The size and number of tumor nodules in the lungs of these mice was examined via H&E-stained sections as an evaluation of tumor burden 1–4 weeks post-tumor inoculation. Black arrowheads indicate established microscopic tumor nodules. **(b–d)** These Renca-HA-bearing mice were adoptively transferred with 3×10^6 CFSE-labeled naive HA-specific CD4 or CD8 T cells from TCR Tg mice, 1 week post-tumor inoculation. As controls, none tumor-bearing BALB/c mice without additional manipulation (no tumor) or immunized with vaccinia virus expressing HA antigen (Vacc-HA) were also transferred with the same number of CFSE-labeled HA-specific T cells. Their percentage and proliferation indicated as CFSE dilution were analyzed via FACS 5 days after the T cells transfer. **(b)** Representative FACS plots of HA-specific CD4 T cells in the spleen of tumor-bearing and control mice. **(c)** To examine the immune responsiveness of these HA-specific T cells, they were relabeled with CFSE and cultured in the presence or absence of HA peptide for 3 days. At the end the 3-day cultured, these T cells were stained with antibodies to identify HA-specific CD4 T cell population and their level of CFSE were analyzed. **(d)** The percentage of HA-specific CD4 and CD8 T cells in tumor-draining and non-draining lymphoid tissues at 3–10 days after the transfer was summarized ($n=3$).

As expected, HA-specific T cells (gated on Tg TCR⁺ population) from Renca-HA-bearing mice did not proliferate upon HA re-stimulation, whereas those from nontumor-bearing mice proliferated moderately and those from Vacc-HA-immunized mice proliferated vigorously (Figure 1c).

Similarly, initial HA-specific CD8 T cell expansion was also observed in tumor-bearing mice, although at a lower extend compared to CD4 T cells when cotransferred to the same tumor-bearing host at an equal number (Figure 1d), followed by tolerance development and gradual decrease in numbers in Renca-HA-bearing mice (Figure 1d). This suggests that the failure of hosts to control tumor growth is due to the establishment of immune tolerance in tumor-bearing hosts.

In vivo derived antigen-presenting DCs from LV-transduced DC progenitors overcome tumor-induced tolerance and promote systemic antigen-specific T-cell activation

Because we have previously demonstrated that *in vivo* derived DCs from LV-transduced DC progenitors, either BM or HSC, are much more potent in activating antigen-specific T cells compared with conventional approaches,^{17,27} we wanted to examine whether this level of stimulation is sufficient to override the tolerogenic environment in tumor-bearing mice. We transplanted GFP or HA gene-modified BM to BALB/c mice bearing Renca-HA tumors established for 7 days. These mice were treated with GM-CSF or PBS followed with adoptive transfer of HA-specific CD4 or CD8 T cells, 1 week after BMT. The percentage of HA-specific T cells in their spleen (Figure 2a), tumor-draining hilar nodes (Figure 2b) and peripheral blood (Figure 2c) was analyzed via FACS. Indeed, 5 days post-T-cell transfer, higher percentages of HA-specific CD4 and CD8 T cells were observed in all the lymphoid-hematopoietic compartments of HA-BMT mice, especially in the mice that also received GM-CSF treatment (Figure 2). More importantly, the *in vitro* proliferation assay revealed that HA-specific T cells from the spleen of HA-BMT mice proliferated vigorously upon re-stimulation with 10 µgml⁻¹ HA peptide in culture (Figure 2d). Even in the absence of additional HA stimulation, those cells seemed to continuously proliferate to the same extend as those T cells from nontumor-bearing mice upon HA stimulation in culture (Figure 2d). In contrast, HA-specific T cells from GFP-BMT tumor-bearing mice lost their responsiveness to HA re-stimulation confirming their immune tolerant status (Figure 2d). Of particular interest, GM-CSF treatment appeared to enhance the total number of HA-specific T cells in tumor-bearing mice without augmenting their effector function on per cell basis (Figure 2). This suggests that GM-CSF treatment alone may enhance the magnitude of T-cell activation, but may not provide a long-term effect in overcoming tumor-induced tolerance alone.

Since ligands for TLR, such as CpG-containing ODN, have been demonstrated to be important factors in mediating DC activation and subsequently augmenting T-cell function to overcome tumor-induced tolerance,^{34–36}

we further explored whether inclusion of DC-activating ODN in combination with GM-CSF treatment in HA-BMT mice would further enhance the function of activated HA-specific T cells. Although ODN treatment alone did not impose significant impacts on the number of *in vivo* derived DCs, ODN in combination with GM-CSF imposed a significant increase in SP-DC number in treated mice (Figure 3a). Furthermore, HA-specific CD4 T cells activated in ODN- and GM-CSF-treated mice produced higher level of interferon (IFN)-γ (Figure 3b) and HA-specific CD8 T cells possessed better CTL killing per clonotypic cell as revealed by *in vitro* assays (Figure 3c), implying their improved effector function compared with those in HA-BMT mice treated with PBS or GM-CSF alone. Together, these results suggest that combination of GM-CSF and ODN treatment not only enhanced the magnitude of T-cell expansion, but also further improved T-cell effector function to overcome tumor-induced tolerance.

