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Lentiviral Vector-mediated Autonomous Differentiation of Mouse Bone Marrow Cells into Immunologically Potent Dendritic Cell Vaccines

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Approaches facilitating generation of dendritic cell (DC) vaccines for clinical trials and enhancing their viability, bio-distribution, and capacity to stimulate antigen-specific immune responses are critical for immunotherapy. We programmed mouse bone marrow (BM) cells with lentiviral vectors (LV-GI4) so that they produced granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) in an autonomous manner. DC/LV-GI4 cells underwent autonomous trans-differentiation to yield typical phenotypic characteristics of DCs. DC/LV-GI4 cells that self-differentiated either *ex vivo* or *in vivo* showed persistent and robust viability and stimulated high influx of DCs into draining lymph nodes (LNs). The immunostimulatory efficacy of DC/LV-GI4 cells was evaluated using MART1 and TRP2 as co-expressed melanoma antigens. Mice vaccinated with DC/LV-GI4 cells that self-differentiated *in vitro* or *in vivo* produced potent antigen-specific responses against melanoma, which correlated with protective and long-term therapeutic anti-tumor effects. Thus, DC precursors can be genetically engineered after a single *ex vivo* manipulation, resulting in DC vaccines with improved activity.

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INTRODUCTION

Dendritic cells (DCs) are the most potent antigen-presenting cells for initiating T- and B-cell immune responses.¹ DCs have been tested as antigen-presenting cells in several clinical trials, with promising results.² In the past, DCs have been produced conventionally *ex vivo* by culturing bone marrow (BM)-derived precursors in the presence of recombinant growth factors. The hematopoietic growth factors that most potently induce generation of DCs are granulocyte-macrophage colony-stimulating

factor (GM-CSF) and interleukin-4 (IL-4). GM-CSF regulates cellular proliferation, differentiation, and up-regulation of major histocompatibility complex (MHC) and co-stimulatory molecules and is required to generate cells with morphologic characteristics of DCs from mouse BM cultures.³ In combination with GM-CSF, IL-4 is required to promote differentiation of human peripheral monocytes to DCs⁴ and to promote DC maturation at low levels of GM-CSF in mouse BM-derived DC culture systems.⁵

In recent years, *ex vivo* generation of DCs from peripheral blood or BM precursor cells has evolved as a potent adjuvant to boost and enhance anti-tumor immune responses.⁶ Cultured DCs are currently being tested in several tumor immunotherapy clinical trials. However, conventional production of DCs using *ex vivo* cultures of hematopoietic precursors with recombinant stimulatory factors involves considerable manipulation under "Good Manufacturing Practices" conditions, which is costly in terms of time and resources. More important, a significant limitation in the production of DC-based vaccines is the need for 1–2 weeks of *ex vivo* culture to generate patient-customized vaccines, which presents problems in terms of variable cell viability, total yield, and preparation-to-preparation consistency. Furthermore, *ex vivo* cultured DCs are short lived after they are administered. In both mice and humans, the vast majority of *ex vivo*-derived DCs (95–99%) administered subcutaneously (s.c.) fail to migrate from vaccination sites to regional lymph nodes (LNs) or to the spleen.^{7,8} The loss in DC viability during the extended culture period and/or their poor engraftment and bio-distribution *in vivo* remain critical issues for the success of immunotherapeutic applications.

To simplify and optimize the generation of DC vaccines, and to improve their functionality *in vivo*, we have genetically modified DC precursor cells to produce GM-CSF/IL-4. Our strategy has been to use third-generation self-inactivating lentiviral vectors, which efficiently transduce quiescent hematopoietic precursor cells, stably integrate in the genome, and do not express

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additional antigenic or cytotoxic genes. We previously established the proof-of-concept that lentiviral vector-mediated expression of GM-CSF and IL-4 in CD14⁺ purified human monocytes was sufficient to promote their differentiation into cells with typical morphology, immunophenotype of immature DCs. These genetically programmed cells displayed greater viability and immune potency *in vitro* than traditionally grown DCs.⁹

In this study we have evaluated the biology of genetically programmed DCs *in vivo* by applying this strategy to non-adherent murine BM cells, a cell population traditionally used to generate DC precursors in mice. Here we show that this approach allows (i) autonomous trans-differentiation of murine BM precursors to functionally active DCs; (ii) rapid re-administration of genetically programmed precursors shortly after harvest, which undergo *in vivo* differentiation associated with enhanced engraftment in the host; (iii) prolonged *in vivo* viability through an autocrine mechanism of GM-CSF/IL-4 production/signaling; and (iv) adjuvant immune effects through GM-CSF/IL-4-mediated paracrine stimulation of “bystander” host antigen-presenting cells.

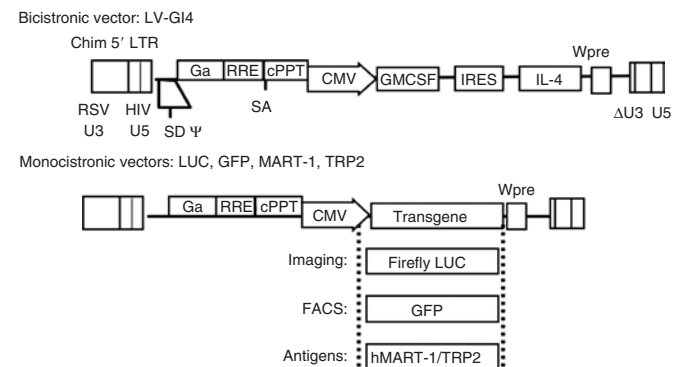
RESULTS

Delivery of GM-CSF, IL-4, and marking genes or antigenic genes into mouse BM

We used two types of lentiviral vectors in transductions of BM cells (Figure 1a). The bicistronic lentiviral vector LV-GI4 contains an internal ribosome entry site, allowing simultaneous expression of GM-CSF and IL-4 to mediate autonomous DC differentiation. The monocistronic vectors express marking genes (firefly luciferase for optical imaging and green fluorescent protein (GFP) for flow cytometry) or model melanoma antigens for functional analyses (MART1, TRP2).

We employed a traditional system for *in vitro* differentiation of mouse DCs based on non-adherent BM cells cultivated in the presence of recombinant GM-CSF/IL-4. This method leads to differentiation of DCs with phenotypic and functional characteristics similar to those of human monocyte-derived DCs: positivity for CD11c/CD11b, expression of MHC and co-stimulatory molecules, and high T-cell stimulation capacity. Thus, BM cells collected from mice femurs were seeded at low density on plastic plates for adherence. For the generation of conventional DCs, non-adherent cells were cultured under standard conditions with recombinant mGM-CSF and mIL-4 and transduced with lentiviral vectors (Figure 1b). For the generation of genetically programmed DCs, non-adherent cells were pre-conditioned with rGM-CSF/rIL-4 for 8 hours, a strategy previously shown to increase transduction efficiency and to eliminate the overgrowth of macrophages. This pre-conditioning was followed by overnight lentiviral transduction, which was performed at high viral concentrations (with 5 μ g p24 equivalent/ml; approximately 2.5×10^8 particles/ml used to transduce 5×10^6 cells; equivalent to a multiplicity of infection of 50). Using this method, we consistently obtained transduction efficiencies of more than 90% (assessed by GFP expression; data not shown) and measurable production of GM-CSF (average 27 ng/10⁶ cells/ml/24 hours) and IL-4 (average 3 ng/10⁶ cells/ml/24 hours). After transductions, cells were maintained *in vitro* to self-differentiate into DC/LV-GI4 or were injected directly into mice

