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Intravenous Injection of a Lentiviral Vector Encoding NY-ESO-1 Induces an Effective CTL Response

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Lentiviral vectors can efficiently transduce a variety of nondividing cells, including APCs. We assessed the immunogenicity of a lentiviral vector encoding the melanoma Ag NY-ESO-1 in HLA-A2 transgenic mice. Direct i.v. injection of NY-ESO-1 lentivirus induced NY-ESO-1157–165-specific CD8⁺ cells, detected ex vivo with an A2/H-2Kb chimeric class I tetramer. These NY-ESO-1157–165-specific CD8⁺ cells could be expanded by boosting with an NY-ESO-1 vaccinia virus and could kill NY-ESO-1157–165 peptide-pulsed targets in vivo. Such direct lentiviral vector injection was similar in potency to the injection of in vitro-transduced dendritic cells (DC). In addition, human monocyte-derived DC transduced by the NY-ESO-1 lentivirus stimulated an NY-ESO-1157–165-specific CTL clone. These data suggest that direct lentiviral transduction of DC in vivo might provide a powerful immunotherapeutic strategy. The Journal of Immunology, 2004, 172: 1582–1587.

Effective protective or therapeutic vaccination remains a significant clinical problem for many infectious diseases; for example, AIDS (1). Vaccination has also been proposed as a highly specific and nontoxic cancer treatment, but, again, effective protocols await development (2). Dendritic cells (DC)³ are the natural initiators of an immune response, so effective vaccination requires mobilization of DC to present Ag (3). For example, DNA vaccination probably results in both gene and Ag uptake by DC and also activation of DC, as DNA stimulates Toll-like receptors (4). Adaptively transferred DC have been shown to be highly effective cellular adjuvants in mice, stimulating protective T cell responses against pathogens and tumors (5, 6). A similar approach is being applied to human tumor immunotherapy (7–9). In these protocols the DC must be in some way engineered to present specific Ags. This could be achieved by loading the DC with exogenous protein Ag, but delivery of Ag genes to DC is also an attractive idea, because it might allow long term, high level presentation of the endogenously expressed Ag. In addition, endogenous presentation allows more efficient loading of antigenic peptides onto MHC class I molecules.

The development of recombinant viral vector systems for gene therapy has prompted examination of their efficacy in gene delivery to DC and in direct immunization. Adenovirus vectors were shown to deliver Ag genes to human (10) or mouse (11) DC in vitro. The endogenously synthesized Ag was efficiently presented to CD8⁺ T cells; however pre-existing immunity to viral proteins expressed by the vector prevented effective immunization (11). Retroviral vectors based on murine leukemia virus (MLV) have also been used to express Ags in human DC, which could be efficiently presented to CD8⁺ T cells (12–14). However, MLV-based vectors only infect dividing cells, so the human DC had to be generated from CD34⁺ hemopoietic progenitor cells. Injection of MLV-based vector into mice could stimulate immunity (15) and resulted in some transduction of DC, but at low efficiency (16).

Like retroviral vectors, lentiviral vectors based on HIV-1 do not encode any viral proteins. This eliminates problems of pre-existing immunity and avoids competition in the generation of anti-vector vs anti-transgene CTL. Lentiviral vectors can infect nondividing, human peripheral blood-derived DC, and transduced human DC expressing antigenic peptides can stimulate specific CTL responses in vitro (17, 18). An advantage of lentiviral vectors is that they do not activate DC constitutively, like adenoviral vectors (19), or block their activation, like herpes simplex viral vectors (20). Previous studies have used lentiviral vectors expressing a tumor Ag to infect mouse DC in vitro before injection, and CTL responses (18) and tumor protection were established in the mice (21). Direct injection of lentiviruses in mice has been reported to transduce APCs and B cells in spleen (22) and DC in a draining lymph node (23). Direct injection of lentiviral vectors expressing peptide epitopes or a HLA-Cw3 transgene in HLA-A2 transgenic mice has been shown to induce lytic activity against peptide-pulsed targets (24) and peptide or transgene-specific CTL responses (23).