The enhanced antigen-specific T-cell activation and effector function in GM-CSF- and ODN-treated HA-BMT mice is translatable to improved survival of tumor-bearing mice

To examine whether our observed improvement in the activation of HA-specific T cells could directly result in therapeutic efficacy of treating established metastatic epithelial tumors, we first treated BALB/c mice bearing 7-day Renca-HA pulmonary metastases with HA or GFP gene-modified BM. To facilitate post-BMT recovery and immune function, these mice were also infused with 1 × 10⁷ fresh splenocytes from naive BALB/c mice as outlined in Figure 4a. After 1 week, these mice were treated with GM-CSF and/or ODN and were monitored daily for their healthiness. Upon any signs of difficulties in breathing, they were terminated as tumor-related mortality, which was confirmed by autopsy. The survival of all treated mice was plotted as Kaplan–Meier survival curve with at least 10 mice in each group (Figure 4). In the absence of any treatment (control), more than 90% of the tumor-bearing mice died of pulmonary metastases by day 33 post-tumor inoculation (Figure 4a). Interestingly, even when mice were treated with reporter gene-modified BM in the form of BMT, that is, GFP-BMT or GFP-BMT + GM-CSF + ODN, a moderate improvement in survival at early time course, such as 1 week posttreatment (21 days post-tumor inoculation), was observed (Figure 4a). This is in agreement with a previous report that homeostatic T-cell proliferation in lymphopenia hosts associated with BMT maneuver enhanced anti-tumor immunity, which has been employed clinically for cancer treatment. Unfortunately, this effect appeared to be short lived and was not sustained beyond day 40 post-tumor inoculation (Figure 4a). On the other hand, mice received HA gene-modified DC progenitors in addition to BMT had extended half-life to 45+ days compared to ~40 days of GFP-BMT (Figure 4a). This result suggests that *in vivo* derived HA-expressing DCs further augmented HA-specific immune activation besides BMT-induced T-cell homeostatic proliferation. Furthermore, inclusion

Figure 2 Strong stimulatory signals from *in vivo* derived HA-expressing DCs in HA-BMT mice enhanced HA-specific T-cell activation in tolerogenic tumor-bearing mice. BALB/c mice were first inoculated i.v. with 1×10^6 Renca-HA to establish pulmonary metastasis for 7 days followed with transplantation of GFP- or HA-modified BM cells. These mice were treated with GM-CSF and adoptively transferred with 3×10^6 HA-specific CD4 and CD8 T cells 1 week post-BMT. The percentage of HA-specific CD4 T cells in the spleen (**a**) and tumor-draining hilar nodes (**b**) was examined 7 days after the transfer via antibody staining against colonotypic marker and FACS analysis ($n=3$). (**c**) HA-specific CD8 T cells in peripheral blood of the treated mice were examined using HA-specific pentamer staining via FACS ($n=3$). (**d**) Responsiveness of HA-specific splenic T cells of the above-treated tumor-bearing mice to HA-antigen stimulation in culture (HA-10) in comparison to no antigen control (HA-0) was determined as ^3H -thymidine incorporation through proliferation assay.

Figure 3 Immune adjuvant ODN not only enhanced the number of *in vivo* derived antigen-presenting DCs, but also augmented DC quality for improved T-cell activation and effector function. **(a)** BALB/c mice were treated with either PBS or GM-CSF producing cells followed with i.v. injection of 100 μg CpG-containing ODN on days 4 and 6 post-GM-CSF treatment. SP-DCs were harvested on day 7 and the total number SP-DCs in each treated group was determined ($n=3-5$). Asterisk (*) represents significant difference from PBS treatment, whereas † denotes significant difference from GM-CSF treatment ($P<0.05$, *t*-test). **(b-c)** BALB/c mice engrafted with GFP- or HA-modified BM were treated with PBS or GM-CSF in combination with ODN treatment followed with adoptive transfer of HA-specific CD4 and CD8 T cells. The ability of IFN- γ production by these activated HA-specific CD4 T cells in the presence (HA 10) or absence (HA 0) of HA antigen **(b)** and the CTL killing capacity of HA-specific CD8 T cells in their spleens were determined via ELISA or a newly developed FACS-based CTL assay,³² respectively ($n=3-5$, * and † represent significant difference from nonpeptide control (HA 0) in **(b)** or PBS-treated HA-BMT group in **(c)**, $P<0.05$).

of GM-CSF and ODN treatment in HA-BMT mice extended their survival by one more week (up to 50% survival by day 53 vs 10% survival by day 33 in control group; Figure 4a). Therefore, a combination of BMT, HA gene transfer and DC activation regimen significantly improved survival of tumor-bearing mice.