a Co-transduction system



b Experimental design

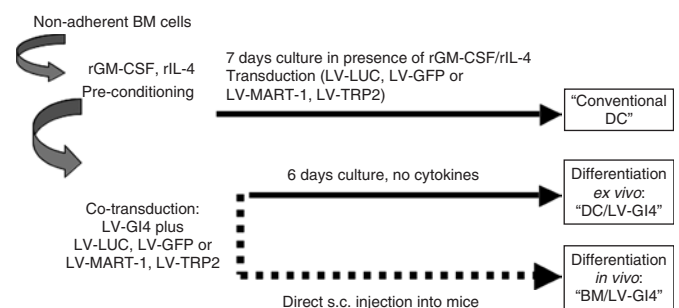


Figure 1 Lentiviral vectors and experimental design. (a) Diagram representing the bicistronic [simultaneously expressing granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4] and monocistronic vectors [expressing luciferase or green fluorescent protein (GFP) marking genes or expressing MART1 and TRP2 tumor antigens]. The long terminal repeats (LTRs), the splice donor site (SD), the splice acceptor site (SA), the packaging signal Ψ , the cytomegalovirus (CMV) enhancer/promoter element, the truncated and out-of-frame gag gene (Ga) upstream to the Rev responsive element (RRE), the central poly-purine and termination sequence (cPPT), the woodchuck hepatitis element (Wpre), and the self-inactivating mutation in the 3' LTR (Δ U3) are indicated (not to scale). The bicistronic construct contains an internal ribosome entry site (IRES) upstream of the mouse IL-4 open reading frame. (b) Experimental design for the different transduction protocols: generation of conventional DCs, *in vitro* self-differentiated DC/LV-GI4 cells, or *in vivo* differentiated BM/LV-GI4 cells.

for testing differentiation *in vivo*, in which case they were called BM/LV-GI4 (Figure 1b).

Autonomous trans-differentiation of BM cells into DCs after lentiviral transduction

The immunophenotypic characteristics of conventional DCs and cells transduced with LV-GI4 were analyzed by flow cytometry at different times during the culture (7 and 14 days). Both types of cultures generated cells that were myeloid (CD11b⁺) and expressed high levels of a primary DC marker (CD11c) and relevant immunologic markers (CD80 and MHC class II), confirming the myeloid DC phenotype (Figure 2). This immunophenotypic pattern was reproducible and stable for triplicate similar experiments (data not shown) and correlated with typical DC morphologic features (Supplementary Figure S1).

In an attempt to increase the purity of self-differentiated DCs and eliminate other possible contaminating cells in the

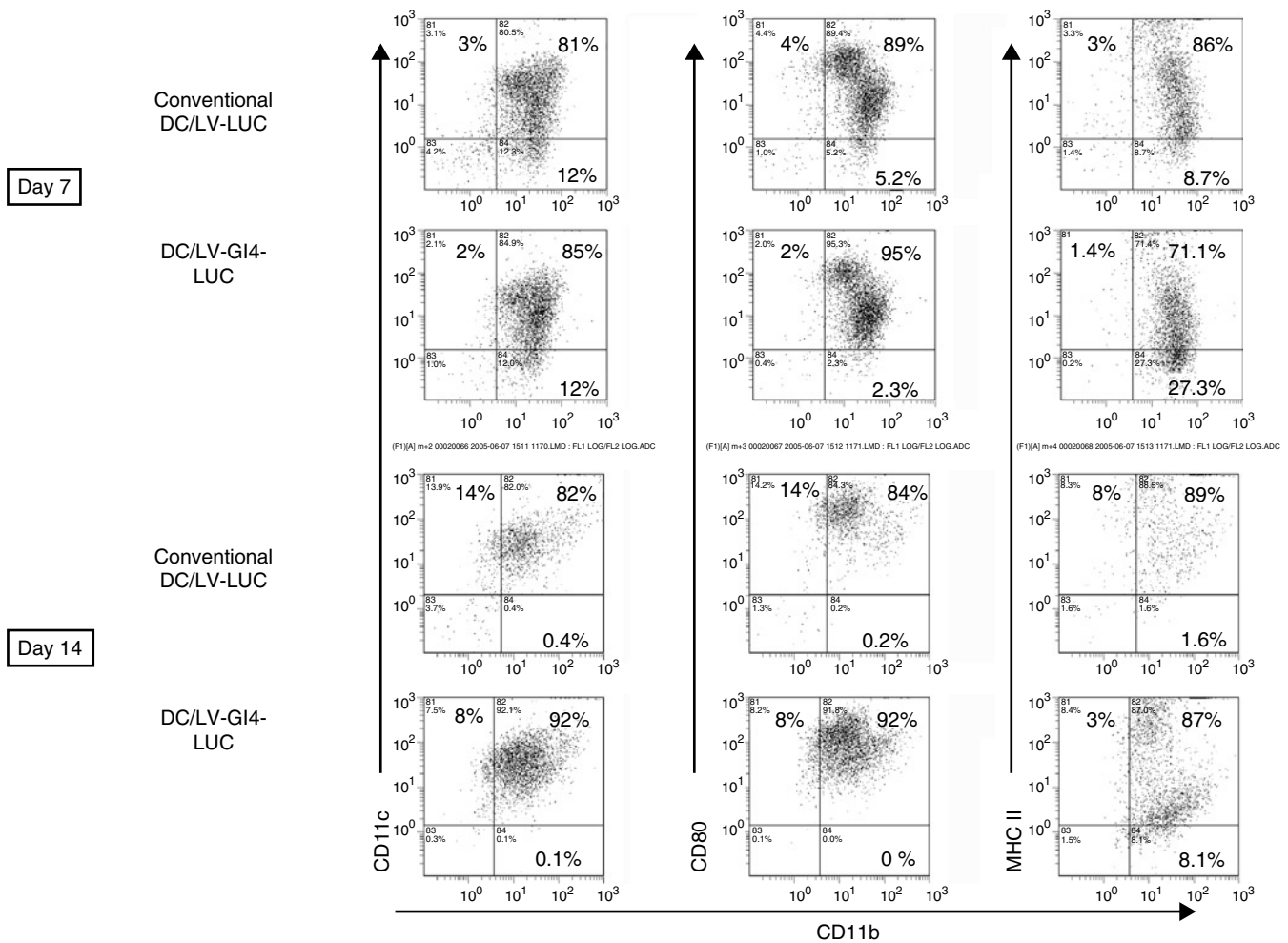


Figure 2 Characteristics of DC/LV-GI4 cells self-differentiated *ex vivo*. Flow cytometry analyses of DC/LV-GI4-LUC on days 7 and 14 of culture recapitulate immunophenotype of conventional DC/LV-LUC: CD11c^{high}, CD11b^{low}, CD80⁺, MHCII⁺.

preparation, we compared cultures derived from non-adherent BM cells with BM cultures immuno-depleted of B, T, and natural killer cells. Conventional DCs and DC/LV-GI4 cells generated using these two protocols were maintained *in vitro* for 7 days before flow cytometry analyses of CD11c, CD11b, CD80, CD86, MHCI, MHCII, CD40, CD19, and CD3 expression (Supplementary Table S1 and Supplementary Figure S2). The frequency of contaminating B (CD19⁺) and T (CD3⁺) cells was overall very low in the DC/LV-GI4 cultures with or without the prior immuno-depletion step (3–4%, Supplementary Table S1 and Supplementary Figure S2). Thus, mouse non-adherent BM cells exposed to lentiviral vectors for co-expression of GM-CSF and IL-4 developed into an overall homogeneous, pure cell population displaying similar immunophenotype to conventional DCs grown in the presence of GM-CSF/IL-4.

