ORIGINAL ARTICLE

Induction of effective therapeutic antitumor immunity by direct in vivo administration of lentiviral vectors

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Ex vivo lentivirally transduced dendritic cells (DC) have been described to induce CD8⁺ and CD4⁺ T-cell responses against various tumor-associated antigens (TAAs) in vitro and in vivo. We report here that direct administration of ovalbumin (OVA) encoding lentiviral vectors caused in vivo transduction of cells that were found in draining lymph nodes (LNs) and induced potent anti-OVA cytotoxic T cells similar to those elicited by *ex vivo* transduced DC. The cytotoxic T-lymphocyte (CTL) response following direct injection of lentiviral vectors was highly effective in eliminating target cells in vivo up to 30 days after immunization and was efficiently recalled after a boost immunization. Injection of

lentiviral vectors furthermore activated OVA-specific CD4⁺ T cells and this CD4 help was shown to be necessary for an adequate primary and memory CTL response. When tested in therapeutic tumor experiments with OVA⁺ melanoma cells, direct administration of lentiviral vectors slowed down tumor growth to a comparable extent with the highest dose of *ex vivo* transduced DC. Taken together, these data indicate that direct in vivo administration of lentiviral vectors encoding TAAs has strong potential for anticancer vaccination.

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Introduction

Most cancer immunotherapy strategies are based on the expression of tumor-specific antigens. Owing to mutations or dedifferentiation, cancer cells express a range of tumor-associated antigens (TAAs) that have limited expression in normal tissues. These TAAs include cancer-testis antigens, products of mutated genes and rearrangements unique to tumor cells, overexpressed tissue-specific antigens and exogenous viral proteins.^{1,2} These tumor-associated target antigens can be delivered in an immunogenic fashion and induce successful and safe antitumor cell mediated immunity. A key player in these strategies is the dendritic cell (DC), the professional antigen-presenting cell of the immune system, fully equipped to prime naive T cells.^{3,4} The development of techniques to generate large amounts of DC *ex vivo* has encouraged the optimization of DC vaccines.^{5–7} Various methods of loading DC with antigenic peptides, proteins, DNA or mRNA and viral vectors have been established.^{8–13} Efficient gene delivery to human DC has been achieved with a variety of viral vectors derived from adenovirus,^{8,14} adeno-associated virus,¹³ onco-retrovirus,^{10,11,15} pox viruses such as vaccinia,^{9,16} and canary

pox^{17,18} and lentivirus.^{19–22} Although adenovirus- and vaccinia virus-derived vectors result in efficient transduction and immune activation, they express viral proteins that evoke a potent antivector immune response, which might dampen the immune response against the tumor antigen in repeated vaccination regimens. Together with the fact that most humans have pre-existing immunity against these viruses, their applicability for DC vaccination is rather limited in their current form.²³

Lentiviral vectors are efficient gene delivery vectors, that can transduce non-dividing, monocyte-derived DC as well as murine bone marrow derived DC, without affecting phenotype or function.^{19,21,22,24–26} These viral vectors are attractive tools for immunotherapy, providing high and stable gene expression in both dividing and non-dividing cell types. Furthermore, the second-generation lentiviral vectors contain a series of safety features placing them among the safest viral gene delivery vehicles currently available.^{21,26–31} Another important issue for immunotherapy is that pre-existing immunity to lentiviral vectors is rare, which would enable direct *in vivo* administration.

DC-based tumor vaccines have already found their way from bench to bedside. Early phase I clinical trials support the idea that DC presenting TAAs can initiate an antitumor response in patients. So far, however, objective clinical responses occur in only a minority of patients. In about 20% of the patients treated with various immunotherapy regimens there was some evidence of tumor regression; this corresponds to only

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$\pm 3\%$ according to RECIST criteria.^{17,32–38} Preparation of patient-specific DC vaccines by isolation, transduction and preparation of autologous DC from patient blood is very labor intensive and time consuming, with considerable financial cost. This process could be overcome by targeting the patient's DC *in vivo* by lentiviral vectors encoding TAA. Intravenous administration of lentiviral vectors was shown to efficiently transduce DC and B cells in the spleen.³⁹ Esslinger *et al.*⁴⁰ reported specific transduction of DC in the draining lymph nodes (LNs) after subcutaneous injection of lentiviral vectors. Direct *in vivo* administration has furthermore been shown to result in efficient CD8⁺ T-cell responses against the melanoma-associated antigens MART-1⁴⁰ and NY-ESO-1 in HLA-A2 transgenic mice with a similar potency compared to *ex vivo* transduced DC.⁴¹

In this study, we describe the use of lentiviral vectors for *in vivo* transduction of DC in C57BL/6 mice. Using ovalbumin (OVA) as a model antigen, we characterized important parameters of the immune response following direct administration of lentiviral vectors. We show that direct *in vivo* administration of lentiviral vectors induces cytotoxic T-lymphocyte (CTL) responses against OVA that persist longer compared to *ex vivo* transduced DC and efficient activation of specific CD4⁺ T cells. Moreover, we report effective therapeutic tumor immunity against MO4 tumor after direct *in vivo* administration of lentiviral vectors.