To further delineate the necessity and contribution of GM-CSF and ODN regimen in addition to HA gene transfer, we treated HA-BMT mice with GM-CSF or ODN alone and compared their survival with those of combinational regimens (Figure 4b). Interestingly, neither GM-CSF nor ODN treatment alone seemed to provide additional survival benefits of HA-BMT-treated tumor-bearing mice (Figure 4b). However, the GM-CSF and ODN combination treatment provided additive or synergistic effects on improving the survival of tumor-bearing mice (Figure 4b). It is worth noting that the full therapeutic effect of GM-CSF and ODN combination treatment requires HA gene transfer to DC progenitors because when HA antigen gene was replaced by GFP in GFP-BMT mice, GM-CSF + ODN combination treatments did not support sustained effects on survival beyond 40 days post-tumor inoculation (Figure 4b).

The improved therapeutic benefit of GM-CSF and ODN combinational treatment in tumor-bearing mice appeared to correlate with reduced tumor burden and enhanced number of tumor-infiltrating T cells

It has been shown clinically that the prognosis and therapeutic efficacy of immunotherapy correlate well with the increased number of tumor-infiltrating T cells because effective tumor elimination relies heavily on direct interaction of effector T cells with tumor cells. Therefore, efficient recruitment of tumor antigen-specific T cells to tumor sites is crucial for epithelial tumor treatment. Thus, we investigated whether the above-observed therapeutic benefits in GM-CSF and ODN-treated HA-BMT mice were the result of enhanced T-cell recruitment to solid tumor mass, which led to the reduction in tumor burden. At 1 and 3 weeks post-cytokine treatment, some of the Renca-HA-bearing mice that received GFP- or HA-transduced DC progenitors and treated with or without GM-CSF and ODN were terminated and their tumor burdens and T-cell infiltration in the lungs were examined via immunohistochemical staining (Figure 5). In agreement with the survival results, HA-BMT in combination with the GM-CSF + ODN treatment seemed to be the most potent regimen in suppressing tumor growth at 1

week posttreatment (3 weeks post-tumor inoculation; Figure 5a). Unsurprisingly, BMT alone also provided moderate effects on reducing tumor burdens compared to tumor-alone control mice during this early period of the treatment (Figure 5a and b). This also appeared to be associated with increases in CD3⁺ T cells within the tumor masses as the result of BMT-mediated homeostatic proliferation (Figure 5b, dark-brown cells), which supported our observed moderate improvement in survival of BMT treatment of tumor-bearing mice during early treatment period (Figure 4). This BMT-mediated T-cell homeostatic expansion and reduction in tumor burden could be further enhanced by HA antigen gene transfer in the context of BMT, especially in combination with GM-CSF treatment (Figure 5a and b).

Similar to the survival results (Figure 4), the lack of sustainability of BMT alone on T-cell infiltration and

reduction in tumor burden was more obvious 3 weeks posttreatment (Figure 5c, GFP-BMT). To quantitatively evaluate the changes in tumor burdens and level of T-cell infiltration in treated mice, we took images of lung sections of those treated mice and employed a computer-assisted program to calculate total areas occupied by the lungs and tumors and calculated the percentage of tumor covered area vs lungs area of all the accumulative 10 or more sections per treatment group (Figure 5d). In strong agreement with our observed improvement in therapeutic efficacy, HA-BMT in combination with GM-CSF + ODN treatment resulted in the highest level of tumor suppression and densest T-cell infiltration within the much reduced tumor masses (Figure 5d). Although GM-CSF treatment in combination with BMT (both HA-BMT and GFP-BMT) appeared to present slight advances in increasing tumor-infiltrating T cells, they did

Figure 4 HA gene-modified BM in combination with GM-CSF and ODN treatment significantly improved survival of tumor-bearing mice. BALB/c mice carrying 7-day established pulmonary metastatic Renca-HA were transplanted with HA- or GFP-modified BM. They were treated with GM-CSF and/or ODN one week later. Healthiness of these tumor-bearing mice was monitored daily. Upon the occurrence of any obvious difficulties in breathing, they were terminated and recorded as tumor-related mortality. (a) Survival of GFP-BMT or HA-BMT mice treated with or without GM-CSF + ODN was compared with the nontreated control. Statistical analysis was performed using one-way ANOVA (analysis of variance, $n=11-14$). Asterisk (*) denotes significant difference from the control group ($P<0.05$). (b) The effects of GM-CSF or ODN alone on improvement in survival of GFP-BMT or HA-BMT mice were examined by comparing them with mice received combination treatment. Asterisk (*) denotes significant difference from the control group ($P<0.05$, one-way ANOVA).