In vitro analyses of cellular viability—through direct viable cell counts and MTS assays—showed high viability during the first 2 weeks and subsequently progressive loss of viability (data not shown). In contrast, conventional DCs maintained in the absence of recombinant GM-CSF and IL-4 lost viability drastically during the first week of culture (data not shown), confirming our previous results with human monocyte-derived DCs.⁹

DC/LV-GI4 cells are more viable and produce higher influx of CD11c⁺ cells into LNs than conventional DCs

Bioluminescence imaging analyses were conducted to compare non-invasively the engraftment, viability, and bio-distribution of DCs and DC/LV-GI4 *in vivo*. BM cells from C57BL/6 mice were grown as conventional DCs or transduced with LV-GI4 on the first day of culture. Both groups were subsequently transduced with the LV-LUC vector on day 5 of culture. Equal amounts of viable cells from each group (5 × 10⁶ cells) were harvested on day 7 of culture and injected s.c. into the hind flank of C57BL/6 mice. The fate of luciferase-marked cells was then followed *in vivo*. To correlate the number of transduced cells with the bioluminescence signal obtained, serial dilutions of the cells were first imaged in plates containing the D-luciferin substrate. The minimum number of cells to produce detectable levels of bioluminescence was found to be approximately 1 × 10³ cells/100 μl (data not shown). The *in vivo* survival kinetics and spatio-temporal distribution of the transduced cells was then monitored by intraperitoneal injection of luciferin followed by live bioluminescence imaging of the whole animal on days 1 and 7 after injection. The bioluminescence signal emitted by sites on the skin injected with DC/LV-GI4-LUC was five to ten times higher than the signal

detectable in sites injected with conventional DCs, indicating higher viability of genetically programmed DCs (**Figure 3a**). We observed that mice injected with DC/LV-GI4-LUC showed noticeable bioluminescence signals co-localizing with inguinal LNs (**Figure 3a**). Histological analyses showed a conspicuous enlargement of LNs for mice injected with DC/LV-GI4. This was associated with a more intense staining for CD11c, as detected by immunohistochemical analysis of LNs, than in untreated mice or mice injected with conventional DCs (**Figure 3b**).

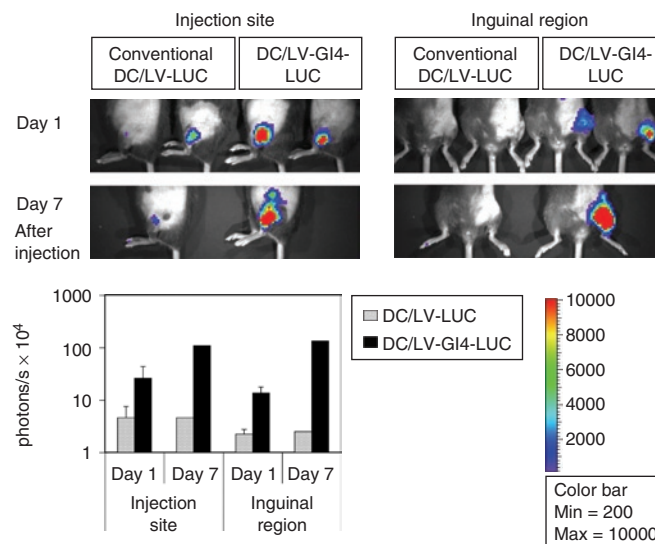
To demonstrate quantitatively and qualitatively the influx of cells into the LNs, DC/LV-GFP and DC/LV-GI4-GFP (2×10^6 cells) were injected into the inguinal region of C57BL/6 mice (two mice per group). Two days later, mice were killed and the LN analyzed. Mice injected with DC/LV-GI4-GFP showed a very pronounced and consistent weight increase of the adjacent LN compared with control mice (**Table 1**). The weight of the contralateral LN was in general not affected (**Table 1**). Higher frequencies of CD11c⁺GFP⁺ cells (quantified by flow cytometry analyses) were detectable in the adjacent LNs of DC/LV-GI4-GFP-injected mice than in LNs of DC/LV-GFP-injected mice (**Table 1**).

BM/LV-GI4-LUC cells differentiate into DCs *in situ*, and stimulate influx of CD11c⁺ cells into LNs

We next tested whether BM cells exposed to LV-GI4 would be capable of self-differentiation *in vivo*, bypassing the *ex vivo* cell culture period. BM cells were transduced with the LV-LUC marking vector only or with LV-GI4 plus LV-LUC. One day after transduction, 5×10^6 cells from each treatment group were injected s.c. into the hind left flanks of C57BL/6 mice. Mice were subjected to bioluminescence imaging on days 7 ($n = 4$), 14 ($n = 3$), and 42 ($n = 2$). One mouse per group was killed on day 7, and one on day 13, for tissue collection. Injection of BM/LV-GI4-LUC yielded two to five times higher bioluminescence signals at the injection site on days 7 and 14 after injection and ten times higher signals on day 42 after injection (**Figure 4a**). The measurements for each treatment group were compared using a mixed-model procedure and yielded significant results ($P = 0.0408$), indicating that cells co-expressing GM-CSF/IL-4 were more abundant and/or viable than cells expressing luciferase only. Analogous to what was observed previously for DC/LV-GI4-LUC injections, mice injected with BM/LV-GI4-LUC showed significant spread of the bioluminescence signal toward the inguinal region ($P = 0.0063$). Imaging analyses performed with explanted LN confirmed the presence of a bioluminescence signal (**Supplementary Figure S3**), indicating the migration of luciferase⁺ cells from the injection site to the adjacent draining LN.

Cryopreserved tissues obtained from these mice were used to confirm the DC identity of the cells in skin and LN by immunohistochemical staining for co-detection of CD11c and luciferase. The skin injected with BM/LV-GI4-LUC showed cells labeled with both luciferase and CD11c, which were not detectable in skin of mice injected with BM/LV-LUC (**Figure 4b**). We also performed immunohistochemical analyses of skin after s.c. injections into the ears using cells transduced with LV-GFP. Two days after injection with BM/LV-GI4-GFP, a conspicuous accumulation of cells in the dermis that stained for both GFP and CD11c was observed, whereas ears injected with BM/LV-GFP

a Bioluminescence imaging analyses



b Immunohistochemistry analyses of lymph nodes

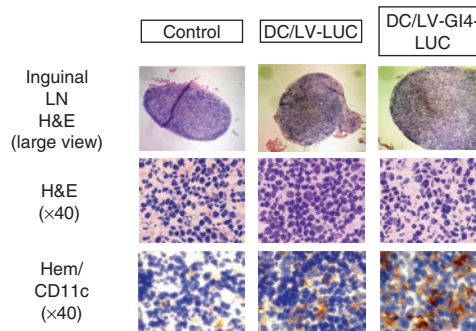


Figure 3 *In vivo* viability and lymphadenopathy after injection of conventional DC/LV-LUC or DC/LV-GI4-LUC s.c. into C57BL/6 mice. **(a)** The panel shows photographs obtained by optical imaging analyses of the injection site and inguinal region at different times. High levels of luciferase signal are shown in red and low levels in blue, as indicated by the reference color bar. The histogram shows the quantified values of bioluminescence signal on a logarithmic scale for conventional DC/LV-LUC (dots) or DC/LV-GI4-LUC (black). Mixed-model statistical analyses comparing the bioluminescence signal between mice in each study group with 12 measurements total did not yield significant values ($P = 0.0744$). **(b)** Histopathological (hematoxylin and eosin) and immunohistochemical (hematoxylin and anti-CD11c) analyses of inguinal lymph nodes (LNs) demonstrating that at 7 days after DC/LV-GI4-LUC injection, a conspicuous lymphadenopathy is observed, which is associated with higher frequency of CD11c⁺ cells, likely DCs.

showed very few GFP⁺ cells in the dermis, which did not stain for CD11c (**Figure 4c**). These results showed that BM precursors transduced with LV-GI4 were capable of self-differentiation into DCs *in vivo*.