Results

Detection of OVA mRNA in draining LNs after direct administration of OVA encoding lentiviral vectors indicates *in vivo* transduction

In this study, we explored the potential of lentiviral vectors for direct *in vivo* transduction of DC in order to induce transgene-specific immunity. We used second-generation VSV-G pseudotyped lentiviral vectors encoding a truncated form of chicken OVA fused to the first 80 amino acids of invariant chain (in order to target MHC class II presentation⁴²) driven by the cytomegalovirus (CMV) promoter.^{22,43}

In situ transduction after direct injection of lentiviral vectors was assessed using reverse transcription-polymerase chain reaction (RT-PCR). Mice were injected with 1×10^7 293T transducing units (TUs) lenti-OVA subcutaneously in the footpads. On different time points after injection, the draining LNs were isolated for mRNA isolation and cDNA synthesis. Amplification of OVA-specific sequences in RT-PCR was very strong 2 days after injection and remained detectable until day 10, indicating the presence of stably transduced cells in the draining LNs. By day 25 after injection, OVA transcripts could no longer be detected. In comparison, RT-PCR on draining LNs after injection of increasing amounts of *ex vivo* transduced DC in the footpads showed only weak OVA amplification after injection of 1×10^6 DC (data not shown and Dullaers *et al.*⁴³).

Using enhanced green fluorescent protein (eGFP) as a reporter for flow cytometry, we could detect transgene-positive cells in the draining LNs after direct injection of lentivirus (data not shown). The proportion of eGFP⁺ cells was, however, very low ($<0.07\%$) and passive uptake of eGFP protein at the site of injection could not

Figure 1 RT-PCR detection of OVA transcripts in draining LNs after direct injection of lentiviral vectors. Mice were injected in the hind limb footpads with 1×10^7 TU Lenti-OVA and draining LNs were isolated on the indicated time points for mRNA extraction and cDNA synthesis. RT-PCR for OVA was performed on this cDNA in order to detect transduced cells. Mix controls and naive mice gave no amplification of OVA.

be excluded. The RT-PCR-based detection indicates that LN cells were effectively transduced and actively transcribing the transgene cassette for up to at least 10 days after injection (Figure 1).

In vitro cytolytic activity of CTL induced by directly injected lentiviral vectors is similar to that induced by *ex vivo* transduced DC

To evaluate their capacity to induce antigen-specific CTL reactive against OVA⁺ tumor cells *in vitro*, we compared direct *in vivo* injected lentiviral vectors with *ex vivo* transduced DC. As we described earlier,²² up to 70–80% of mouse bone marrow-derived DCs are transduced *in vitro* and these OVA-expressing DC are potent inducers of specific CTL upon immunization. Mice were injected subcutaneously in the footpads with either 1×10^5 *ex vivo* Lenti-OVA transduced DC or with 2×10^7 TU Lenti-OVA directly (dir). Seven days after the immunization, we isolated the draining LNs and restimulated them *in vitro* for 5 days with EG.7-OVA. Then we tested the *in vitro* cytolytic activity against EG.7-OVA and EL4 tumor cells. DC Lenti-OVA induced $52.0 \pm 6\%$ lysis of EG.7-OVA cells whereas Lenti-OVA dir induced $45.9 \pm 8\%$ lysis at effector-to-target (E:T) ratio 100:1 (mean of eight mice) (Figure 2). This difference was not statistically significant for any of the E:T ratios tested (all $P > 0.08$). It should be noted that consistently more cells were found in LNs isolated from Lenti-OVA dir mice (6.7×10^6 cells, $n = 8$) than from DC Lenti-OVA mice (3.4×10^6 cells, $n = 8$) ($P = 0.007$). This suggested that direct injection of lentiviral vectors activated more T cells than *ex vivo* transduced DC.

Direct injection of lentiviral vectors induces a high number of interferon- γ producing cells

The frequency of activated T cells after direct injection of lentiviral vectors and *ex vivo* transduced DC was determined in an *ex vivo* interferon (IFN)- γ enzyme linked immunospot assay (ELISPOT) assay. Mice were immunized subcutaneously in the interscapular area with the indicated numbers of *ex vivo* transduced DC or Lenti-OVA dir. One week later, IFN- γ -producing OVA-specific T cells in the spleen were quantified directly *ex vivo* by ELISPOT using spleen APC pulsed with OVA-K^b (SIINFEKL) peptide as stimulators. As shown in Figure 3, a clear dose-response relation was found for both regimens, although no maximum plateau was reached in this dose range. Directly injected lentiviral vectors (2419 ± 797 spots/ 10^6 spleen cells) induced at the highest dose tested 3 times as many spots as the highest DC dose (816 ± 82 spots/ 10^6 spleen cells). This confirmed that at

Figure 2 *In vitro* cytolytic activity. Mice were immunized with either 1×10^5 *ex vivo* transduced or control DC or 2×10^7 TU lenti-OVA. Seven days later, LN cells were isolated and restimulated with E.G7-OVA cells. A chromium release assay was performed to evaluate the cytotoxicity against EL4 and E.G7-OVA cells. The EL4 and E.G7-OVA lysis induced by the generated CTL is shown (one out of two similar experiments).

