In vivo administration of a lentiviral vaccine targets DCs and induces efficient CD8+ T cell responses

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The present study evaluates the potential of third-generation lentivirus vectors with respect to their use as in vivo–administered T cell vaccines. We demonstrate that lentivector injection into the footpad of mice transduces DCs that appear in the draining lymph node and in the spleen. In addition, a lentivector vaccine bearing a T cell antigen induced very strong systemic antigen-specific cytotoxic T lymphocyte (CTL) responses in mice. Comparative vaccination performed in two different antigen models demonstrated that in vivo administration of lentivector was superior to transfer of transduced DCs or peptide/adjuvant vaccination in terms of both amplitude and longevity of the CTL response. Our data suggest that a decisive factor for efficient T cell priming by lentivector might be the targeting of DCs in situ and their subsequent migration to secondary lymphoid organs. The combination of performance, ease of application, and absence of pre-existing immunity in humans make lentivector-based vaccines an attractive candidate for cancer immunotherapy.


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Nonstandard abbreviations used: oligodeoxynucleotide (ODN); cytotoxic T lymphocyte (CTL); 1,4-diazabicyclo-(2.2.2)-octane (DABCO); peripheral blood lymphocyte (PBL); bone marrow–derived DC (BMDC); expression-forming unit (EFU); incomplete Freund’s adjuvant (IFA).

Introduction

The prospects of an antitumor vaccine depend on its ability to induce robust and sustained tumor-specific T cell responses. Since the initiation of such responses is crucially dependent on the presentation of the antigen by DCs, current tumor vaccines are designed to target DCs ex vivo. One approach consists of pulsing DCs in vitro with antigenic peptides before transferring them back to the patient (1–3). In an analogous approach, DCs are transduced with an antigen-recombinant viral vector (4, 5). Transduction is thought to result in better antigen processing and more sustained antigen presentation than pulsing (4, 6). However, the generation and ex vivo manipulation of DCs is laborious and costly. Therefore, a cell-free vaccine that could be easily administered yet would retain the efficacy of the DC-based approach would be a significant improvement. However, as the majority of recombinant viral vectors are derived from viruses originally designed to prevent viral infections, pre-existing immunity to the parental virus might interfere with their ability to induce potent antitumor immune responses (7).

In this context, the advent of new, efficient gene delivery vehicles may provide a solution. We and others recently introduced lentiviral vectors (lentivectors) as tools for the ex vivo transduction of DCs and the induction of T cell responses (8–11). These third generation lentivectors are designed for in vivo gene therapy and to our knowledge exhibit the most advanced safety features so far included in any viral vector system (12) and thus may be suitable for in vivo administration as a vaccine.

This study characterizes the properties of an in vivo administered lentivector-based vaccine with regard to its cellular targets and its ability to induce antigen-specific CD8+ T cell responses. We observed that lentivector administration transduced DCs that were found in the draining lymph node and in the spleen. In addition, the vaccine induced potent T cell responses as demonstrated by up to 40% antigen-specific cells among the CD8+ subset and high levels of specific cytotoxicity.

Comparative vaccination with transduced DCs demonstrated that in vivo administration of lentivector induces equally strong antigen-specific T cell responses. Moreover, when compared with another cell-free approach, administration of antigenic peptides in combination with CpG oligodeoxynucleotides (ODNs) emulsified in adjuvant, lentivector induced a 3- to 10-fold higher amplitude of response.

Methods

Mice. B6D2F1 mice (Harlan, Zeist, the Netherlands) were used for Cw3 vaccination. HLA-Cw3 transgenic mice were used as source of antigen-transgenic DCs (13).
HLA-A*0201/Kb transgenic mice were used for vaccinations with Melan-A/ELA26-35 (14). These mice were originally provided by Harlan Sprague-Dawley (Indianapolis, Indiana, USA). Mouse experiments were carried out with permission of the “Service Vétérinaire” of the Canton de Vaud, Swiss Confederation.