Subsequently, we investigated the effects of self-differentiating BM/LV-GI4 cells in the adjacent LN. LNs from mice injected with BM/LV-GI4-LUC were macroscopically bigger than those from the control mice (injected with BM/LV-LUC) and contained a higher frequency of CD11c⁺ cells (**Figure 4d**). Similar to effects observed with DC/LV-GI4-GFP injections, C57BL/6 mice injected with BM/LV-GI4-GFP also showed weight increase of the adjacent LN and detectable CD11c⁺GFP⁺ cells (**Table 1**).

Table 1 Analyses of inguinal lymph nodes 2 days after cell injections

	Adjacent LN weight (mg)	Contralateral LN weight (mg)	FACS % CD11c ⁺ GFP ⁺
CTL #1	2.5	2.5	0
CTL #2	2.3	2.4	0
DC/LV-GFP #1	2.2	1.9	0
DC/LV-GFP #2	2.1	1.7	0.49
DC/LV-GI4-GFP #1	4.3	3.2	1.03
DC/LV-GI4-GFP #2	3.0	1.9	0.62
BM/LV-GI4-GFP #1	2.8	2.4	0.12
BM/LV-GI4-GFP #2	2.1	2.0	1.63

Abbreviations: CTL, control; DC, dendritic cell; GFP, green fluorescent protein; LN, lymph node; LV, lentiviral vector.

As a confirmatory *in situ* analysis, immunohistochemistry for co-localization of the marker GFP and CD11c in LN sections was performed. LN obtained from LV-GI4-GFP-injected mice showed regions containing large cells (seen by hematoxylin and eosin staining) that were CD11c⁺GFP⁺, confirming that self-differentiated DCs migrated to the LN (Figure 4e).

Serum specimens collected from mice on days 1, 3, and 7 after injection with BM/LV-GI4 showed no detectable GM-CSF and IL-4. The lymphadenopathy was transient, lasting for at least 1 week after vaccination.

DC/LV-GI4 and BM/LV-GI4 cells as potent protective and therapeutic vaccines against melanoma

We evaluated the vaccine potential of genetically programmed DCs in the well-characterized B16 transplantable mouse melanoma model, used extensively in pre-clinical immunotherapy studies. Vectors expressing the melanoma antigens MART1 or TRP2 were used. Western blots of transduced cells confirmed the expression of MART1 and TRP2 proteins in DCs at the expected molecular size (data not shown).

Eight mice per cohort were (i) injected on days -13 and -7 with phosphate-buffered saline (PBS) control ("PBS"); (ii) vaccinated with a prime/boost on days -13 and -7 with 1×10^5 conventional DCs maintained in culture for 7 days with recombinant GM-CSF and IL-4 and transduced with LV-MART1 ("DC/LV-MART1(2x)"); a vaccination schedule known to produce high survival rates); or (iii) vaccinated once on day -20 with 1×10^5 BM cells transduced with LV-GI4 plus LV-MART1, maintained in culture for only 1 day and allowed to differentiate *in vivo* for an additional 6 days ("BM/LV-GI4-MART1"). On day 0, mice were challenged with 1×10^4 B16 cells and survival was followed until tumors reached 1.5 cm in diameter, at which time mice were humanely killed. Under these experimental conditions, we observed a rate of two out of eight survivors for the PBS negative control and six out of eight survivors for prime/boost vaccinations with the conventional DC-LV-MART1 positive control (log rank test $P = 0.0282$). In contrast, all eight mice survived in the group vaccinated once with BM/LV-GI4-MART1, showing the best protective results in terms of survival distribution (log rank test $P = 0.0022$) (Figure 5a). Statistical analyses comparing the survival distribution between BM/LV-GI4-MART1 and DC/LV-MART1 yielded no significance (log rank test $P = 0.1435$),

although BM/LV-GI4-MART1 vaccine protected all mice in the cohort. (Graphs showing the kinetics of tumor growth over time are included as Supplementary Figure S4a.)

We performed additional triplicate experiments challenging mice with a higher tumor dose to compare the efficiency of genetically programmed DC vaccines differentiated *ex vivo* or *in vivo*. Mice ($n = 5$ per cohort) were vaccinated s.c. on the hind flanks: (i) on day -13 with PBS control ("PBS"); (ii) on day -13 with 1×10^5 DC/LV-GI4-MART1; or (iii) on day -20 with 1×10^5 BM/LV-GI4-MART1, maintained in culture for only 1 day. On day 0, all mice were injected with a lethal dose of B16 melanoma (5×10^4 cells). The Kaplan-Meier plot of the merged triplicate experiments ($n = 15$ for each treatment) demonstrates that vaccination with BM/LV-GI4-MART1 produced approximately 27% survivors, whereas the PBS control and DC/LV-GI4-MART1 groups produced approximately 7% survivors (Figure 5b). A log rank test comparing the survival distribution of the PBS control group with that of the other vaccination groups showed statistically significant protection for BM/LV-GI4-MART1 ($P = 0.0154$) and DC/LV-GI4-MART1 ($P = 0.0465$). (Graphs showing the kinetics of tumor growth over time are included as Supplementary Figure S4b.)

A melanoma treatment model was evaluated using different modalities of DC vaccines and the extensively tested GM-CSF-transduced B16 tumor vaccine. C57BL/6 mice were challenged s.c. with 1×10^4 B16 cells on day 0 and vaccinated with 1×10^5 cells on days 4, 8, 12, and 16. The treatment groups ($n = 8$) consisted of (i) no vaccine ("PBS"); (ii) conventional DC vaccines transduced for MART1 and TRP2 co-expression, maintained in culture for 7 days and treated with sCD40L for 24 hours before injection; (iii) DC/LV-GI4 vaccines co-expressing MART1 and TRP2, maintained in culture for 7 days and treated with sCD40L for 24 hours before injection; (iv) BM/LV-GI4 vaccines co-expressing MART1 and TRP2, and injected 24 hours after transduction; (v) B16 cells expressing GM-CSF. The PBS control was the only group showing tumor development in five of eight mice (the average survival period for control mice developing tumors was 37.2 ± 9 days; Table 2). All mice in the other cohorts remained tumor free for at least 80 days after challenge (Table 2). Subsequently, the memory response effects of each immunization modality was evaluated by re-challenging all mice that survived the initial challenge with a high dose of melanoma cells (1×10^5 B16 cells) on day 90. Age-matched naïve mice were used as controls. Tumor occurrence, volume, and survival were followed for 70 days (Supplementary Figure S5). Six out of eight mice in the control group developed tumors early and died within the first 3 weeks after re-challenge. Three out of eight mice vaccinated with conventional DCs expressing TRP2 and MART1 showed tumors developing 5 weeks after re-challenge. One out of eight mice vaccinated with B16/GM-CSF or DC/LV-GI4-TRP2, MART1 showed tumors developing 3 weeks after re-challenge. The only group showing 100% long-term protection was the cohort vaccinated with lentivirus-programmed DCs self-differentiated *in vivo* (BM/LV-GI4-TRP2, MART1).