Figure 3 Quantification of IFN- γ -producing specific T cells by ELISPOT. Mice were immunized with increasing amounts of *ex vivo* transduced DC or Lenti-OVA directly. Seven days later, splenocytes were isolated and restimulated with $1 \mu\text{M}$ SIINFEKL peptide in anti-IFN- γ -coated nitrocellulose microtiter plates. Eighteen hours later, bound IFN- γ was detected and spots were developed. Values represent the mean number of spots $\pm$ standard deviation of two mice normalized for the number of spots in unstimulated cultures.

these doses, direct injection of lentiviral vectors activated more OVA-specific T cells compared to injection of *ex vivo* transduced DC ($P = 0.002$).

Potent *in vivo* cytotoxic activity following direct *in vivo* administration of lentiviral vectors

Having shown that direct injection of lentiviral vectors results in similar *in vitro* CTL activity and induction of higher numbers of IFN- γ -producing T cells compared to *ex vivo* transduced DC, we wanted to expand these results by testing both regimens for their *in vivo* cytolytic activity. The *in vivo* CTL assay measures specific depletion of carboxyfluorescein diacetate succinimide ester (CFSE) labeled, OVA/ K^b peptide (SIINFEKL) pulsed syngeneic splenocytes. Figure 4a shows typical flow cytometric results for the day 7 response of the highest dose of each regimen. The percentage specific killing of target cells induced by increasing amounts of *ex vivo* transduced DC and Lenti-OVA dir was plotted in Figure 4b and d, respectively. *In vivo* cytolytic activity induced by Lenti-OVA dir and *ex vivo* transduced DC reached a maximum at, respectively, 5×10^6 TU and 1×10^5 DC.

To verify that the observed immune response was the result of actual *in vivo* transduction, mice were injected with 1×10^7 TU of heat-inactivated ($15' 70^\circ\text{C}$) Lenti-OVA (Figure 4c). This treatment is a variant of pasteurization, which has been shown to eliminate reverse transcriptase activity.⁴⁴ Heat inactivation of the vector particles totally abrogated their potential to induce CTL, ruling out the possibility that contaminating vector DNA or

co-precipitated OVA protein in the vector stock were involved in CTL induction.

Effective CTL memory following direct injection of lentiviral vectors

Important for a protective immune response is the establishment of a memory pool to prevent relapse. We followed the evolution of the primary CTL response for two doses of DC Lenti-OVA and Lenti-OVA dir using the *in vivo* CTL assay (Figure 5a). We observed a gradual decline of SIINFEKL-specific lysis from day 7 towards day 30 after DC Lenti-OVA immunization. No remarkable difference was observed between 1×10^5 and 5×10^5 DC Lenti-OVA. Both conditions resulted in 20–30% specific lysis 30 days after immunization. Injection of 1×10^6 TU Lenti-OVA dir was significantly inferior to 1×10^7 TU, with day 30 specific lyses of 38.6 and 80.3%, respectively ($P < 0.001$). The CTL responses induced with the highest dose of lentiviral vectors decreased very little. The difference in day 30 specific lysis between 1×10^7 TU Lenti-OVA dir and 5×10^5 DC Lenti-OVA was statistically significant ($P = 0.004$).

The presence of an effective memory pool should lead to a more rapid and effective response to a second antigen exposure. In order to test functional memory, animals were given a second immunization 150–200 days after the initial immunization and tested for cytolytic activity on days 1 and 7 after the boost (Figure 5b). Mice that did not receive a second immunization exhibited low but reproducible cytolytic activity 150 days after immunization with both DC Lenti-OVA (11.2%)

Figure 4 Primary *in vivo* cytolytic activity. Mice were immunized with increasing amounts of *ex vivo* transduced DC or Lenti-OVA directly. One week later, mice were intravenously injected with a 1:1 mix of unpulsed CFSE^{low} and peptide-pulsed CFSE^{high} spleen cells. Flow cytometry on peripheral blood cells was performed to evaluate specific lysis of peptide pulsed targets. Panel a shows typical flow cytometric results for each group. The percentage specific killing 7 days after immunization with increasing amounts of *ex vivo* transduced DC and Lenti-OVA dir is plotted in panels b and d, respectively. Panel c shows *in vivo* cytolytic activity after injection of 1×10^7 TU Lenti-OVA that were either heat inactivated or not. Results in (b–d) represent mean specific lysis $\pm$ standard deviation for 3–9 mice per group after normalization for values in non-immunized mice.

and Lenti-OVA dir (13.2%). One day after the boost immunization, only mice that had received Lenti-OVA dir showed increased cytolytic activity (38.6%) whereas mice that received DC Lenti-OVA had baseline levels (10.1%). Seven days after the boost, both DC Lenti-OVA and Lenti-OVA dir elicited a potent memory response with 61.1 and 65.0% mean specific lysis, respectively. Littermates of these mice that received only an immunization on day 150 showed no cytolytic activity on day 1 (data not shown).