Figure 5 *In vivo* derived HA-expressing DCs from LV-modified DC progenitors recruited T cells to tumor masses and reduced tumor burdens. BALB/c mice were first inoculated with 1×10^6 Renca-HA cells through i.v. injection. Seven days after tumor establishment, mice were engrafted with GFP- or HA-transduced BM followed by GM-CSF and/or ODN treatment one week later, whereas control tumor-bearing mice did not receive any treatments. Lungs from some of the mice were collected at 1 (a, b) and 3 (c, d) weeks posttreatment (3 weeks and 5 weeks post-tumor inoculation, respectively). (a) H&E staining at 1 week posttreatment was performed to examine the size and number of tumor nodules. Black arrowhead indicates a microscopic tumor nodule. (b, c) Tumor-infiltrating T cells were examined via immunohistochemical staining against the CD3 surface marker, shown as brown stained cells. These lung tissues were counterstained with hematoxylin to visualize the size of tumor nodules. ND denotes not determined due to lack of identifiable tumor nodules (representative images of at least three mice in each treatment group). (d) To quantitatively determine the tumor burden and tumor-infiltrating T cells at 3 weeks posttreatment, photomicrographs of entire representative lung sections were obtained using a Nikon Eclipse E600 upright brightfield microscope (Nikon Instruments Inc.). The images were loaded to Spot Advanced imaging software (Diagnostic Instruments Inc.), where the borders of tumor nodules were traced (Figure 5d). The ratio of total tumor nodule area to total lung area was calculated from these images using the Spot Advanced imaging program. Quantification of CD3⁺ cell area was performed on a total of 10 nodules per section through thresholding DAB⁺ signals and masking subsampling with Slidebook software (Intelligent Imaging Innovations). Total CD3 area/tumor nodule area was then calculated.

not provide long-term effect on tumor burden reduction (Figure 5d) or therapeutic efficacy (Figure 4).

Collectively, these results suggest that GM-CSF and ODN treatments improved quantity and quality of *in vivo* derived HA-presenting DCs, which subsequently augmented systemic expansion of HA-specific T cells and more importantly their mobilization to metastatic tumors to control tumor growth even in tolerogenic environment.

Discussion

We demonstrate that appropriately activated, *in vivo* derived tumor antigen-expressing DCs from engineered DC progenitors can be very efficient in activating antigen-specific T cells, even in a tolerogenic environment. This strong antigen-specific T-cell activation supports T-cell recruitment to epithelial tumors leading to reduction in tumor burden and improvement in long-term survival of

tumor-bearing mice. This therapeutic efficacy relies on the additive effects of three critical components: (1) BMT of tumor antigen-modified DC progenitors, (2) DC expansion and activation via cytokine and adjuvant, and (3) homeostatic proliferation of naive or antigen-experienced T cells in lymphopenia hosts. Although each of the three regimens has been employed for clinical and experimental cancer treatment, including this study, each regimen alone has been shown incapable of promoting strong, sustained immune activation in tumor-bearing hosts for satisfactory therapeutic efficacy.^{37–40} In fact, decades of research on developing simple and effective means for cancer treatment without severe adverse effects has proven to be unproductive due to the complexity of cancer, which further signifies the necessity of developing complex strategies, such as this tripartite approach, for concerted and/or additive effects of multicomponents to treat aggressive tumor.^{2,19,33,41}

HSC/BMT has been the treatment of choice for hematological malignancy and has been used for the treatment of inherited and acquired severe hematological or immunological disorders.^{17,42–45} However, it has not been routinely used for the treatment of epithelial tumors. One of the major limitations of BMT-based therapeutics for cancer treatment is related to a long period of immune deficiency post-BMT. This delay in immune reconstitution has been overcome in this study with the combination of total BM as DC progenitors and GM-CSF as we have demonstrated in a previous report.²⁷ This improvement warrants early initiation of DC differentiation and activation regimens within 1 week post-BMT. Even though the Flt-3 ligand treatment in BMT setting seems to provide equally potent DC differentiation at a later time post-BMT,^{17,27} an additional 2-week waiting time required for its potent DC differentiation and activation unavoidably results in losing valuable time for experimental therapeutics and clinical application.