Our aim was to develop HIV-1-based vectors that efficiently expressed a tumor Ag in mouse DC. As an Ag we chose NY-ESO-1 (25), a cytoplasmic protein (26) expressed in melanoma and other tumors. NY-ESO-1 is highly immunogenic, eliciting a spontaneous immune response in 50% of patients with NY-ESO-1-expressing cancers (reviewed in Ref. 27). NY-ESO-1 elicits a combined Ab and T cell response (28). Several epitopes of NY-ESO-1 presented by HLA class II molecules (29–32) and HLA class I molecules (28, 33, 34) have been identified. Previous work from our group has shown that priming of HLA-A2 (A2) transgenic mice with plasmid DNA and recombinant vaccinia virus encoding the A2-restricted epitope NY-ESO-1157–165 elicits a strong NY-ESO-1157–165–specific CTL response (35).
Materials and Methods

Lentiviral vector production

The green fluorescence protein (GFP)-expressing HIV vector pHR5IN-CSGW was provided by A. Thrasher (Institute of Child Health, London, U.K.) in pHR5IN-NY, an NY-ESO-1 cDNA replaces GFP. To make virus, 293T cells were cotransfected with pHR5IN-NY, pCMVR8.91, and MDG plasmids (37) as previously described (38). Unenveloped NY-ESO-1-lentivirus was produced by transfection without MDG. Culture supernatants were concentrated by ultracentrifugation. Titer were determined on 293T cells by measurement of GFP or NY-ESO-1-expression, using a FACScan and CellQuest software (BD Biosciences, Mountain View, CA). NY-ESO-1 was detected in cells fixed with 4% paraformaldehyde and permeabilized in 0.1% saponin using an anti-NY-ESO-1 Ab (gift from Dr. G. Spagnoli, University Hospital, Basel, Switzerland) (26) and goat anti-mouse Texas Red conjugate (Molecular Probes, Eugene, OR).

Infection of 45 cells and immunohistochemical analysis

Cells from the EBV-transformed, HLA-A2+ B cell line .45 were infected with GFP- or NY-ESO-1-expressing vector at MOI 20. Two weeks later, when >90% of the cells were positive for NY-ESO-1 expression, total protein was separated on a 12% denaturing SDS-polyacrylamide gel. Expression of NY-ESO-1 was detected with the anti-NY-ESO-1 Ab and goat anti-mouse HRP (Harlan, Indianapolis, IN).

Transduction of DC

Mouse DC were prepared from bone marrow as previously described (39). Human monocytes were isolated with CD14 beads (Miltenyi Biotec, Auburn, CA) and differentiated into DC in RPMI 1640 with 10% FCS, IL-4 (50 ng/ml), and GM-CSF (1000 U/ml). Day 4–5 immature human or murine DCs were transduced with GFP-, NY-ESO-1-, or NY-ESO-1-noEnv lentiviruses at MOI 40. DCs were analyzed for GFP, NY-ESO-1, CD11c fluorescence microscopy (Axiovert 100 fl, Zeiss, Oberkocken, Germany) with a MRC 1024 Confocal (Bio-Rad, Hercules, CA) expression after 5 days by FACScan and CellQuest software (BD Biosciences, Mountain View, CA). For CD11c-expressing, CD11c(+) and -negative (CD11c(-)) cells were also detected by anti-CD11c mAbs. GFP expression after lentiviral transduction of mouse DC cultures (105) or 9 days after lentiviral injection in the tail vein. PBL were prepared from blood samples using RBC lysis buffer (Invitrogen, Carlsbad, CA). Cells were resuspended in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FCS. PBL samples were stained with NY-ESO-1 tetramer for 20 min at 37°C, then cells were costained with anti-CD8α (Caltag Laboratories, Burlingame, CA), washed, and analyzed on a FACSCalibur using CellQuest software (BD Biosciences).