Directly in vivo administered lentiviral vectors are strong activators of type 1 CD4⁺ T helper cells in vivo As CD4⁺ T helper cells play an important role in CTL memory and tumor immunity, we wanted to determine the capacity of both regimens to activate CD4⁺ T cells *in vivo*. To this end, naive mice were adoptively transferred

with CFSE labeled OT-II TCR transgenic CD4⁺ T cells and subsequently immunized with either 5×10^5 DC Lenti-OVA or 1×10^7 TU Lenti-OVA dir in the footpads. The draining LNs were harvested 4 days later for analysis. Figure 6a shows typical flow cytometric results for each group. Proliferation of OT-II TCR transgenic CD4⁺ T cells, visualized by the dilution of CFSE content in the V α 2 positive population (gate R1), was observed in both DC Lenti-OVA and Lenti-OVA dir immunized mice. As a positive control, we included DC pulsed with the OVA/I-A^b peptide. We can conclude from this experiment that both DC Lenti-OVA and Lenti-OVA dir induced *in vivo* proliferation of OVA-specific CD4⁺ T cells.

To monitor the type of immune response induced by either vaccination regimen, draining LN cells were cultured with or without the OVA/I-A^b peptide for 3 days. LN cells from both Lenti-OVA dir and DC Lenti-OVA mice proliferated well and produced high amounts

Figure 5 Time course of the CTL response following immunization and boost with DC Lenti-OVA or Lenti-OVA dir. Mice were immunized with the indicated amounts of *ex vivo* transduced DC or Lenti-OVA directly. *In vivo* CTL activity was evaluated at different time points until day 30 (a). At 150 days after the first immunization, secondary CTL responses were evaluated on days 1 and 7 after a booster (b). Values represent mean specific lysis $\pm$ standard deviation for 3–6 mice per group after normalization for values in non-immunized mice.

of IFN- γ in response to antigenic restimulation, to the same magnitude as cells from peptide DC mice (Figure 6b). In contrast, no IL-4 could be detected in cultures of LN cells from DC Lenti-OVA or Lenti-OVA dir mice, whereas small amounts of IL-4 were detected when mice were immunized with peptide pulsed DC. These data suggest that lentiviral vectors, both directly administered as well as used for *ex vivo* transduction of DC, induce Th1 polarized immune responses, which are favorable in case of cancer immunotherapy.

CTL priming and memory is totally dependent on CD4 help after immunization with transduced DC but only partially after direct injection of lentiviral vectors

We wanted to investigate whether CD4⁺ T-cell-mediated help is responsible for the superior memory CTL response observed after direct injection of lentiviral vectors. To this end, we compared both primary and memory *in vivo* CTL responses in mice that were either treated with an anti-CD4 antibody or not. The efficiency of depletion was analyzed by flow cytometry on

peripheral blood cells. On the day of immunization, we observed complete CD4⁺ T-cell depletion, which remained complete for at least 1 week (data not shown). Mice were immunized in the interscapular area 2 days after CD4 depletion with either 1×10^5 DC Lenti-OVA or 1×10^7 TU Lenti-OVA dir. These doses were chosen on the basis of the kinetics of the CTL response (Figure 5).

The impact of CD4⁺ T-cell depletion on both primary (day 7) and memory (day 30) response was striking (Figure 7). For *ex vivo* transduced DC, the primary CTL response in CD4⁺ T-cell-depleted mice was reduced to 5.6% mean specific lysis (16 times less than that in non-depleted mice). The memory response was reduced to 1.1% mean specific lysis (30 times less than that in non-depleted mice) (both $P < 0.001$). For directly injected lentiviral vectors, the effect of CD4 depletion on the primary CTL response was less pronounced, the mean specific lysis being 50.4% (2 times less than that in non-depleted mice) ($P = 0.03$). The memory response after CD4 depletion, however, was strongly reduced, with the mean specific lysis being 11.8% (7 times less of that in non-depleted mice) ($P < 0.001$). Taken together, these results confirm that DC vaccination needs CD4⁺ T-cell help for adequate priming and maintaining of CTL. Direct injection of lentiviral vectors is only partially dependent on CD4⁺ T-cell help for the priming of CTL, but needs CD4 help for persistence of the memory pool.

Directly in vivo administered lentiviral vectors induce therapeutic tumor immunity

As we were able to activate both OVA-specific CD8⁺ and CD4⁺ T cells with a single injection, we wanted to investigate whether this immune response could slow down the outgrowth of pre-existing OVA-expressing tumor cells. Groups of mice were inoculated with a lethal dose of MO4, that is, 2×10^5 cells, as tested earlier in a prophylactic setting,²² and subsequently immunized in the interscapular area on days 3, 10 and 17 with either $1 \times 10^5/5 \times 10^5$ DC Lenti-OVA or $2 \times 10^6/1 \times 10^7$ TU Lenti-OVA dir. Tumor growth and survival were followed for 60 days after tumor engraftment and the survival data were plotted in a Kaplan–Meier cumulative survival graph (Figure 8). We observed tumors developing in the non-immunized mice from day 10 on which all reached the exclusion criteria of 15 mm diameter by day 20. In most of the immunized mice, small palpable tumors were registered on day 17, the time of the third immunization. The mice that received the lowest dose (1×10^5) of transduced DC developed rapidly growing tumors and they all reached the exclusion criteria by day 38. This prolonged survival compared to naive mice was significant ($P = 0.013$). Mice that received 5×10^5 *ex vivo* transduced DC reached the exclusion criteria on average 10 days later ($P = 0.009$). Both groups of animals that were injected with lentiviral vectors directly had a highly significant prolonged survival compared to naive mice (both $P < 0.001$). Eventually all the mice in the 2×10^6 TU lenti-OVA group had to be killed by day 45, whereas in the 1×10^7 TU lenti-OVA group some mice survived the tumor challenge. In the depicted experiment in Figure 8 one out of eight mice survived tumor free for over 60 days, in a comparable experiment this was three out of 10. One surviving animal tested on day 250 after tumor