It is necessary to point out that BMT treatment alone, in the absence of HA gene transfer, moderately expanded HA-specific T cells in tumor-bearing mice at the early period of treatment (Figures 2 and 5). This was mostly contributed by lymphopenia-driven T-cell homeostatic proliferation in BMT hosts. In fact, recent studies show that infusion of naive T cells to lymphopenia hosts enhances antitumor immunity and provides short-term therapeutic efficacy in controlling tumor progression.^{37–40} This agrees well with our observation of a short-term T-cell expansion and tumor infiltration during early treatment period in mice transplanted with mock- or GFP-transduced BM (Figure 5). Furthermore, it is also worth noting that adoptive T-cell transfer for their homeostatic proliferation post-BMT not necessarily requires a pool of tumor antigen-specific T cells, that is, naive polyclonal T cells from non-antigen-experienced hosts would provide similar therapeutic effects as shown in this study and previously.¹⁷ Regardless, this BMT-mediated homeostatic proliferation is short lived and could not support strong stimuli to overcome tolerance and maintain sustained T-cell activation in tumor-bearing hosts (Figures 4 and 5).

Tumor antigen (HA) gene delivery and integration to DC progenitors in conjunction with *in vivo* DC differentiation further enhances BMT-mediated HA-specific T-cell activation. More importantly, this approach supports better antigen presentation and activation of CD8 T cells although marked activation of antigen-specific CD4 T cells was also achieved. This can be explained by our understanding of MHC class I presentation of endogenous (integrated transgene) antigen to CD8 T cells being more efficient than MHC II presentation to CD4 T cells. On the other hand, an exogenous antigen presentation, such as HA on Renca-HA cells, through MHC II molecules on APCs to CD4 T cells is more efficient than that of MHC I-mediated presentation to CD8 T cells. This explains why we observed higher HA-specific CD4 T cell expansion than CD8 T cells in Renca-HA tumor-bearing mice (Figure 1c). This enhanced activation of tumor-specific CD8 T cell is crucial for improved therapeutic efficacy as increased CTL frequency and function are necessary for tumor elimination as shown in our previous study and the studies of others.^{17,46}

GM-CSF, as a DC promoting cytokine, has been widely employed in cancer immunotherapy experimentally and clinically.^{20,31,47–52} Our previous study demonstrated that GM-CSF treatment significantly expanded the number of antigen-expressing DCs for antigen presentation.²⁷ However, the effects of GM-CSF on enhanced activation of antigen-specific T cells appeared to be short lived in tumor-bearing HA-BMT mice in the absence of DC activation adjuvant. This is in agreement with previous report that in the absence of additional activation signal, GM-CSF may not provide strong activation signaling sufficient to overcome tumor-induced tolerance for a lasting antitumor immunity even though its effects on DC differentiation were not compromised.⁵³ We have shown that only when GM-CSF and ODN treatments are combined in HA-BMT mice, T-cell infiltration to tumors is sustained and significant reduction in tumor size and improved therapeutic efficacy are achieved (Figures 4 and 5). This is similar to previous reports that ODN-mediated DC activation is crucial for effective tumor elimination in other therapeutic settings.^{34–36} In light of recent discovery on contributions of PD-1 and PD-L1 interaction to tumor-induced immune tolerance^{54,55} and detectable expression of PD-L1 on Renca-HA cells, it will be of great interests and importance to examine whether our observed enhanced antitumor immunity is related to the interruption of the PD-1/PD-L1 pathway or other mechanisms in future studies. Nevertheless, these studies suggested that in the absence of additional maneuvers beyond BMT, early T-cell homeostatic proliferation mediated by BMT could not provide strong enough stimuli to overcome tumor-induced tolerance. To achieve long-lasting effects in eliminating established tumor and significant therapeutic efficacy, improving quantity and quality of antigen-presenting DCs to support great numbers of effector T cells and maintaining their immune function in a tolerogenic environment is essential.

In summary, this study shows that *in vivo* derived tumor antigen-expressing DCs from engineered DC progenitors can be very efficient in activating antigen-specific T cells, even in a tolerogenic environment. Through combination of three clinically employed, but individually less effective, regimens with further modification to provide persistent antitumor immunity, this approach can improve therapeutic efficacy in an otherwise tolerogenic environment for solid tumors treatment and represents a promising avenue for potential clinical translation.

Acknowledgements

We thank Ms Jennifer Aguirre and Jennifer Simkin for technical assistance in immunohistochemistry staining and image acquisition. This research was supported by funds from Louisiana Gene Therapy Consortium, Stanley S Scott Cancer Center and grants from the Susan Komen Breast Cancer Foundation and National Institutes of Health to YC (CA112065 and P20RR021970). Gina Washington was supported by an NCI summer student scholarship.