CTL killing, ELISPOT assay

The human HLA-A2.01 (A2)-positive B cell line .45 transduced with lentiviruses (see above) was labeled with 3H (3H) and incubated with a CTL clone specific for the A2-restricted NY-ESO-1 epitope 157–165 (41). Specific lysis was determined according to this formula: (experimental release – spontaneous release)/(total release – spontaneous release) × 100. Transduced human DC (105; see above) were incubated with 106 NY-ESO-1-specific CTL clone in anti-IFN-γ (MabTech, Nacka, Sweden)-coated ELISPOT plates (Millipore, Watford, U.K.). Plates were developed according to the manufacturer's directions.

In vivo killing assay

Freshly isolated splenocytes from HHD mice were incubated in RPMI 1640 medium with 1 μM peptide for 2 h and labeled with CFSE (Molecular Probes, Eugene, OR). Labeled cells were injected at 105 cells/mouse into the tail vein with a control population without peptide that had been labeled with a different concentration of CFSE. Disappearance of peptide/fluorescence-labeled cells was tracked using FACS analysis of freshly isolated PBL 5 h after the injection. The level of specific cytotoxicity was calculated relative to the labeled unpulsed population using the following calculation: 100 × (100 – (percentage pulsed/percentage unpulsed)).

Results

Transduction of mouse DC ex vivo and in vivo

The HIV-1-based vector pHR5IN-CSGW was developed for high level, sustained transgene expression in human hematopoietic stem cells and their progeny (36). Fig. 1A shows that this vector transduced mouse bone marrow-derived DC cultures. Preferential GFP expression in the CD11c+ cells was seen, with up to 50% of CD11c+ cells expressing GFP. Transduction in vivo was then examined by injection of 5 × 107 293T infectious units (i.u.) in the tail vein, followed by analysis of GFP expression in spleen cells. Fig. 1B demonstrates that CD11c+ GFP+ cells were also detected in vivo (a typical mouse is shown); 0.3 and 0.4% of CD11c+ cells purified from spleen expressed GFP after 9 days in duplicate experiments. A similar percentage of GFP+/CD11c+ cells was detected in spleen between 5 and 12 days after GFP lentiviral vector injection (data not shown). The CD11c+ cells were transduced by

Some mice were primed with plasmid DNA encoding full-length NY-ESO-1 or boosted by injecting 106 PFU recombinant vaccinia virus encoding full-length NY-ESO-1 into the tail vein. PBL were prepared from blood samples using RBC lysis buffer (Invitrogen, Carlsbad, CA). Cells were resuspended in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FCS. PBL samples were stained with NY-ESO-1 tetramer for 20 min at 37°C, then cells were costained with anti-CD8α (Caltag Laboratories, Burlingame, CA), washed, and analyzed on a FACSCalibur using CellQuest software (BD Biosciences).

A

in vitro transduced

B

untransduced

C

untransduced

non-DC (unit)

DC (unit)

non-DC (EnV1)

DC (EnV1)

non-DC (EnV2)

DC (EnV2)

non-DC (GFP)

DC (GFP)
the lentiviral vector, as demonstrated by the detection of GFP DNA in these cells (Fig. 1C). Injection of a control vector preparation without viral envelope did not result in GFP DNA detection (Fig. 1C). A previous study injected a higher dose of an essentially identical lentiviral vector in the tail vein of mice and demonstrated long term transduction of both APCs and B cells in spleen (22). It is therefore likely that CD11c\(^+\) B cells are also transduced in our experiments. Injection of a lentiviral vector in the footpad transduced DC in the draining lymph node (23).