DC/LV-GI4 and BM/LV-GI4 vaccines elicit the highest antigen-specific immune responses

To correlate the immunization effects obtained with DC/LV-GI4 and BM/LV-GI4 vaccines with the antigen-specific immune

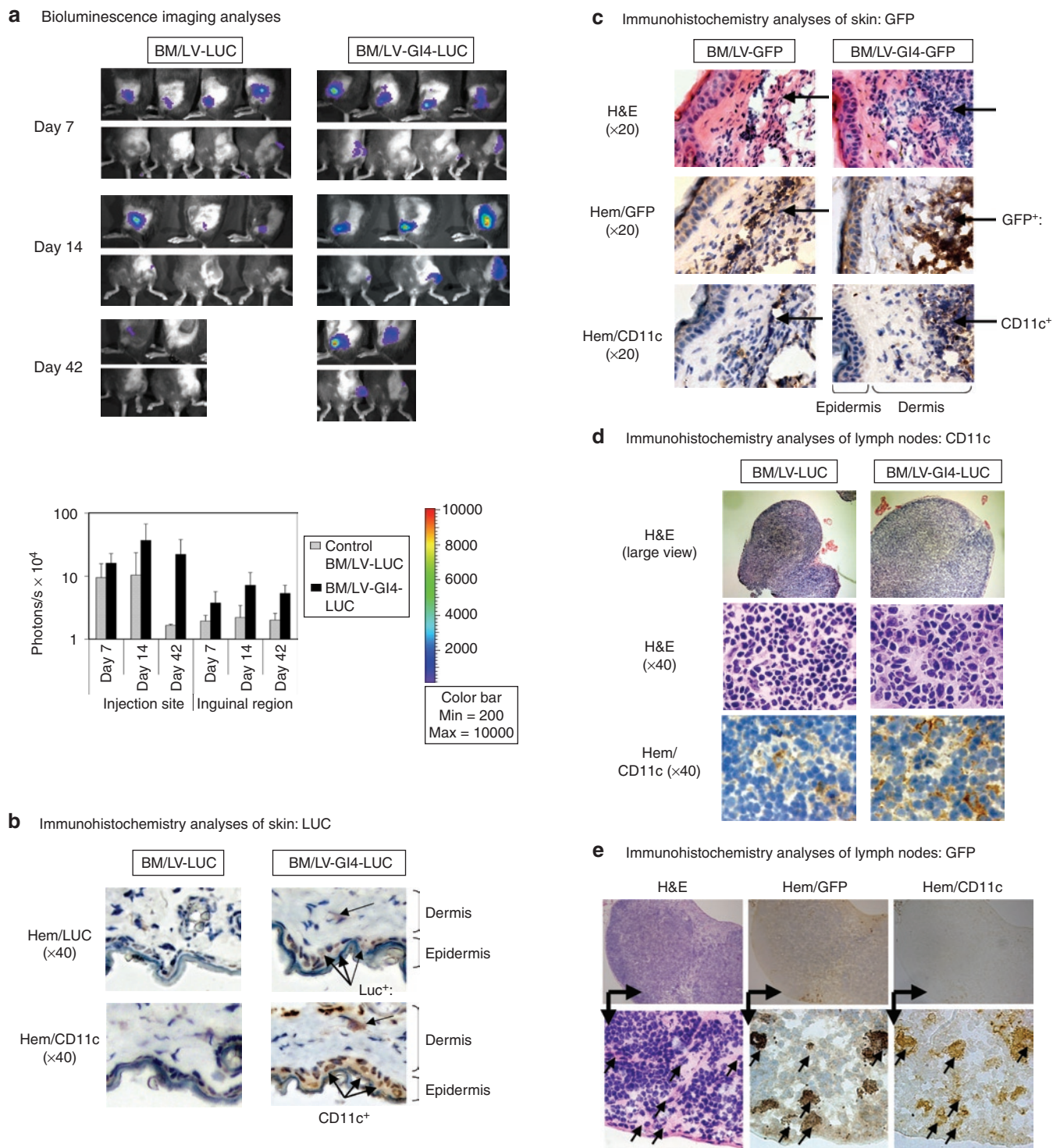


Figure 4 *In vivo* superior viability, self-differentiation, and migration of BM/LV-GI4-LUC cells injected subcutaneously (s.c.) into C57BL/6 mice. **(a)** Photographs obtained by optical imaging analyses of the injection site and inguinal region of mice at different times. High levels of luciferase signal are shown in red and low signals in blue, as indicated by the reference color bar. The histogram shows the quantified values of bioluminescence signal on a logarithmic scale for BM/LV-LUC (dots) or BM/LV-GI4-LUC cells (black). Mixed-model statistical analyses comparing the bioluminescence signal between mice in each study group with 18 measurements total yielded significant values (injection site $P = 0.0408$ and inguinal region $P = 0.0063$). **(b)** Immunohistochemical (hematoxylin and anti-CD11c or anti-LUC) analyses of injected skin (flanks) showing the presence of CD11c⁺LUC⁺ cells in the epidermis and dermis of mice injected with BM/LV-GI4-LUC (arrows). **(c)** Immunohistochemical (hematoxylin and anti-CD11c or anti-GFP) analyses of injected skin (ears) showing the accumulation of CD11c⁺GFP⁺ cells in the dermis of mice injected with BM/LV-GI4-GFP (arrows). **(d)** Histopathological (hematoxylin and eosin) and immunohistochemical (hematoxylin and anti-CD11c) analyses of inguinal lymph nodes (LNs) demonstrating that at 7 days after injection with BM/LV-GI4-LUC, a conspicuous lymphadenopathy is observed, which is associated with increased frequency of CD11c⁺ cells. **(e)** Large view ($\times 4$, upper panels) and high-magnification view ($\times 100$, lower panels) of immunohistochemical analyses (hematoxylin, anti-CD11c, and anti-GFP) of LN obtained from mice injected with BM/LV-GI4-GFP showing co-localization of large cells positive for CD11c and GFP (arrows).

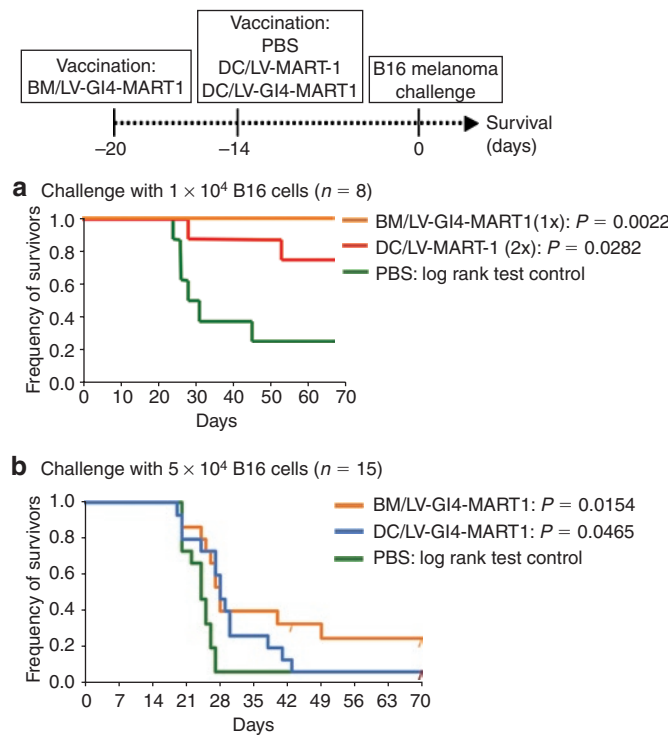


Figure 5 Protection against B16 melanoma challenge after vaccination with BM/LV-GI4-MART1. Upper panel shows the experimental schedule for vaccinations and challenge. **(a)** Sub-lethal B16 challenge dose (1×10^4 cells): phosphate-buffered saline (PBS) injection control (green), two vaccinations with day-7 conventional DC/LV-MART1 gene (red), or vaccination with day-1 BM/LV-GI4-MART1 (orange). Kaplan-Meier analyses (frequency of survivors over time) is shown for the different treatment groups. Significance values (P) were calculated by log rank test relative to the PBS control. **(b)** Lethal B16 challenge dose (5×10^4 cells): PBS injection control (green), vaccination with day-7 DC/LV-GI4-MART1 (blue), or vaccination with day-1 BM/LV-GI4-MART1 (orange).