References

- 1 Finn OJ. Cancer vaccines: between the idea and the reality. *Nat Rev Immunol* 2003; **3**: 630–641.
- 2 Pardoll D. Does the immune system see tumors as foreign or self? *Annu Rev Immunol* 2003; **21**: 807–839.
- 3 Powell Jr DJ, Dudley ME, Hogan KA, Wunderlich JR, Rosenberg SA. Adoptive transfer of vaccine-induced peripheral blood mononuclear cells to patients with metastatic melanoma following lymphodepletion. *J Immunol* 2006; **177**: 6527–6539.
- 4 Sato E, Olson SH, Ahn J, Bundy B, Nishikawa H, Qian F et al. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proc Natl Acad Sci USA* 2005; **102**: 18538–18543.
- 5 Zhang L, Conejo-Garcia JR, Katsaros D, Gimotty PA, Massobrio M, Regnani G et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N Engl J Med* 2003; **348**: 203–213.
- 6 Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 2004; **10**: 942–949.
- 7 Cuenca A, Cheng F, Wang H, Brayer J, Horna P, Gu L et al. Extra-lymphatic solid tumor growth is not immunologically ignored and results in early induction of antigen-specific T-cell anergy: dominant role of cross-tolerance to tumor antigens. *Cancer Res* 2003; **63**: 9007–9015.
- 8 O'Neill D W, Adams S, Bhardwaj N. Manipulating dendritic cell biology for the active immunotherapy of cancer. *Blood* 2004; **104**: 2235–2246.
- 9 Bancheau J, Paczesny S, Blanco P, Bennett L, Pascual V, Fay J et al. Dendritic cells: controllers of the immune system and a new promise for immunotherapy. *Ann NY Acad Sci* 2003; **987**: 180–187.
- 10 Baggers J, Ratzinger G, Young JW. Dendritic cells as immunologic adjuvants for the treatment of cancer. *J Clin Oncol* 2000; **18**: 3879–3882.
- 11 Wang HY, Fu T, Wang G, Zeng G, Perry-Lalley DM, Yang JC et al. Induction of CD4(+) T cell-dependent antitumor immunity by TAT-mediated tumor antigen delivery into dendritic cells. *J Clin Invest* 2002; **109**: 1463–1470.
- 12 Timmerman JM, Czerwinski DK, Davis TA, Hsu FJ, Benike C, Hao ZM et al. Idiotype-pulsed dendritic cell vaccination for B-cell lymphoma: clinical and immune responses in 35 patients. *Blood* 2002; **99**: 1517–1526.
- 13 Fong L, Engleman EG. Dendritic cells in cancer immunotherapy. *Annu Rev Immunol* 2000; **18**: 245–273.
- 14 Bonifaz LC, Bonnyay DP, Charalambous A, Darguste DI, Fujii S, Soares H et al. *In vivo* targeting of antigens to maturing dendritic cells via the DEC-205 receptor improves T cell vaccination. *J Exp Med* 2004; **199**: 815–824.
- 15 Merad M, Sugie T, Engleman EG, Fong L. *In vivo* manipulation of dendritic cells to induce therapeutic immunity. *Blood* 2002; **99**: 1676–1682.
- 16 Esslinger C, Chapatte L, Finke D, Miconnet I, Guillaume P, Levy F et al. *In vivo* administration of a lentiviral vaccine targets DCs and induces efficient CD8(+) T cell responses. *J Clin Invest* 2003; **111**: 1673–1681.
- 17 Cui Y, Kelleher E, Straley E, Fuchs E, Gorski K, Levitsky H et al. Immunotherapy of established tumors using bone marrow transplantation with antigen gene-modified hematopoietic stem cells. *Nat Med* 2003; **9**: 952–958.
- 18 Diehl L, den Boer AT, Schoenberger SP, van der Voort EI, Schumacher TN, Melief CJ et al. CD40 activation *in vivo* overcomes peptide-induced peripheral cytotoxic T-lymphocyte tolerance and augments anti-tumor vaccine efficacy. *Nat Med* 1999; **5**: 774–779.
- 19 Sotomayor EM, Borrello I, Tubb E, Rattis FM, Bien H, Lu Z et al. Conversion of tumor-specific CD4+ T-cell tolerance to T-cell priming through *in vivo* ligation of CD40. *Nat Med* 1999; **5**: 780–787.
- 20 Borrello I, Pardoll D. GM-CSF-based cellular vaccines: a review of the clinical experience. *Cytokine Growth Factor Rev* 2002; **13**: 185–193.
- 21 Jaffee EM, Hruban RH, Biedrzycki B, Laheru D, Schepers K, Sauter PR et al. Novel allogeneic granulocyte-macrophage colony-stimulating factor-secreting tumor vaccine for pancreatic cancer: a phase I trial of safety and immune activation. *J Clin Oncol* 2001; **19**: 145–156.
- 22 Eggert AA, Schreurs MW, Boerman OC, Oyen WJ, de Boer AJ, Punt CJ et al. Biodistribution and vaccine efficiency of murine dendritic cells are dependent on the route of administration. *Cancer Res* 1999; **59**: 3340–3345.
- 23 Eggert AA, van der Voort R, Torensma R, Moulin V, Boerman OC, Oyen WJ et al. Analysis of dendritic cell trafficking using EGFP-transgenic mice. *Immunol Lett* 2003; **89**: 17–24.
- 24 Cayeux S, Richter G, Becker C, Pezzutto A, Dorken B, Blankenstein T. Direct and indirect T cell priming by dendritic cell vaccines. *Eur J Immunol* 1999; **29**: 225–234.
- 25 Ruedl C, Koebel P, Bachmann M, Hess M, Karjalainen K. Anatomical origin of dendritic cells determines their life span in peripheral lymph nodes. *J Immunol* 2000; **165**: 4910–4916.
- 26 Cui Y, Golob J, Kelleher E, Ye Z, Pardoll D, Cheng L. Targeting transgene expression to antigen-presenting cells derived from lentivirus-transduced engrafting human hematopoietic stem/progenitor cells. *Blood* 2002; **99**: 399–408.
- 27 Zhao P, Liu W, Cui Y. Rapid immune reconstitution and dendritic cell engraftment post-bone marrow transplantation