Direct immunization with lentiviral vector

To address whether in vivo transduction resulted in the induction of Ag-specific CTL, HLA-A2 transgenic (HHD) mice were injected with escalating doses of lentiviral vector encoding the tumor testis Ag NY-ESO-1. CTL responses were monitored in the blood by staining of PBL with a chimeric A2Kb/peptide tetramer (35, 42) (Fig. 2). At the highest dose, NY-ESO-1\(_{157-165}\)-specific CD8\(^+\) cells could be detected in peripheral blood after injection; typical mice and a summary are shown in Fig. 2A. When the same group of animals was boosted with NY-ESO-1 recombinant vaccinia virus, NY-ESO-1\(_{157-165}\)-specific CD8\(^+\) cells could be detected in all three groups of mice (Fig. 2A). Control mice injected with NY-ESO-1 vaccinia alone or mice boosted with irrelevant vaccinia virus showed only a weak NY-ESO-1\(_{157-165}\)-specific response (mean responses, 0.025% CD8\(^+\) cells after NY-ESO-1 vaccinia alone, 0.25% CD8\(^+\) cells after NY-ESO-1 lentivirus, followed by irrelevant vaccinia virus). The NY-ESO-1\(_{157-165}\)-specific CD8\(^+\) cells induced by lentiviral vector priming were effective CTL, as demonstrated by their ability to kill NY-ESO-1\(_{157-165}\) peptide-pulsed target cells in vivo (Fig. 2B).

FIGURE 2. A. NY-ESO-1\(_{157-165}\)-specific CD8\(^+\) cells 8 days after injection of the number of lentiviruses shown and 8 days after boosting of the same mice with NY-ESO-1 vaccinia viruses. Typical mice from a group of three are shown, with the mean response of each group. B. Detection of NY-ESO-1\(_{157-165}\) peptide-pulsed splenocytes (R2) and unpulsed splenocytes (R3) 5 h after injection into immunized mice.
50% of 10^6 mouse DC. As a control, unenveloped virus was also
expressed with NY-ESO-1 lentivirus. Expression of NY-ESO-1 was detected
with APC. The human EBV-transformed B cell line .45 was transduced
of HHD mice 8 days after injection of 10^6 DC, transduced as indicated, and
8 days after boosting of the same mice with 10^6 NY-ESO-1 vaccinia vi-

\[ \text{NY-ESO-1} \]

ruses. Typical mice from a group of three are shown, with the mean re-
response of each group.

**FIGURE 3.** NY-ESO-1_{157-165}-specific CD8^+ cells in peripheral blood
of HHD mice 8 days after injection of 10^6 DC, transduced as indicated, and
8 days after boosting of the same mice with 10^6 NY-ESO-1 vaccinia vi-

**Immunization with ex vivo-transduced DC**

Direct injection of 5 \times 10^5 (293T i.u.) lentiviruses was able to
prime an effective response. We therefore examined the efficiency of
NY-ESO-1 lentiviral vector-transduced DC as immunogens. Because human and mouse DC are relatively refractory to lenti-
viral vector transduction, 4 \times 10^7 i.u. were required to infect
\sim50% of 10^6 mouse DC. As a control, unenveloped virus was also
used in a mock infection of DC, as phagocytic DC can ingest and
present proteins from lentiviral vector preparations. Fig. 3 shows that
NY-ESO-1_{157-165}-specific CD8^+ cells could be detected in
peripheral blood of mice that received NY-ESO-1-transduced DC. This
response could also be boosted with NY-ESO-1 vaccinia vi-

**Discussion**

We compared immunization with lentiviral vectors expressing an
Ag used either to modify DC ex vivo or to transduce DC and other
cells in situ after i.v. injection. Both routes of immunization re-