responses, we initially assessed the immune responses against TRP2. Two independent vaccination experiments were performed. In the first experiment, only the TRP2 transgene was expressed in DC, DC/LV-GI4, and BM/LV-GI4 vaccines. In the second experiment, both the TRP2 and MART1 transgenes were expressed. C57BL/6 mice ($n = 2$) were injected with (i) no vaccine ("PBS"); (ii) transduced DC (experiment 2: treated with sCD40L for 24 hours before injection); (iii) co-transduced DC/LV-GI4 (experiment 2: treated with sCD40L for 24 hours before injection); (iv) co-transduced BM/LV-GI4 injected 24 hours after transduction; (v) B16 cells expressing GM-CSF (experiment 2 only). Two weeks (experiment 1) or one week (experiment 2) after the last vaccination, spleens were harvested. The splenocytes were re-stimulated *in vitro* with the immuno-dominant peptide epitope, TRP2₁₈₀₋₁₈₈ SVYDFVWL, which binds to C57BL/6 MHC class I (H-2K^b), and stained for surface CD3/CD8 and intracellular IFN- γ . For both experiments, mice vaccinated with DC/LV-GI4 or BM/LV-GI4 showed the highest levels of CD3⁺/CD8⁺/IFN- γ ⁺ reactive T cells (Table 2). On the other hand, mice vaccinated with conventional DCs showed near-to-baseline levels of CD3⁺/CD8⁺/IFN- γ ⁺ T cells, comparable to the control "PBS" groups. Additional evidence for the potent presentation of TRP2₁₈₀₋₁₈₈ by DC/LV-GI4 or BM/LV-GI4 vaccines was that these animals also

Table 2 Analyses of anti-TRP2 specific immune responses (experiments 1 and 2) and survival outcome for B16 melanoma treatment model (experiment 2)

	Experiment #1 Antigenic gene: TRP2 No sCD40L		Experiment #2 Antigenic genes: TRP2 + MART1DC Maturation with sCD40L		B16 Challenge ($n = 8$)% Survival (80 d) Survival period
	FACS IFN- γ ICS	FACS TRP2 Tetr	FACS IFN- γ ICS	FACS TRP2 Tetr	
CTL#1	0.8	1.5	1.9	1.1	37.5% (3/8)
CTL#2	NA	NA	2.0	1.0	37.2 \pm 9 ($n = 5$) 80 d ($n = 3$)
DC/LV#1	0.9	2.2	2.5	1.8	100% (8/8)
DC/LV#2	1.2	2.1	1.5	1.3	80 d ($n = 8$)
DC/LV-GI4#1	1.2	3.6	8.6	3.9	100% (8/8)
DC/LV-GI4#2	1.9	3.7	9.3	2.8	80 d ($n = 8$)
BM/LV-GI4#1	1.7	3.2	3.8	1.5	100% (8/8)
BM/LV-GI4#2	1.4	2.7	4.5	NA	80 d ($n = 8$)
B16/GM#1	NA	NA	2.6	1.7	100% (8/8)
B16/GM#2	NA	NA	3.1	1.4	80 d ($n = 8$)

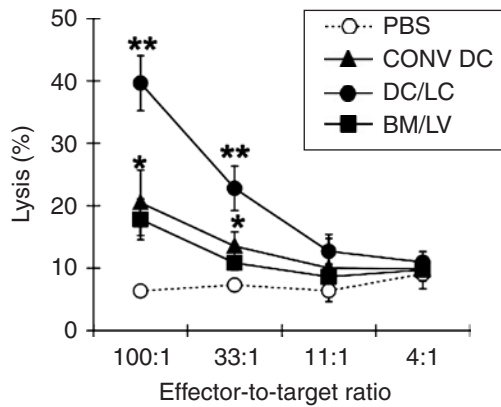
Abbreviations: CTL, control; DC, dendritic cell; GM, granulocyte macrophage; LV, lentiviral vector.

possessed the highest frequencies (approximately 3%) of TRP2-specific CD8⁺ T cells, as evaluated by flow cytometry of splenocytes stained with monoclonal antibodies against CD3/CD8 α and tetramers specific for TRP2 peptide (Table 2).

To confirm the stimulation of the cytotoxic T-lymphocyte effector function against target cells expressing MART1, we performed microtoxicity assays. C57BL/6 mice ($n = 3$) were immunized (i) on day 14 with PBS; (ii) on day 14 with conventional DC/LV-MART1; (iii) on day 14 with DC/LV-GI4-MART1; or (iv) on day 20 with BM/LV-GI4-MART1 injected 24 hours after transduction. On day 0, splenocytes were harvested, re-stimulated *in vitro* with irradiated EL-4 cells co-expressing CD80 and MART1, and assayed in a chromium release test using B16 cells as targets. At effector-to-target ratios of 100:1 and 33:1, effectors of mice vaccinated with DC/LV-GI4 displayed significantly ($P < 0.01$) higher lysis capability than those of mice in the other groups (Figure 6). Vaccination with conventional DCs or with BM/LV-GI4 resulted in similar levels of cytotoxic T-lymphocyte activity. At an effector-to-target ratio of 100:1, both vaccines were significantly better than the PBS control ($P < 0.01$) (Figure 6).

Examination of vaccinated mice maintained long term showed no pathology

Nine months after vaccination, two mice vaccinated with DC/LV-MART1, four mice vaccinated with BM/LV-GI4-MART1, and four mice vaccinated with BM/LV-GI4-TRP2 were killed for pathological analyses. None of the mice showed signs of pathology in the skin, spleen, liver, LNs, or thymus. LNs and spleen were in the normal size range. Peripheral blood smears were analyzed for the frequency of different hematopoietic lineages and for presence of blasts. The frequencies of the different lineages were found to be normal (Supplementary Table S2). No leukemia was



* $P < 0.01$ between BM/LV or ConvDC versus PBS
 ** $P < 0.01$ between DC/LV versus BM/LV, ConvDC, or PBS

Figure 6 MART1-specific effector function after vaccination with DC/LV-GI4-MART1 or BM/LV-GI4-MART1. Chromium release assay was performed with splenocytes at several effector-to-target ratios after mice were vaccinated with phosphate-buffered saline (PBS) (open circles), conventional DC/LV-MART1 (triangles), DC/LV-GI4-MART1 (closed circles), or BM/LV-GI4-MART1 (squares).

found. LNs adjacent to the vaccination sites were analyzed using real-time quantitative polymerase chain reaction for the presence of integrated lentivirus. In parallel to amplification of lentiviral sequences, amplification of β -actin was used as internal control. Non-vaccinated mice were used as negative controls. Except for one mouse vaccinated with DC/LV-MART1, all other mice were negative for latently integrated virus (**Supplementary Table S2**). Eight additional mice (four mice vaccinated with BM/LV-GI4-MART1 and four vaccinated with BM/LV-GI4-TRP2) have been maintained for 13 months after vaccination and no pathologies have developed.