- with heterogeneous progenitors and GM-CSF treatment. *Exp Hematol* 2006; **34**: 951–964.
- 28 Trono D. Lentiviral vectors: turning a deadly foe into a therapeutic agent. *Gene Therapy* 2000; **7**: 20–23.
 - 29 Miyoshi H, Smith KA, Mosier DE, Verma IM, Torbett BE. Transduction of human CD34+ cells that mediate long-term engraftment of NOD/SCID mice by HIV vectors. *Science* 1999; **283**: 682–686.
 - 30 Case SS, Price MA, Jordan CT, Yu XJ, Wang L, Bauer G *et al*. Stable transduction of quiescent CD34(+)CD38(–) human hematopoietic cells by HIV-1-based lentiviral vectors. *Proc Natl Acad Sci USA* 1999; **96**: 2988–2993.
 - 31 Borrello I, Sotomayor EM, Cooke S, Levitsky HI. A universal granulocyte-macrophage colony-stimulating factor-producing bystander cell line for use in the formulation of autologous tumor cell-based vaccines. *Hum Gene Ther* 1999; **10**: 1983–1991.
 - 32 Chen K, Chen L, Zhao P, Marrero L, Keoshkerian E, Ramsay A *et al*. FL-CTL assay: fluorolysometric determination of cell-mediated cytotoxicity using green fluorescent protein and red fluorescent protein expressing target cells. *J Immunol Methods* 2005; **300**: 100–114.
 - 33 Zhou G, Lu Z, McCadden JD, Levitsky HI, Marson AL. Reciprocal changes in tumor antigenicity and antigen-specific T cell function during tumor progression. *J Exp Med* 2004; **200**: 1581–1592.
 - 34 Blazar BR, Krieg AM, Taylor PA. Synthetic unmethylated cytosine-phosphate-guanosine oligodeoxynucleotides are potent stimulators of antileukemia responses in naive and bone marrow transplant recipients. *Blood* 2001; **98**: 1217–1225.
 - 35 Atkins H, Davies BR, Kirby JA, Kelly JD. Polarisation of a T-helper cell immune response by activation of dendritic cells with CpG-containing oligonucleotides: a potential therapeutic regime for bladder cancer immunotherapy. *Br J Cancer* 2003; **89**: 2312–2319.
 - 36 Yang Y, Huang CT, Huang X, Pardoll DM. Persistent Toll-like receptor signals are required for reversal of regulatory T cell-mediated CD8 tolerance. *Nat Immunol* 2004; **5**: 508–515.
 - 37 Kondo M, Wagers AJ, Manz MG, Prohaska SS, Scherer DC, Beilhack GF *et al*. Biology of hematopoietic stem cells and progenitors: implications for clinical application. *Annu Rev Immunol* 2003; **21**: 759–806.
 - 38 Storb RF, Champlin R, Riddell SR, Murata M, Bryant S, Warren EH. Non-myeloablative transplants for malignant disease. *Hematology (Am Soc Hematol Educ Program)* 2001; **1**: 375–391.
 - 39 Dummer W, Niethammer AG, Baccala R, Lawson BR, Wagner N, Reisfeld RA *et al*. T cell homeostatic proliferation elicits effective antitumor autoimmunity. *J Clin Invest* 2002; **110**: 185–192.
 - 40 Hu HM, Poehlein CH, Urba WJ, Fox BA. Development of antitumor immune responses in reconstituted lymphopenic hosts. *Cancer Res* 2002; **62**: 3914–3919.
 - 41 Zou W. Immunosuppressive networks in the tumour environment and their therapeutic relevance. *Nat Rev Cancer* 2005; **5**: 263–274.
 - 42 Aiuti A, Slavin S, Aker M, Ficara F, Deola S, Mortellaro A *et al*. Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. *Science* 2002; **296**: 2410–2413.