**Lentivector vaccines.** The HLA-Cw3 cDNA was cloned by RT-PCR from P 815 cells stable-transfected with HLA-Cw3 (15) and inserted into lentiviral transfer vector pIRES-EGFPpRRLSin18.PPT.CMV. A minigene encoding the H-2Kd–restricted cytotoxic T lymphocyte (CTL) antigen of HLA-Cw3 (Cw3194–203) (15–17) was inserted in pIRES-EGFPpRRLSin18.PPT.CMV (8). Likewise, a minigene encoding the immunodominant peptide analogue of the HLA-A*0201–restricted Melan-A CTL antigen (ELA26-35) was cloned in pIRES-EGFPpRRLSin18.PPT.CMV. The mutation at position 27 (Ala to Leu) results in increased immunogenicity of the peptide analogue as compared with the wild type (18). Both antigen minigenes contained a start and a stop codon. Preparation of concentrated stocks of third-generation lentivectors was performed as described (8, 19).

**PCR detection of integrated virus.** PCR detection of lentivector integration into tissue was performed on genomic DNA from whole lymph nodes and spleen using a seminested approach with GFP-specific primers (forward, 5′-GGCCACAAGTTCAGCGTGTCC-3′; reverse, 5′-CTTTGAGTCCTTTATTTGATT-3′). Lymph nodes and spleens were infused with permeabilization buffer (0.5% Tween 20, 100 mM Tris, 0.1 mg/ml BSA, pH 7.5) and frozen in liquid nitrogen before homogenization. Translation products were diluted in TNE buffer, and genome was extracted by the phenol–chloroform method. DNA was quantitated by spectrophotometry, and integration was detected in 2% agarose gels. The following primers were used: (forward) 5′-CAAATGGGCGGTAGGCGTGTA-3′ and (reverse) 5′-GGCCACAAGTTCAGCGTGTCC-3′.

**Histology.** Lymph nodes and spleens were embedded in Tissue Tek OCT compound (Miles, Elkhart, Indiana, USA) and snap frozen in 2-methylbutane (Merck, Zurich, Switzerland). Cryostat sections (7 µm) were either analyzed directly for GFP fluorescence or fixed in 5% paraformaldehyde for histochemical detection of GFP with rabbit anti-GFP mAb (Aurora, Madison, Wisconsin, USA) and Alexa Fluor 594 goat anti-rabbit IgG antibody (Invitrogen, Carlsbad, California, USA). Sections were examined under a Zeiss Axiosplan microscope equipped with a cooled charge-coupled device camera and a Personal Computer and analyzed with Axiovision software (Zeiss).

**Flow cytometry.** Flow cytometry was performed on a FACSCalibur using Cellquest software (Becton Dickinson, San Jose, California, USA). Peripheral blood lymphocytes (PBLs) obtained by tail bleedings were incubated with phycoerythrin-conjugated H2-Kd/Cw3194–203 or HLA-A*0201/ELA26–35 tetramers (prepared in our laboratory) for 30 minutes at 20°C and then washed before incubation with Cyochrome-conjugated anti-CD8 mAb 53-6.7 (eBioscience, San Diego, California, USA) and FITC-conjugated anti-Vβ10 (B21.5), and APC-conjugated anti-CD62L (Mel-14, prepared in our laboratory) for 20 minutes at 4°C. Erythrocytes were lysed using the FACS Lysing solution (Becton Dickinson).

**Transduction of bone marrow–derived DCs.** Bone marrow–derived DCs (BMDCs) cultured in the presence of GM-CSF (20) were transduced at day 5 of culture by adding concentrated virus (MOI, 100; vector concentration, 5 × 10⁴ expression-forming units [EFUs] per microliter) to the culture medium as described (8). Three days later, the cells were extensively washed and administered to mice.

**Vaccination.** Peptide vaccination was carried out by subcutaneously injecting 50 µg of peptide and 50 µg of CpG ODNs emulsified in 50 µl of incomplete Freund’s adjuvant (IFA) and 50 µl of PBS into the base of the tail. Recombinant lentivectors were administered by subcutaneously injecting 2 × 10⁴ EFUs in 100 µl of PBS either into the footpad or into the base of the tail. DC-based vaccination was carried out by administering 5 × 10⁵ DCs containing 25–30% lentivector-transduced or Cw3-transgenic DCs into each hind footpad of mice.

