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Intravenous Injection of a Lentiviral Vector Encoding NY-ESO-1 Induces an Effective CTL Response¹

Michael J. Palmowski,* Luciene Lopes,[†] Yasuhiro Ikeda,[†] Mariolina Salio,*
Vincenzo Cerundolo,* and Mary K. Collins^{2†}

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-1_{157–165}-specific CD8⁺ cells, detected ex vivo with an A2/H-2K^b chimeric class I tetramer. These NY-ESO-1_{157–165}-specific CD8⁺ cells could be expanded by boosting with an NY-ESO-1 vaccinia virus and could kill NY-ESO-1_{157–165} 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-1_{157–165}-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). Adoptively 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-1_{157–165} elicits a strong NY-ESO-1_{157–165}-specific CTL response (35).

*Tumour Immunology Unit, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, United Kingdom; and [†]Department of Immunology and Molecular Pathology, Windeyer Institute, University College, London, United Kingdom

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² Address correspondence and reprint requests to Dr. Mary K. Collins, Department of Immunology and Molecular Pathology, Windeyer Institute, 46 Cleveland Street, London, U.K. W1T 4JF. E-mail address: mary.collins@ucl.ac.uk

³ Abbreviations used in this paper: DC, dendritic cell; GFP, green fluorescence protein; i.u., infectious unit; MLV, murine leukemia virus; VSV, varicella-zoster virus.

Materials and Methods

Lentiviral vector production

The green fluorescence protein (GFP)-expressing HIV vector pHRSIN-CSGW was provided by A. Thrasher (Institute of Child Health, London, U.K.) (36). In pHRSIN-NY, an NY-ESO-1 cDNA replaces GFP. To make virus, 293T cells were cotransfected with pHRSIN-NY, pCMV8.91, and pMDG plasmids (37) as previously described (38). Unenveloped NY-ESO-1-lentivirus was produced by transfection without pMDG. Culture supernatants were concentrated by ultracentrifugation. Titers 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 immunoblotting 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).

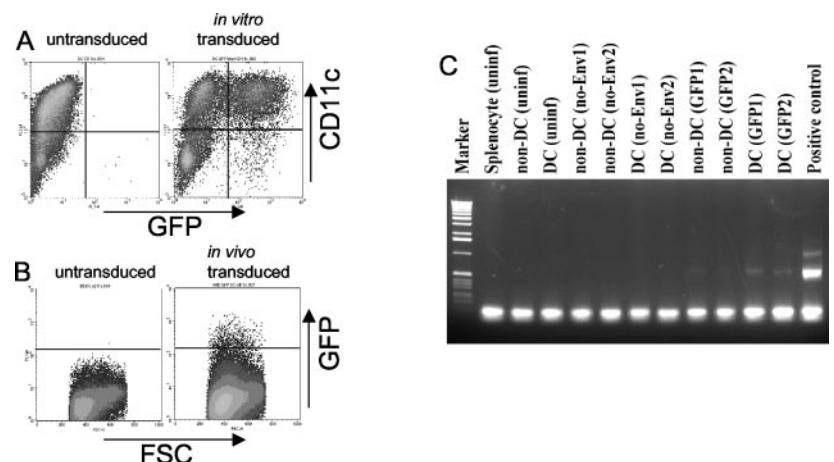
Transduction of DC

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 (BD PharMingen, San Diego, CA), and CD1a (eBioscience, San Diego, CA) expression after 5 days by fluorescence microscopy (Axiovert 100 (Zeiss, Oberkochen, Germany) with a MRC 1024 Confocal (Bio-Rad, Hercules, CA)) or FACScan. Mouse DC were cultured for 4 days after transduction and incubated with 20 μ g/ml CpG, to induce maturation before injection. Human DC were cultured for 4 days after transduction, then matured by cultivation with CD40 ligand-expressing J558L cells (gift from Dr. P. Lane, Birmingham, U.K.) before use in an ELISPOT assay. In some experiments mouse DC were purified from splenocytes using CD11c microbeads (Miltenyi Biotec). DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Detection of the enhanced GFP sequence was conducted by nested PCR using enhanced GFP-specific primers and Ex-Taq (Takara, Ohtsu, Japan). Primer pair F1 and R1 was used for the first reaction with 0.4 μ g of the total cellular DNA as a template. Primer pair F2 and R2 was used for the second reaction with 4 μ l of the first PCR reactions as template: F1, atgtgtgacgaagggcggagagctg; R1, tagtggttgtcggcagcagcacg; F2, ggtgtgtccatctctgtctgag; and R2, tctgtgtagtgtctggcagctgc.