DISCUSSION

We developed an approach to manipulate DC precursors *ex vivo* with one hit of gene transfer, in order to generate autonomously differentiated and long-lived DCs. We previously showed that co-expression of GM-CSF and IL-4 was sufficient and necessary in the monocyte-derived DC system to promote differentiation of cells with typical DC morphology and immunophenotype.⁹ Using an analogous strategy, we now extend the generation of self-differentiating DCs to a mouse pre-clinical system, using a bicistronic vector for simultaneous GM-CSF/IL-4 expression and a monocistronic vector for co-expression of marking genes or tumor-associated antigens. BM cells transduced *ex vivo* with lentiviral vectors for GM-CSF and IL-4 co-expression readily differentiated *in vitro* into a homogeneous population, showing typical DC morphology and immunophenotype. DC/LV-GI4 cells were more viable both *in vitro* and *in vivo* than DCs cultured *ex vivo* with recombinant cytokines. BM cells co-expressing GM-CSF/IL-4 that were injected s.c. on the day after transduction engrafted, differentiated into DCs *in vivo*, maintained viability, and migrated to LN. Examination of LNs after DC/LV-GI4 and BM/LV-GI4 injections showed transient and local lymphadenopathy correlated with increased number of CD11c⁺ cells, indicating paracrine bystander

effects toward host DCs. Despite these observed bystander effects, we have not observed any significant increase in the levels of circulating GM-CSF and IL-4 after s.c. vaccination of mice with DC/LV-GI4 or BM/LV-GI4 (data not shown). Therefore, the effect of the genetically programmed DCs is most likely the local recruitment of DCs through effects in the injection site's milieu. This mechanism is therefore distinct from those previously reported in studies of systemic infusion of recombinant GM-CSF and IL-4 in mice and humans increasing DC number and function *in vivo*.^{10,11}

Ultimately, we examined the immunostimulatory capability of genetically programmed DCs, using TRP2 and MART1 as tumor-associated melanoma antigens in the B16 model. BM/LV-GI4-MART1 vaccines that self-differentiated *in vivo* and were used as a single-dose vaccine before tumor challenge produced consistently higher rates of survival than conventional DCs transduced with lentiviral vectors for MART1 expression. Interestingly, genetically programmed DCs expressing MART1 were more effective vaccines when allowed to self-differentiate *in vivo*, thereby demonstrating that the *ex vivo* culture can be bypassed. We were able to demonstrate the long-term therapeutic effects of DC/LV-GI4 and BM/LV-GI4 co-expressing TRP2 and MART1 against pre-implanted tumor. Antigen-specific immune responses (against TRP2 and MART1) were higher for genetically programmed DCs than for conventional DCs or B16 vaccines.

To our knowledge, despite the fact that GM-CSF gene delivery has been shown to have significant effects in autologous cancer cell¹²⁻¹⁴ and DC vaccines,¹⁵ this is the first demonstration that *ex vivo* lentiviral transduction of hematopoietic precursors for GM-CSF/IL-4 co-expression leads to the direct, autonomous trans-differentiation of these cells into DCs *in vivo*. Of note, carcinogenesis resulting from persistent production of growth factors, lentiviral vector insertional mutagenesis, or spread of replication-competent lentiviral vectors is a relevant concern for the clinical development of genetically programmed DCs. However, long-term observation of C57BL/6 mice immunized with BM/LV-GI4 has shown no signs of injurious effects. Therefore, this efficient and simpler methodology for DC production could be applied in large-scale immunization approaches against cancer and infectious diseases.

MATERIALS AND METHODS

Cell culture. The murine melanoma cell lines B16-F0 and B16/GM-CSF (clone D5, kindly provided by Dr. Bernie Fox, Oregon Health and Science University, Portland, OR) were cultured in Dulbecco's modified Eagle's medium plus 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μ g/ml), 100 mM 2-mercaptoethanol, and 2 mM L-glutamine.

Lentiviral vector production. The lentiviral vectors in this study were derived from the pRRL-sin-cPPT-hCMV-GFP-pre vector, and large-scale production was performed by transient co-transfection of 293T cells as described elsewhere.¹⁶ The murine GM-CSF and IL-4 cDNAs were obtained from the National Gene Vector Laboratories. Dr. Lily Wu (University of California at Los Angeles; UCLA) kindly provided a lentivector backbone containing the firefly luciferase gene (pCCL-sin-cPPT-CMV-fluc). Dr. James Economou and Dr. Robert Prins (UCLA) provided the hMART1 and mouse TRP2 transgenes, respectively. The structural integrity of all constructs was reconfirmed by restriction digestion and sequencing analysis of the transgenes. Lentiviral titer was determined by assessing

viral p24 antigen concentration by enzyme-linked immunosorbent assay (Coulter Immunotech, Miami, FL), and hereafter expressed as micrograms of p24 equivalent units per milliliter.

Culture of conventional DCs, transduction. BM was aseptically harvested from femurs of C57BL/6 mice, washed, and incubated in 10-cm-diameter plastic dishes with Roswell Park Memorial Institute medium (RPMI) + 10% fetal calf serum overnight. Non-adherent cells were plated at 5×10^6 cells/well of 6-well plates in RPMI with 10% fetal calf serum in the presence of recombinant GM-CSF and IL-4 (50 ng/ml each; R&D Systems, Minneapolis, MN) as previously described.¹⁷ For conventional DC growth, additional GM-CSF and IL-4 were added on day 3 of culture, and subsequently every 3 days if required. Transduction was carried out on day 5 of culture with lentiviral vectors (5 μ g p24 equivalent/ml) and protamine sulfate (5 μ g/ml) added to $2-5 \times 10^6$ cells and incubated for 16 hours at 37°C, after which the cells were washed twice with PBS and used for analyses or vaccinations. When indicated, soluble murine CD40 ligand (500 ng/ml; R&D Systems, Minneapolis, MN) was added to induce DC maturation. For immuno-depletion of granulocytes and T, B, and natural killer cells, BM cells were treated with antibodies (1 μ g/million cells) against GR-1, B220, Thy 1.2, and NK1.1 (all from BD/Pharmingen, San Jose, CA) and cell lysis was induced with 10% rabbit complement (Sigma, St. Louis, MO) in RPMI for 30 min.

Ex vivo production of genetically programmed DCs. Non-adherent BM cells were obtained as above and pre-conditioned with murine GM-CSF and IL-4 (50 ng/ml each; R&D Systems, Minneapolis, MN) for 8 hours in RPMI + 10% fetal calf serum medium before lentiviral transduction. Lentiviral vectors (5 μ g p24 equivalent in 1 ml) and protamine sulfate (5 μ g/ml) were added to $2-5 \times 10^6$ cells and transduction was carried out for 16 hours at 37°C, after which time the cells were washed twice with PBS and used for analyses or vaccinations.

Analysis of transgene expression after transduction. GM-CSF and IL-4 secretion by DC/LV-GI4 was measured by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN). Expression of GFP by DCs and DC/LV-GI4 was examined directly by flow cytometry in the fluorescein isothiocyanate channel. Immuno-detection of MART1 in DCs was confirmed by Western blotting using commercially available antibodies (Novocastra, Newcastle, UK).

Flow cytometry analyses of surface antigens. Immunostaining was performed with commercially available fluorescent immunconjugated monoclonal antibodies against CD11c, CD11b and CD80, CD86, MHCI, MHCII, CD40, CD3, CD19, and respective isotype controls (BD/Pharmingen, San Jose, CA). Flow cytometry was performed using a Beckman Coulter flow cytometry apparatus (Fullerton, CA). For each set of analyses, 1×10^4 cells were acquired. To establish background for fluorescence and to set gates for data acquisition, cells stained with isotype antibodies were used as baseline reference. Care was taken to analyze cells that were in the viable white blood cell gate as indicated by forward and side scatter characteristics.