Figure 6 *In vivo* CD4⁺ T-cell activation. Naive mice were adoptively transferred with CFSE labeled OT-II CD4⁺ T cells and subsequently immunized. Four days later, draining LNs were isolated and the cells were evaluated in flow cytometry for dilution of the CFSE label (a). Based on the profile of the non-modified DC immunized mice, CFSE⁺Vα2⁺ cells were gated in CFSE^{high}(R2) and CFSE^{low}(R1) fractions representing naive and divided cells, respectively. Part of the LN cells was cultured with or without OVA/I-A^b peptide to evaluate proliferation, IFN- γ and IL-4 secretion (b).

Figure 7 Role of CD4⁺ help in CTL activation. Mice were either i.p. injected with 200 μ g anti-CD4 antibody or not, 2 days before immunization with *ex vivo* transduced DC or lentiviral vectors dir. Primary (day 7) and memory (day 30) CTL responses were evaluated with *in vivo* CTL assay. The mean specific lysis $\pm$ standard deviation for each group is shown (4–8 mice per group) after normalization for values in non-immunized mice.

challenge showed still active CTL with 29% specific lysis of peptide pulsed target cells.

Direct injection of lentiviral vectors was furthermore significantly better than the lowest dose of *ex vivo* transduced DC ($P=0.004$ and 0.001). The difference in survival between both groups of mice that received lentiviral vectors directly and the highest dose DC group was, however, not significant.

Discussion

In this report, we expand on the data of Esslinger *et al.*,⁴⁰ that is, that direct administration of lentiviral vectors *in vivo* causes transduction of DC, resulting in strong CTL responses against the transgene. We show that directly administered lentiviral vectors not only induce more effective memory CTL responses as compared to

Figure 8 Induction of therapeutic tumor immunity. Mice (eight per group) were challenged with a lethal dose of MO4 tumor cells (2×10^5 cells) and subsequently immunized with either 1×10^5 / 5×10^5 *ex vivo* transduced DC or 2×10^6 / 1×10^7 lentiviral vectors directly on days 3, 10 and 17 (arrows). Survival of the mice was evaluated until day 70 post-tumor challenge and plotted in a Kaplan-Meier cumulative survival plot.

ex vivo transduced DC, but they also provide better therapeutic antitumor immunity *in vivo*, thus strengthening the idea that direct immunization with lentiviral vectors could be a potent tool for cancer immunotherapy.

After direct injection of OVA encoding lentiviral vectors, we could detect OVA mRNA in draining LN, indicating that cells had been effectively transduced and actively transcribed the transgene cassette. OVA transcripts were abundantly present on day 2, could still be detected, although at lower levels, on day 10 and were no longer detectable on day 25 after injection. The disappearance of OVA transcription is probably the result of different mechanisms. First, OVA-expressing cells will likely be eliminated by the resulting immune response. Second, OVA expression could be silenced at the transcriptional level due to methylation of CpG sites in the CMV promoter sequence as was documented for adenoviral vectors or naked plasmid DNA.^{45–47} In addition, possible emigration of transduced cells from the LNs could result in the disappearance of the OVA signal.

Earlier studies^{40,41} report that after subcutaneous injection of lentiviral vectors, the majority of transduced cells in the draining LNs have a DC phenotype. The presence of transduced cells in the LNs is most likely the consequence of transduction at the site of injection followed by migration into the draining LNs, as it is unlikely that free viral particles that reached the LNs would exclusively transduce DC. As we use VSV-G pseudotyped viral particles, we assume that other cell types, such as macrophages, fibroblasts and muscle cells, are also transduced at the injection site. The question whether subcutaneous injection results also in systemic delivery of the virus and which cell types are exactly transduced, is the subject of ongoing research. In this report, we have focused on the analysis of the resulting immune response.

We observed in *in vitro* cytotoxicity assays that direct immunization with OVA encoding lentiviral vectors induced CTL similar to immunization with *ex vivo* transduced DC. We noticed, however, that upon isola-

tion, the LNs of mice injected with lentiviral vectors consistently contained more cells than those of mice immunized with *ex vivo* transduced DC. This suggested that a higher absolute number of effectors were induced in these mice. We tested this hypothesis using IFN- γ ELISPOT, as this assay allows a quantitative comparison without *in vitro* restimulation. Indeed, we found a substantially higher proportion of SIINFEKL-specific IFN- γ -producing cells after immunization with lentiviral vectors compared to *ex vivo* transduced DC at most doses tested.