**CTL assays.** In vivo CTL assays were performed as described previously (21). Briefly, splenocytes from syngeneic mice were pulsed or left nonpulsed with Cw3 antigenic peptide (RYLKNGKETL). The pulsed fraction was then labeled in PBS containing CFSE at 0.6 µM, and the nonpulsed fraction was labeled in 0.04 µM CFSE. Both fractions were adjusted to similar cell content (50:50 ratio), pooled, and injected into the tail vein at 10⁷ cells per mouse on day 8 after vaccination with Cw3 cDNA lentivector. Twelve hours later, mice were bled, and the disappearance of peptide-pulsed cells was determined by FACS analysis. By comparing the ratio of the pulsed (high fluorescence intensity) to the nonpulsed fraction (low fluorescence intensity), the percentage of specific killing was established according to the following equation: 100 – [(percentage pulsed) × (percentage nonpulsed)] / 100.

**Results**

**Targeting of DCs in situ by lentivector.** Viruses can be potent inducers of T cell immunity but also dispose of many ways to avoid immune recognition. This has to be taken into account when evaluating the potential of a
recombinant virus for the purpose of vaccination. As T cell responses critically depend on the presentation of viral antigens by the MHCs of professional APCs (22, 23), the tropism of a recombinant virus may determine its immunogenicity. The lentivector used in this study was pseudotyped with vesicular stomatitis virus glycoprotein and thus is taken up through the normal endocytotic pathways. Therefore, it is able to transduce a wide variety of cells in vitro, including DCs (8, 9).

We analyzed the tissue distribution and the target cells after administration of lentivector vaccine into one footpad of mice by a sensitive PCR assay and by immunohistochemical analysis of lymph nodes and spleen. For PCR analysis, genomic DNA was isolated from the site of injection, the draining (popliteal) lymph node, the spleen, and — as controls — the mandibular lymph nodes and the tip of the tail. Amplification of GFP-specific sequences included in the lentivector vaccine was detected, apart from in the injection site, in only the draining popliteal lymph node and the spleen (Figure 1a).

Monitoring GFP fluorescence on frozen sections of total lymph nodes revealed the presence of transduced cells in the cortex of the draining lymph node but not in lymph nodes of nontreated animals or in the contralateral lymph node of lentivector-treated mice (Figure 1b, upper panels). This fluorescence was found 2–3 days after administration of lentivector. Sections prepared 10 days after lentivector administration did not show any GFP fluorescence (data not shown). The observation that integrated vector persisted at later time points, as revealed by PCR, might be due to the greater sensitivity of this assay.

To identify which cell types were transduced in vivo by lentivector, we combined immunofluorescence analysis of GFP expression with markers specific for DCs, macrophages, and B cells. Double-positive cells were observed with an antibody against the DC marker CD11c (Figure 1b, lower panels) but not with antibodies to either the B cell marker CD45R/B220 or the macrophage marker CD11b (Figure 1b) in any of the sections analyzed (n = 30). Although the majority of GFP-positive cells were CD11c positive, about 10% of GFP-positive cells were negative for all of the markers tested.

**Induction of CTL responses upon lentivector administration.** We then tried to elucidate whether the observed in vivo targeting of DCs by lentivector vaccine correlated with efficient induction of CTL responses. To that end, we vaccinated mice with a lentivector bearing a CTL-defined antigen derived from human HLA-Cw3 (referred to throughout the text as Cw3194–203). The ensuing response was characterized by monitoring the CD8+ T cell response specific to H-2 Kd-restricted...
Cw3\textsubscript{194–203} in PBLs of vaccinated mice by staining with H-2 K\textsuperscript{d} Cw3\textsubscript{194–203} tetramers (17, 24). In combination with tetramer staining, we used antibodies against the V\textbeta\textsubscript{10} T cell receptor segment, since the majority of Cw3-specific cells are V\textbeta\textsubscript{10}+ (25). A lentivector bearing the cDNA of HLA-Cw3 (Cw3 cDNA\textsubscript{lv}) (Figure 2a) was administered by injecting 2.5×10\textsuperscript{7} EFUs into the hind footpads of mice. As a negative control, the same amount of a lentivector expressing an irrelevant antigen (Melan-A) was used (Figure 2a).

The vaccines were well tolerated, and a swelling at the site of infection was usually resolved after 1 day. Administration of Cw3 cDNA lentivector resulted in the induction of a massive T cell response (Figure 2b), peaking at day 9 and characterized by the presence of 10–40% antigen-specific cells (H-2 K\textsuperscript{d} Cw3\textsubscript{194–203} tetramer-positive) among total CD8\textsuperscript{+} cells (Figure 2b).