NY-ESO-1 presentation by lentiviral vector-transduced human B
cells and DC

To show that this approach might ultimately be used in clinical
settings, we wanted to demonstrate that the NY-ESO-1 lentivirus
could induce NY-ESO-1_{157-165} peptide presentation in human
APC. The human EBV-transformed B cell line .45 was transduced
with NY-ESO-1 lentivirus. Expression of NY-ESO-1 was detected
by Western blot (Fig. 4A), and FACS analysis showed that \sim90% of
cells were NY-ESO-1-positive by intracellular staining (data not
shown). NY-ESO-1_{157-165} peptide presentation by the B cells was
demonstrated in a \text{^51}Cr release assay using an NY-ESO-1_{157-165}-
specific, HLA-A2-restricted CTL clone (Fig. 4A). We then used
the NY-ESO-1 lentiviral vector to transduce human HLA-A2^+, monocyte-derived DC, using a protocol that we previously reported
can transduce up to 30% of DC without affecting their viability or
ability to mature (38). Fig. 4B shows cytoplasmic expression of
NY-ESO-1 in \sim30% of the transduced CD11c^+ DC. To demonstrate
that the transduced DC could present an epitope from the cytoplasmic
NY-ESO-1 protein, we used an NY-ESO-1_{157-165}-specific CTL
clonal by tetramer sorting from peripheral blood of a melan-
oma patient. Fig. 4C shows that the transduced DC could stimu-
ulate IFN-\gamma secretion by this NY-ESO-1_{157-165}-specific CTL
clonal in an ELISPOT assay. These data show that both immature
and mature DC stably modified to express a cytoplasmic protein
can present an epitope from that protein to CD8^+ T cells. Previous
reports using lentiviral vectors (17) or varicella-zoster virus
(VSV)-G-pseudotyped HIV-1 (43, 44) to modify human DC have
examined presentation of CTL epitopes engineered for secretion
into the endoplasmic reticulum (17, 18) or HIV-1 Gag that buds
from the cell (43, 44).

Lentiviral vectors are attractive for prime/boost protocols be-
cause there are no pre-existing neutralizing Abs to heterologous
envelopes, such as VSV-G, that might inhibit CTL priming (45).
Furthermore, as the vector encodes only the immunizing Ag, trans-
duced APC will not express viral proteins that might inhibit prim-
ing due to competition by CTL at the APC (46). Heterologous
prime/boost may be more efficient than homologous boost with
lentivirus, as pre-existing anti lentiviral vector responses have been
shown to inhibit immunization (22). Clearly, lentiviral vector safety
will require rigorous testing before clinical trials, as there is
potential for similar insertional mutagenesis to that seen with ret-
roviral vectors (47). However, transduction of nondividing DCs is
likely to be less oncogenic than transduction of rapidly proliferat-
ing hemopoietic stem cells; targeting vector to DCs may also en-
hance its safety. Although it is clear from our data that DC trans-
duced ex vivo can prime an immune response, we cannot be sure
that the CD11c^+ cells transduced after i.v. injection are the cells
responsible for immune stimulation. Again, surface or transcrip-
tional targeting of NY-ESO-1 expression to DC will resolve this
question.

HIV-1 itself infects DC in vitro and in patients, which may serve
as a reservoir of infected cells (48), and also traffics to lymphoid
tissue bound to the DC surface (49). To evade the immune response, wild-type HIV-1 has been reported to modulate DC function by a number of strategies, including Nef and Tat induction of cytokine and chemokine production in the absence of maturation (50, 51). It has been proposed that this serves to attract T cells, permitting HIV-1 transmission from DC without stimulating an immune response. HIV-1 viruses deleted in envelope (44) or envelope Nef, Vif, Vpr, and Vpu (43) and pseudotyped with VSV-G have been shown to infect DC in vitro and stimulate Gag-specific T cells. The lentiviral vectors we used in this study are further deleted for Tat, Rev, and HIV-1 Gag and Pol proteins, which will permit HIV-1 transmission from DC without stimulating an immune response. Wild-type HIV-1 has been reported to modulate DC function by a number of strategies, including Nef and Tat induction of cytokine and chemokine production in the absence of maturation (50, 51).

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