To further compare the CTL responses induced with both regimens, we performed CTL assays with peptide pulsed splenocytes as *in vivo* targets. We observed a clear dose-response for both directly injected lentiviral vectors and *ex vivo* transduced DC and did not see a difference in early CTL responses. After immunization with transduced DC, however, cytolytic activity decreased more rapidly even with the highest dose used. Day 30 CTL responses after immunization with the highest dose of lentiviral vectors were significantly higher than after *ex vivo* transduced DC. This is most probably the result of prolonged priming of naive T cells in Lenti-OVA dir injected mice as OVA expression in the LNs could be detected up to 10 days after immunization. Moreover, when the secondary CTL response was evaluated following a booster 150 after the first immunization, mice that received Lenti-OVA dir showed a prompt reactivation of CTL 1 day later, whereas mice that received DC Lenti-OVA responded more slowly. One week after the second immunization, both regimens showed equal CTL activity. This suggests that direct injection of lentiviral vectors induced more effective memory.

We investigated whether different levels of CD4⁺ could help explain the difference in memory responses observed with the two regimens, as CD4⁺ T cells have been postulated as critical regulators of memory.^{48–50} Investigation of CD4⁺ T-cell activation in OT-II assays taught us that both immunization regimens resulted in activation of OVA-specific CD4⁺ T cells with a type 1 cytokine profile. Inefficient CD4 activation by transduced DC could thus be ruled out. In order to assess the contribution of CD4⁺ T cells to the memory CTL response, we investigated the capacity of both regimens to induce CTL memory in the absence of CD4⁺ T cells. The impact of CD4⁺ depletion on the primary response was very strong for DC vaccination, whereas direct vaccination with lentiviral vectors had reduced, though still important, CTL priming potency. However, the memory response in the absence of CD4⁺ T cells was strongly impaired for both vaccination strategies, indicating that CD4⁺ T-cell help is indeed the main determinant for adequate memory induction. This is consistent with the findings of two recent reports, which show that CD4 help is dispensable for CTL priming, but necessary for inducing and maintaining memory.^{48,49} On the other hand, Smith *et al.*⁵¹ showed in a herpes simplex infection system, that both priming of CTL and memory are dependent on CD4⁺ T-cell help in a cognate and antigen-specific manner. They proposed a licensing system, in which DC are licensed to activate CTL after antigenic recognition by CD4⁺ T cells. The discrepancy in the need for CD4 help in the primary response is probably related to the nature of the infection/vaccina-

tion,^{52,53} the level of antigen presentation and seems to differ for different antigens.⁴⁰ The fact that in our study, immunization with *ex vivo* transduced DC needs CD4 help for CTL priming whereas direct injection of lentiviral vectors (at least partly) does not, could be related to other than bone marrow-derived DC being transduced and more efficient CTL activators in the absence of CD4 help. In this respect, plasmacytoid DC could play a role. This subtype of DC, in response to viral infection, secretes large amounts of type I IFN, which acts on various cell types and promotes NK and CTL cytotoxicity and IFN- γ production⁵⁴ and could play a role in the superiority of directly injected lentiviral vectors over *ex vivo* transduced DC in the absence of CD4 help. Other cells that are possibly transduced after immunization with lentiviral vectors, such as epidermic Langerhans cells and cells of the innate immune system, such as NK cells, NKT cells and polymorphonuclear cells, might contribute to the efficiency of the immune response in the absence of CD4 help. NK cells in particular have been described to mediate priming of CD4-independent tumor-specific CTL through NKG2D triggering.⁵⁵

Another mechanism that could contribute to the difference observed in CTL memory and CD4 dependence is cross-priming. DC (in particular CD8 α^+ DC) could engulf *in situ* transduced dead cells and subsequently cross-present the transgene-derived peptides to CD8 $^+$ T cells.⁵⁶ Cross-priming has also been shown to occur when immunizing with DC, since vaccine DC have been shown to interact with host DC, that are able to cross-present transgene-derived peptides.^{57,58}

To evaluate the tumor protective capacity of the observed CTL responses, we chose to look at therapeutic tumor immunity with OVA-expressing B16 melanoma (MO4). First, because the therapeutic approach is more relevant towards the clinical situation and second, because we have already demonstrated the protective efficacy of *ex vivo* lentivirally transduced DC in a prophylactic setting earlier.²² We used the same number of tumor cells as in those experiments (2×10^5), which is significantly higher than the number of cells used in other therapeutic B16 studies.^{59,60} Three immunizations at weekly intervals with two doses of either *ex vivo* transduced DC or lentiviral vectors *per se*, provided significant protection against outgrowth of the pre-existing tumor. For *ex vivo* transduced DC, the use of 5×10^5 DC resulted in remarkably better (though not significant) survival compared to 1×10^5 DC. For direct injection of lentiviral vectors, we observed no significant difference in between both doses. However, some mice immunized with 1×10^7 TU lentivector remained tumor free for over 70 days. The highest dose of DC was not significantly different compared to both doses of directly injected lentiviral vectors. This OVA-tumor model thus indicates that lentiviral vectors are potent tools for the induction of an antitransgene immune response that is capable of slowing tumor growth, resulting in prolonged survival in all animals and in some cases complete tumor regression.