In order to test the functional capacity of the induced antigen-specific CD8\textsuperscript{+} cells, we performed in vivo CTL assays using as targets syngeneic spleen cells pulsed with antigenic peptide that were transferred to vaccinated mice (21). Before transfer, the pulsed cells were labeled with CFSE, allowing us to monitor their fate in vivo by FACS analysis in tissue such as blood, spleen, and liver. As an internal control, the same number of nonpulsed splenocytes was labeled with a lower intensity of CFSE and co-injected with the pulsed cells. The shift in the ratios of the two CFSE-positive populations provoked by the in vivo elimination of pulsed cells allowed us to calculate the percentage of specific killing that had occurred. Transfer of pulsed and nonpulsed target cells into vaccinated mice was performed 1 day before the peak of the T cell response. Analysis of PBLs, splenocytes, and liver lymphocytes was carried out 12 hours after transfer, and the proportion of H-2 K\textsuperscript{d} Cw3\textsubscript{194–203} tetramer+/CD8\textsuperscript{+} cells, as well as the shift in the ratios of the two CFSE-labeled populations, was determined by FACS analysis.
As demonstrated in Figure 2c, vaccination with Cw3 cDNA lentivector resulted in an almost complete (over 95%) elimination of the pulsed, CFSEhigh population. In marked contrast, vaccination with a lentivector bearing an irrelevant CTL-defined antigen did not result in significant killing. Moreover, the observed killing activity induced by vaccination with Cw3 cDNA lentivector was systemic, as was demonstrated by the simultaneous and almost complete disappearance of pulsed targets in spleen and liver of vaccinated mice.

Comparative efficiency of direct administration of lentivector with transfer of transduced DCs or peptide/CpG/adjuvant vaccination. Having established that administration of lentivector bearing a T cell antigen resulted in strong CTL responses, we next wanted to compare its potency with other currently used vaccine protocols. Among these, administration of ex vivo–transduced DCs has set the standards in experimental T cell vaccination. For ex vivo DC transduction, the same lentivector bearing the cDNA of HLA-Cw3 (Cw3 cDNA lv) was used as for direct in vivo administration. As described previously (8), DC-based vaccination was performed by injecting transduced BMDCs into the hind footpads of mice (8). As a control for the influence of the viral vector on immune responses, we transferred BMDCs of Cw3 transgenic mice.

Kinetic analysis of the response (Figure 3a) revealed that with all three vaccines, the peak of response was reached around day 9. Subsequently, the proportion of Cw3-specific cells diminished rapidly to reach near preimmune levels by day 50. Comparison of the proportion of antigen-specific CD8+ T cells at peak response with the different vaccination approaches demonstrated that direct administration of lentivector resulted, on average, in a higher amplitude of the response (Figure 3b), with maximal responses of up to 42% of antigen-specific cells among total CD8+ T cells.

A further step in the assessment of the lentivector vaccine was its comparison with another cell-free vaccine: peptide/CpG/adjuvant vaccination. We recently demonstrated that vaccination using synthetic antigenic peptide in combination with CpG ODNs emulsified in IFA was able to induce relatively potent T cell responses (26). The comparison of lentivector vaccine with Cw3194–203 peptide/CpG/IFA was done using a lentivector bearing a minigene that encoded the HLA-Cw3 (Cw3194–203)
derived peptide. Both vaccines were administered subcutaneously in the base of the tail because of the relatively high volume of the peptide-adjuvant emulsion. The immune response induced by lentivector or peptide/CpG/adjuvant vaccination was evaluated by monitoring the evolution of parameters such as activation of CD8+ cells and the proportion of Cw3-specific cells among activated and total CD8+ cells (Figure 3c). The most obvious difference between the two vaccines is the 10-fold higher level of activated CD8+ cells induced by the lentivector vaccine. This results in turn in a higher proportion of Cw3-specific cells among total CD8+ cells. Another difference is that 100% of the mice treated with lentivector vaccine mounted a Cw3-specific response, whereas peptide/CpG/adjuvant vaccination achieved a success rate of only 70% (Figure 3d).