Mice and immunization

HHD mice (40) were immunized by injecting lentiviral vectors or bone marrow-derived DC transduced with lentiviral vectors suspended in PBS into the tail vein. Blood samples were taken 8 days after immunization.

FIGURE 1. GFP expression after lentiviral transduction of mouse DC cultures (A) or 9 days after lentiviral injection in the tail vein (B). B, CD11c⁺ cells purified from the spleen of a typical mouse; 0.3 and 0.4% of CD11c⁺ cells expressed GFP after injection of duplicate mice. C, Two mice were injected in the tail vein with 8×10^8 293T i.u. of GFP lentiviruses (GFP1 and GFP2). Control groups were injected with either PBS (uninf) or an equivalent amount of nonenveloped GFP lentivirus particles (no-Env1 and no-Env2). Six days later, spleens were removed and CD11c-positive (DC) and -negative (non-DC) cells were isolated. The GFP sequence was detected by nested PCR as described in *Materials and Methods*.



Some mice were primed with plasmid DNA encoding full-length NY-ESO-1 or boosted by injecting 10^6 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-CD8- α (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 ⁵¹Cr 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)) \times 100. Transduced human DC (10^4 ; see above) were incubated with 10^2 NY-ESO-1_{157–165}-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 μ M peptide for 2 h and labeled with CFSE (Molecular Probes, Eugene, OR). Labeled cells were injected at 10^7 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/fluorochrome-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: $100 \times (100 - (\text{percentage pulsed}/\text{percentage unpulsed}))$. WinMDI 2.8 software (J. Trotter, The Scripps Institute, La Jolla, CA; <http://facs.scripps.edu>) and CellQuest 3.3 software (BD Biosciences) were used to analyze the FACS data.

Results

Transduction of mouse DC ex vivo and in vivo

The HIV-1-based vector pHRSIN-CSGW was developed for high level, sustained transgene expression in human hemopoietic 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 5×10^7 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

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-1₁₅₇₋₁₆₅-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-1₁₅₇₋₁₆₅-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-1₁₅₇₋₁₆₅-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-1₁₅₇₋₁₆₅-specific CD8⁺ cells induced by lentiviral vector priming were effective CTL, as demonstrated by their ability to kill NY-ESO-1₁₅₇₋₁₆₅ peptide-pulsed target cells *in vivo* (Fig. 2B).