 - 43 Down JD, White-Scharf ME. Reprogramming immune responses: enabling cellular therapies and regenerative medicine. *Stem Cells* 2003; **21**: 21–32.
 - 44 Anderson Jr LD, Savary CA, Mullen CA. Immunization of allogeneic bone marrow transplant recipients with tumor cell vaccines enhances graft-versus-tumor activity without exacerbating graft-versus-host disease. *Blood* 2000; **95**: 2426–2433.
 - 45 Saccardi R, Mancardi GL, Solari A, Bosi A, Bruzzi P, Di Bartolomeo P *et al*. Autologous HSCT for severe progressive multiple sclerosis in a multicenter trial: impact on disease activity and quality of life. *Blood* 2005; **105**: 2601–2607.
 - 46 Hanson HL, Kang SS, Norian LA, Matsui K, O'Mara LA, Allen PM. CD4-directed peptide vaccination augments an antitumor response, but efficacy is limited by the number of CD8+ T cell precursors. *J Immunol* 2004; **172**: 4215–4224.
 - 47 Daro E, Pulendran B, Brasel K, Teepe M, Pettit D, Lynch DH *et al*. Polyethylene glycol-modified GM-CSF expands CD11b(high)CD11c(high) but notCD11b(low)CD11c(high) murine dendritic cells *in vivo*: a comparative analysis with Flt3 ligand. *J Immunol* 2000; **165**: 49–58.
 - 48 Basak SK, Harui A, Stolina M, Sharma S, Mitani K, Dubinett SM *et al*. Increased dendritic cell number and function following continuous *in vivo* infusion of granulocyte macrophage-colony-stimulating factor and interleukin-4. *Blood* 2002; **99**: 2869–2879.
 - 49 Maraskovsky E, Brasel K, Teepe M, Roux ER, Lyman SD, Shortman K *et al*. Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. *J Exp Med* 1996; **184**: 1953–1962.
 - 50 Levitsky HI. Augmentation of host immune responses to cancer: overcoming the barrier of tumor antigen-specific T-cell tolerance. *Cancer J* 2000; **6**(Suppl 3): S281–290.
 - 51 Luznik L, Slansky JE, Jalla S, Borrello I, Levitsky HI, Pardoll DM *et al*. Successful therapy of metastatic cancer using tumor vaccines in mixed allogeneic bone marrow chimeras. *Blood* 2003; **101**: 1645–1652.
 - 52 Emens LA, Armstrong D, Biedrzycki B, Davidson N, Davis-Sproul J, Fetting J *et al*. A phase I vaccine safety and chemotherapy dose-finding trial of an allogeneic GM-CSF-secreting breast cancer vaccine given in a specifically timed sequence with immunomodulatory doses of cyclophosphamide and doxorubicin. *Hum Gene Ther* 2004; **15**: 313–337.
 - 53 Hess PR, Boczkowski D, Nair SK, Snyder D, Gilboa E. Vaccination with mRNAs encoding tumor-associated antigens and granulocyte-macrophage colony-stimulating factor efficiently primes CTL responses, but is insufficient to overcome tolerance to a model tumor/self antigen. *Cancer Immunol Immunother* 2006; **55**: 672–683.
 - 54 Okazaki T, Honjo T. The PD-1-PD-L pathway in immunological tolerance. *Trends Immunol* 2006; **27**: 195–201.
 - 55 Thompson RH, Dong H, Lohse CM, Leibovich BC, Blute ML, Cheville JC *et al*. PD-1 is expressed by tumor-infiltrating immune cells and is associated with poor outcome for patients with renal cell carcinoma. *Clin Cancer Res* 2007; **13**: 1757–1761.