Inducing Melan-A–specific CD8+ T cell responses by direct lentivector administration. The efficacy of direct in vivo administration of lentivector as demonstrated in the Cw3 model was then evaluated in a more clinically relevant model system involving a modified CTL-defined antigen derived from the human melanoma-associated differentiation antigen Melan-A/MART-1 (27, 28). This HLA-A*0201–restricted Melan-A antigenic peptide (referred to throughout the text as ELA26–35) was expressed in lentivector vaccines as a minigene (Figure 1a). Vaccination and characterization of the ensuing immune response was then carried out in HLA-A*0201/Kb transgenic mice (A2/Kbmice). In addition to endogenous MHC class I molecules, these mice express a chimeric human/mouse MHC I molecule composed of the α1 and α2 domains of HLA-A2.1 and the α3, transmembrane, and cytoplasmic domains of H-2Kb (14). The murine elements of the chimeric class I molecule are required for efficient interaction with mouse β2-microglobulin and the CD8 coreceptor.

With direct administration of ELA26–35 minigene lentivector, we observed a peak of the response at day 14, whereas with peptide/CpG/IFA vaccination the peak was reached at day 7, similar to the situation in the Cw3 antigen model. A representative example of these peak responses is shown in Figure 4a. Using HLA-A2/ELA26–35 tetramers to detect Melan-A–specific CD8+ T cells, we observed a strong, specific response in mice immunized with ELA26–35 minigene lentivector but not in those vaccinated with an irrelevant vector. A comparison of this response to that obtained with peptide/CpG/IFA vaccination shows that ELA26–35 minigene lentivector induces more than twofold higher levels of ELA26–35-specific cells among total CD8+ cells (Figure 4, a and b) and more than threefold higher levels among total PBLs (Figure 4a). As seen with the Cw3 model, this is a direct consequence of the higher levels of CD8+ T cell activation induced by lentivector and the higher proportion of ELA26–35-specific cells contained in the activated population.

Recall responses to lentivector vaccination. After the characterization of the strong and sustained primary responses induced by ELA26–35 minigene lentivector, we next evaluated whether a secondary challenge with the same vector would be effective. At the same time, we tested whether antivector immunity had been established during primary immunization. To that end, we performed recall immunizations with the same dose of ELA26–35 minigene lentivector 60 days after primary vaccination in mice primed with either ELA26–35 minigene lentivector or with an irrelevant lentivector not bearing the ELA26–35 CTL-defined antigen.
Mice that had been primed with an irrelevant lentivirus were unable to mount a significant ELA26–35-specific T cell response (Figure 5). This indicated that primary immunization with lentivector resulted in the induction of antivector immunity. However, ELA26–35 minigene lentivector–primed mice responded with similar kinetics and amplitude as seen during the primary response (Figure 5 and data not shown).

Dependence of lentivector-induced CTL responses on CD4+ T cell help. Most CD8+ T cell responses are dependent on help mediated by antigen-specific CD4+ T cells (29). To address the role of CD4 T cells in CTL responses induced by lentivector, we vaccinated mice that were depleted or not of CD4+ T cells. Depletion was carried out by injecting the anti-CD4 mAb GK1.5 (30) 2 days before vaccine inoculation. This resulted in less than 2% remaining CD4+ T cells by the day of vaccine inoculation (data not shown). Helper dependency was evaluated both in the Cw3 and the Melan-A/A2/Kb antigen models.

Although vaccination with Cw3 cDNA lentivector was only slightly affected by CD4+ T cell depletion, administration of ELA26–35 minigene lentivector to CD4-depleted mice resulted in dramatically decreased proportions of antigen-specific T cells (Figure 6).

Discussion
Currently, there is no vaccine available that is able to cure cancer. There is, however, evidence that antitumor vaccination can induce specific antitumor T cell responses and even tumor regression (2, 3, 31). In the overwhelming majority of cases, these regressions were only transient, suggesting that the T cell responses induced might not have been strong and sustained enough (32). On the basis of these observations, the possibility exists that an optimized vaccination protocol may lead to stronger and curative antitumor T cell responses.

Toward this end, we evaluated lentiviral vector as a new candidate T cell vaccine. Third-generation lentivector was chosen because of its advanced safety profile, allowing its administration in vivo, and because of the presumed absence of pre-existing antivector immunity in humans. To characterize the properties of lentivector vaccine, we studied its transduced targets and its ability to induce CTL responses in vivo.