Taken together, the advantage of directly injected lentiviruses over *ex vivo* transduced DC in inducing antitumor immunity could primarily be explained by an overall quantitatively better response, a prolonged priming period in the LNs and the cooperation of different unidentified host cells after *in situ* lentiviral

transduction. In this context, NK cells could be key players as they have been implicated to interact with DC and mediate tumor destruction.^{61,62} Identification of the *in situ* transduced cells could unravel the mechanisms leading to the observed therapeutic tumor immunity.

The results of this study need to be extended towards native tumor antigens as OVA is a foreign and very immunogenic antigen. Previous studies reported potent CTL responses against melanoma-associated antigens MART-1,⁴⁰ MAGE-3, tyrosinase, gp100²¹ and NY-ESO⁴¹ after direct injection of lentivirus in HLA-A2 transgenic mice, indicating the strength of the method. It remains to be investigated whether vaccination against these antigens can result in therapeutic tumor immunity.

Provided that the production can be scaled up to meet the demands for clinical use, direct injection of lentiviral vectors is an attractive approach for cancer vaccination strategies. The ease of simply injecting defective viral particles encoding the antigen of choice, without having to isolate and expand DC for each individual patient, represents a valuable alternative for DC-based and other cancer vaccines.

Materials and methods

Mice, cell lines and reagents

Six to eight-week-old female C57BL/6 mice were purchased from Harlan. OT-II mice,⁶³ which are transgenic for a V α 2 $^+$ TCR recognizing the I-A b -restricted OVA_{323–339} peptide, were kindly provided by Professor M Moser (ULB Gosselies, Belgium). They were bred in our germ-free facility and used at 6–9 weeks of age. All animals were maintained and treated according to the institutional guidelines.

The EL4, E.G7-OVA and MO4 tumor cell lines and 293T cells were previously described.²²

The peptides OVA_{323–339} ISQAVHAAHAEINEAGR, binding I-A b and recognized by the OT-II TCR and OVA_{257–264} SIINFELK, binding H2-K b , were purchased from Thermo Electron.

Plasmids and production of lentiviral particles

The multiple attenuated packaging plasmid pCMV Δ R8.9 and the VSV-G encoding plasmid pMD.G were a kind gift from Dr D Trono (University of Geneva, Switzerland). The transfer vector pHR'rip CMV Ii80tOVA-Ires-tNGFR SIN, containing a truncated OVA fused to the first 80 amino acids of invariant chain under the CMV promoter, was previously described.^{22,42} Lentiviral particles produced from these plasmids are referred to as Lenti-OVA. Preparation, concentration and titration of the lentiviral vector stocks were performed as previously described²² with the only adjustment that after ultracentrifugation, lentivectors were resuspended in phosphate-buffered saline (PBS) containing 10 μ g/ml protamine sulphate.

Generation and lentiviral transduction of DC

Murine DC were generated according to the protocol described by Lutz *et al.*⁵ with minor adjustments. Briefly, bone marrow cells were collected from the tibia and femurs. The cells were seeded in six-well plates at 10^6 cells per well in complete medium (CM) containing 20 ng/ml muGM-CSF (prepared in-house). On days 3

and 6, fresh GM-CSF containing CM was added. On day 7, the DC were harvested, washed and resuspended at 10^6 cells/ $100\ \mu\text{l}$ in PBS containing recombinant lentiviral particles at a multiplicity of infection of 15 and $10\ \mu\text{g}/\text{ml}$ protamine sulfate. The cells were plated in a 24-well plate and incubated at 37°C . After 2 h, CM containing $20\ \text{ng}/\text{ml}$ muGM-CSF and $100\ \text{ng}/\text{ml}$ LPS (*Escherichia coli* serotype O55:B5; Sigma-Aldrich) was added to the cells in order to reach a final density of 10^6 cells/ml. The cells were matured for 4 h and then used for immunization.

RT-PCR

Total LN RNA was extracted using the SV Total RNA Isolation System (Promega). Total RNA was converted into first-strand cDNA using random hexamers and SuperScript II reverse transcriptase (Superscript First Strand Synthesis System for RT-PCR, Invitrogen). cDNA was amplified using BIOTAQ™ DNA polymerase (Bio-line) following the manufacturer's instructions. The OVA sequence was amplified with OVA235-5'-sense: 5'-GGGGGATCCATTTGCCAGTGGGACAAT-3' and OVA-3'-antisense: 5'-GGGGATCCGGGGAAACACATCTGC CAA-3' primers. To assess the RNA and cDNA quality and quantity, RT-PCR for the house-keeping gene β -actin was performed using the primers actin1: 5'-TGCTATC CAGGCTGT-GCTAT-3' and actin2: 5'-GATGGAGTT GAAGGTAGTTT-3'. The PCR was performed with annealing temperature of 56°C and 45 cycles.