Mouse injections, vaccinations, tumor inoculation. All procedures involving mice were reviewed and approved by the UCLA Animal Research Committee and followed the UCLA Institutional Animal Care and Use Committee guidelines according to the American Association for Laboratory Animal Science. Cell suspensions in pharmaceutical-grade saline solution were injected in volumes of 100 μ l s.c. into the mouse hind flank with a 27-gauge needle. Mice were immunized with 1×10^5 DCs or B16/GM-CSF irradiated (3,500 rads) cells and challenged with 1×10^4 or 5×10^4 B16 cells. After B16 s.c. challenge, tumor growth was measured daily with a microcaliper, and mice that developed tumors larger than 1.5 cm in diameter were humanely killed. Tumor-free animals were kept under observation for at least 6 months after vaccination and/or challenge.

Histology and immunohistochemistry analysis. After mice were killed, biopsies from tissues (skin at the vaccination site, inguinal LNs) were taken. Tissues were embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA) and cryopreserved to produce frozen sections. Slides containing sections were fixed with paraformaldehyde and stained for hematoxylin and eosin for morphology analysis. Immunohistochemical staining was carried out using commercially available first antibodies for detection of CD11c (hamster anti-mouse; BD/Pharmingen), luciferase (goat polyclonal; Novus, Littleton, CO), and GFP (rabbit polyclonal; Molecular Probes, Eugene, OR).

Real-time quantitative polymerase chain reaction analysis of genomic DNA. Genomic DNA from tissues was purified using a DNeasy Tissue Kit (Qiagen, Valencia, CA). Quantification of the vector copy number was performed in a 25- μ l reaction containing 300 ng of genomic DNA (equivalent to 5×10^4 genomes) using the TaqMan RT-Q-PCR assay to detect lentiviral packaging sequences of human immunodeficiency virus-1 as a universal primer.¹⁸ The oligonucleotides used were LV primers: 5'-ACCTGAAAGCGAAAGGGAAAC-3' and 5'-CACCCATCTCTCTCTCTCTAGCC-3'; LV probe: 5'-FAM-AGCTCTCTCGACGCAGGACTCGGC-TAMRA-3'; β -actin internal control primers: 5'-GGTCGTACCACAGGCATTGT-3' and 5'-CTCGTAGATGGGCACAGTGT-3'; and β -actin probe: 5'-FAM-CCCGTCTCCGGAGTCC-NFQ-3'. Amplifications were carried out in an ABI PRISM 7,700 sequence detector (Perkin Elmer, Wellesley, MA); after the initial denaturation (10 minutes at 95°C), amplification was performed with 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. To calculate the copy number in the samples, a reference curve was prepared by amplifying serial dilutions of LV-CMV-LUC plasmid in a background of genomic DNA of spleen from non-treated mice; the C_t values were plotted against the input plasmid, and a standard reference curve was obtained.

In vivo bioluminescence imaging analyses. Mice were anesthetized with ketamine (100 mg/kg intraperitoneally) and xylazine (10 mg/kg intraperitoneally), and an aqueous solution of D-luciferin (150 mg/kg intraperitoneally) was injected 5 minutes before imaging. Animals were placed into the light chamber of the charge-coupled device camera (IVIS, Xenogen, Cranbury, NJ), and grayscale body surface reference images (digital photograph) were taken under weak illumination. After the light source was switched off, photons emitted from luciferase-expressing cells within the animal body and transmitted through the tissue were quantified over a defined time of up to 5 minutes using the software program Living Image (Xenogen, Cranbury, NJ) as an overlay on Igor (Wavemetrics, Seattle, WA). For anatomical localization, a pseudocolor image representing light intensity (blue, least intense; red, most intense) was generated in Living Image and superimposed over the grayscale reference image.

Analyses of CD8⁺ T-cell responses against TRP2. For the identification of TRP2-specific reactive cells, splenocytes were stained with PerCP-conjugated monoclonal antibody (MAb) for CD3, fluorescein isothiocyanate-conjugated MAb for CD8, and PE-labeled tetramers specific to H-2K^b TRP2₁₈₀₋₁₈₈ and a control non-specific tetramer as described previously.¹⁹ Antibodies were obtained from BD/Pharmingen and tetramers from Beckman Coulter. More than 30,000 CD8⁺ cells were acquired for this assay. For intracellular IFN- γ staining, splenocytes were cultured for 6 hours in 96-well plates at a concentration of 1×10^6 cells/well in 0.2 ml of complete medium with 1 μ l/ml brefeldin A (GolgiPlug; BD/Pharmingen, San Jose, CA) and human IL-2 (50 U/ml). The cells were re-stimulated with TRP2 (TRP2₁₈₀₋₁₈₈ SVYDFVWL) peptide at 5 μ g/ml. The cells were spun down and surface antigens were stained in PBS, supplemented with 2% FBS with PE-conjugated MAb for CD8 and PerCP-conjugated MAb for CD3. After the unbound antibody was washed off, cells were subjected to intracellular cytokine stain using the Cytofix/Cytoperm kit (Pharmingen) according to the manufacturer's instructions. For intracellular IFN- γ staining, the fluorescein isothiocyanate-conjugated monoclonal rat anti-mouse IFN- γ

Ab (clone XMG1.2; Pharmingen) or its isotype control Ab (rat IgG1) was used. Flow cytometry was performed with a Beckman Coulter flow cytometry apparatus. For each set of analyses, 5×10^4 cells were acquired. To establish background for fluorescence and to set gates for data acquisition, cells stained with isotype antibodies were used as baseline reference. Care was taken to analyze cells that were in the viable white blood cell gate as indicated by forward and side scatter characteristics.

Analyses of CD8⁺ T-cell responses against MART1. For *in vitro* microcytotoxicity assays, splenocytes were harvested, pooled, depleted of red blood cells by hypotonic lysis, re-stimulated *in vitro* with irradiated EL-4 cells co-expressing CD80 and MART1 for 96 hours in the presence of 10 U/ml of IL-2, and assayed in a standard 4-hour chromium release test using B16 cells as targets.²⁰

Statistical analysis. A mixed model was used to compare the differences in *in vivo* viability and bio-distribution between the experimental groups (conventional versus genetically programmed DCs). The different days and different locations (injection site and inguinal region) when the measurements were obtained were taken into account in the model. For tumor challenge studies, the probability of disease-free survival was estimated using the Kaplan–Meier method; disease-free survival was plotted and compared using log rank test. All tests were two-sided, and $P < 0.05$ was considered significant. SAS software (SAS Institute, Cary, NC) was used for the statistical analysis.

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SUPPLEMENTARY MATERIALS

Figure S1. Phase contrast microscopic examination of DC/LV-GI4-LUC cells showing typical morphology at several stages of differentiation.

Table S1. Immunophenotypic characterization of conventional DCs and lentivirally programmed DCs (DC/LV-GI4) generated without or with immuno-depletion of B, T, and natural killer cells before transduction. The percentages represent the frequency of cells positive for the different cell-surface markers quantified by flow cytometry analyses.

Figure S2. (a) Immunophenotypic characterization of DCs and DC/LV-GI4 cells generated with an additional immuno-depletion step following the standard protocol with non-adherent BM cells. (b) Detection of contaminating T and B cells in each type of preparation.

Figure S3. Optical imaging analyses demonstrating bioluminescence detected in lymph node (LN) explanted from C57BL/6 mice injected with BM/LV-GI4-LUC. Pictures show the injection site, the inguinal region, and a large view of adjacent LN.

Figure S4. Kinetics of tumor growth after vaccination with DC/LV-MART1, DC/LV-GI4-MART1, or BM/LV-GI4-MART1.

Figure S5. Kinetics of tumor growth and survival rate after challenge of control naïve mice or re-challenge of previously challenged/vaccinated mice with 5×10^4 B16 cells.

Table S2. Post-mortem analyses of mice maintained for 9 months after vaccination.

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