In a recent report, the administration of lentivector into the tail vein of mice resulted in transduction in both the liver and the spleen. Within these organs, and particularly in the spleen, the cellular targets of lentivector included a prominent proportion of cells that displayed some markers characteristic of professional APCs (33). However, no clear evidence for the transduction of bona fide DCs was provided.

In our study, lentivector was injected subcutaneously into the footpads, and integrated vector was found mainly at the site of injection, the draining lymph node, and the spleen. We were further able to demonstrate that most transduced cells in the lymphoid organs were CD11c+ DCs. The presence of transduced DCs in the draining lymph node and the spleen could be the result of transduction of resident DCs in situ or of migration of DCs that had been transduced at the site of injection. In the latter scenario, lentivector-induced maturation would then result in the migration of DCs to the draining lymph node where, upon presentation of the antigen, they would be able to prime naive T cells. In this context, the ability of lentivector to transduce nondividing cells (34) may be crucial for its efficacy as a vaccine.
since immature DCs are thought to be nondividing (35). The observation that the majority of transduced cells found in the spleen and draining lymph nodes are DCs further supports this theory, since it is unlikely that free viral particles reaching the lymph node or spleen would exclusively infect DCs.

Our observations support the idea that T cell responses obtained after lentivector administration are due to direct priming of CTL precursors in situ. In view of the strength of the response as compared with the relative paucity of transduced DCs, an additional involvement of cross-priming by antigen taken up by DCs at the site of injection cannot, however, be excluded.

In order to quantify the efficiency of lentivector vaccination, we chose the direct monitoring of antigen-specific T cells by tetramer staining and, in the initial characterization of the Cw3 cDNA lentivector, an in vivo CTL assay. We show that lentivector administration induces high levels of antigen-specific CD8+ T cells that display a high CTL activity in vivo. Moreover, although quantitative differences in the outcome of distinct vaccination protocols are difficult to evaluate, the comparison with two of the major existing approaches, peptide/adjuvant vaccination and transfer of antigen-transduced DCs, clearly demonstrated that direct administration of lentivector induces a highly effective antigen-specific CD8+ T cell response.

Since most antitumor T cell vaccination protocols rely on a prime boost strategy, we evaluated whether lentivector administration resulted in the induction of antitumor immunity. Because of the failure to induce a Melan-A–specific response in mice that had been primed with an irrelevant lentivector, we presume that antivector immunity had been established. This could be a potentially limiting factor for repetitive lentivector immunization that needs to be examined further. Nevertheless, the induction of antivector immunity did not prevent repetitive vaccination from being effective when the Melan-A minigene vector was used for priming.

As a further step in the characterization of the lentiviral vaccine, we addressed the dependency of the CD8+ T cell response on help by CD4+ T cells. According to a popular model, T cell help is thought to augment CD8+ responses indirectly through the activation of DCs (36–38). This implies that the action of CD4+ T cell help could be substituted by any another mechanism that leads to appropriate DC activation, including transduction with a recombinant vector. When assessing helper dependency in the Cw3 and the Melan-A/A2/Kb antigen models, we found that the two models differ in their dependency on CD4+ T cell help. Whereas with Cw3 no significant difference in the response was observed whether CD4+ T cells were present or not, vaccination with the ELA26–35 minigene lentivector heavily relied on the presence of CD4+ T cells. In the latter case, it appears that a helper epitope must have been provided either by the GFP marker or the viral particle, since the vector expresses only the CTL antigen of Melan-A.

A possible explanation for the divergence in the dependency on CD4+ T cells could be provided by recent data demonstrating that a high frequency of CTL precursors can compensate for CD4 help (39, 40). According to this scenario, the responses seen in the Cw3 model would have been mediated by CD4-independent help, whereas in the Melan-A/A2/Kb model with low precursor frequencies (41), the presence of CD4+ T cells is needed to induce a CD8+ T cell response.

In conclusion, data presented here demonstrate the efficacy of direct in vivo administration of lentiviral vectors for the induction of antigen-specific CTL responses. A decisive factor for this efficacy appears to be the in vivo targeting of DCs. From a practical point of view, our results demonstrate that the time-consuming and costly steps currently used to elicit tumor-specific CTL responses through the transfer of ex vivo–manipulated DCs could be replaced by the much simpler direct in vivo administration of antigen-recombinant lentivectors.

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