ELISPOT assay

Mice were injected subcutaneously in the interscapular area with the indicated amount of DC Lenti-OVA or Lenti-OVA dir. Antigen-specific T cells from vaccinated mice were quantified *ex vivo* in an ELISPOT assay using the BD mouse IFN- γ ELISPOT pair (Pharmingen, BD Biosciences). After coating of the capture antibody on nitrocellulose microplates (MAHA S4510, Millipore) and blocking with 10% normal mouse serum, freshly isolated splenocytes ($1\text{--}5 \times 10^5$ cells/well) were cultured with $1\ \mu\text{M}$ OVA_{257–264} peptide (SIINFEKL) for 18 h. For each cell suspension, Concanavalin A stimulated cells were included as a positive control and unstimulated cells as a negative control. After overnight incubation, IFN- γ was detected with the biotine-conjugated Ab. Spots were developed by with streptavidin ALP (BenderMed Systems) and the BCIP-NBT substrate kit (BioRad). Spots obtained from unstimulated cell cultures were subtracted from the number of spots obtained from peptide stimulated cell cultures.

CTL assays

For *in vitro* restimulation and Cr⁵¹ release assay, naive C57BL/6 mice were immunized subcutaneously in the hind limb footpads with the indicated amounts of Lenti-OVA or Lenti-OVA directly. The draining popliteal LNs were isolated 7 days later. The LN responder cells were restimulated *in vitro* for 5 days with mitomycin C-treated E.G7-OVA cells (OVA-transfected EL4 cells) at a responder/stimulator ratio of 1:2. After restimulation, the cultures were tested for their cytolytic activity against EL4 and E.G7-OVA cells in a standard ⁵¹Cr release assay, as described earlier.¹⁰

In vivo CTL activity was assessed after interscapular immunization with the indicated amounts of DC or TU lentivector using the *in vivo* CTL assay technique as

previously described.⁶⁴ Briefly, splenocytes from naive syngeneic mice were used as target cells for *in vivo* cytotoxicity. One population was pulsed with $5\ \mu\text{M}$ OVA/K^b peptide for 2 h at 37°C and labeled with $5\ \mu\text{M}$ CFSE (Molecular Probes) whereas the control population was incubated for 2 h without peptide and labeled with $0.5\ \mu\text{M}$ CFSE. Both populations were mixed in a 1:1 ratio and 2×10^7 cells of the mixture were injected per mouse via the tail vein. Eighteen hours after injection, peripheral blood was collected from the tail vein. After density gradient centrifugation using Lympholyte M (Sanbio) and additional RBC-lysis, cells were analyzed by flow cytometry. By comparing the ratio of peptide-pulsed (CFSE^{high}) versus unpulsed targets (CFSE^{low}), the percentage of specific killing normalized for the ratio in naive mice was calculated with the following equation: $1 - ((\% \text{ CFSE}^{\text{high}} / \% \text{ CFSE}^{\text{low}})_{\text{immunized}} / (\% \text{ CFSE}^{\text{high}} / \% \text{ CFSE}^{\text{low}})_{\text{non-immunized}})$.

CD4⁺ T-cell activation

Spleen cells from OT-II TCR transgenic mice were isolated, labeled with $10\ \mu\text{M}$ CFSE and adoptively transferred into naive C57BL/6 recipient mice via intraperitoneally (i.p.) injection (equivalent of $3\text{--}5 \times 10^6$ V α 2⁺ transgenic T cells/mouse). Twenty-four hours later, the mice were immunized with either 5×10^5 DC Lenti-OVA or 1×10^7 TU Lenti-OVA dir in the footpads. Four days post-immunization, draining popliteal LNs were isolated. Part of the LN cells was stained for the transgenic T-cell receptor V α 2 with anti-V α 2 (clone B20.1, Pharmingen) and PI. V α 2⁺PI⁺ cells were analyzed for proliferation by dilution of the CFSE label in flow cytometry. Another part of the LN cells was put in culture with or without the OVA/I-A^b OT-II peptide. Proliferation as measured by ³H-thymidine incorporation, IFN- γ secretion as measured with the Ready-Set-Go moIFN- γ ELISA kit (eBioScience) and IL-4 secretion as measured by the moIL-4 Module Set (Bender MedSystems) were evaluated 3 days after restimulation. DCs loaded with the OVA/I-A^b peptide were used as positive control for the CD4⁺ T-cell activation.

In vivo CD4⁺ T-cell depletion

In order to deplete mice from their CD4⁺ T cells, they were i.p. injected with $200\ \mu\text{g}$ anti-CD4 antibody (clone GK1.5, kindly provided by Professor M Moser) 2 days before immunization. The efficiency of depletion was checked on the day of immunization (day 0) and on the day of the CTL assay (day 7) through flow cytometric analysis of peripheral blood.

Tumor rejection experiments

For therapeutic tumor treatment, mice were grafted subcutaneously with 2×10^5 MO4 tumor cells in the interscapular area and subsequently immunized on days 3, 10 and 17 after tumor challenge with either 1×10^5 / 5×10^5 DC Lenti-OVA or 2×10^6 / 1×10^7 TU Lenti-OVA dir. Tumor growth and survival were evaluated until 70 days post-tumor challenge. Mice were killed when the tumor reached a diameter of more than 15 mm.

Statistics

For statistical comparison between two cohorts of animals receiving different treatment regimens, paired Student's *t*-tests were performed. Survival data were

recorded in a Kaplan–Meier survival curve and a log-rank test was used to determine *P*-values. *P*-values exceeding 0.05 were considered significant.

Abbreviations

DC, dendritic cells; CM, complete medium; TU, transducing units; LN, lymph node

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