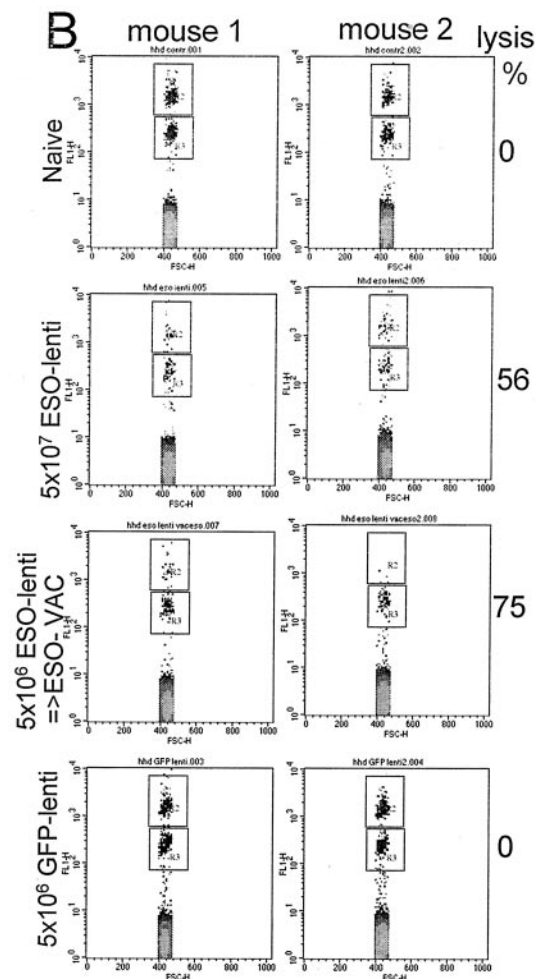
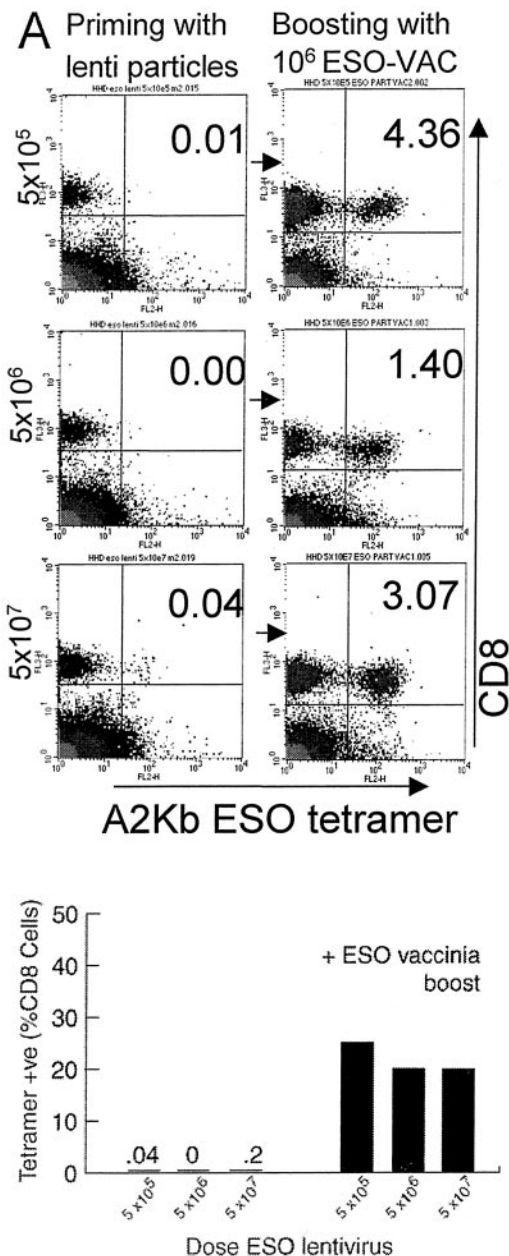


FIGURE 2. A, NY-ESO-1₁₅₇₋₁₆₅-specific CD8⁺ cells 8 days after injection of the number of lentiviruses shown and 8 days after boosting of the same mice with NY-ESO-1 vaccinia viruses. Typical mice from a group of three are shown, with the mean response of each group. B, Detection of NY-ESO-1₁₅₇₋₁₆₅ peptide-pulsed splenocytes (R2) and unpulsed splenocytes (R3) 5 h after injection into immunized mice.

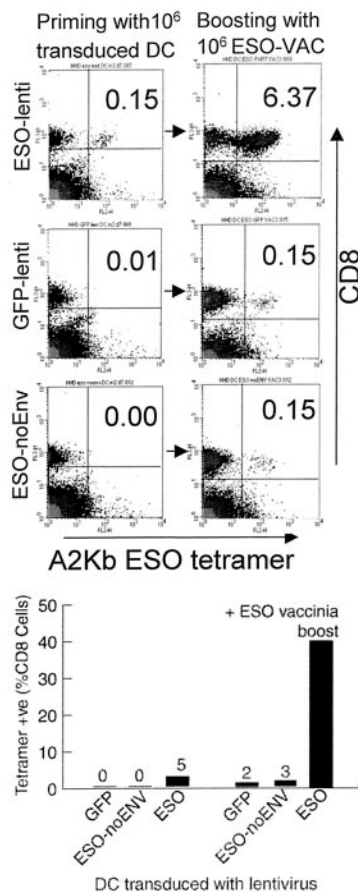


FIGURE 3. NY-ESO-1_{157–165}-specific CD8⁺ cells in peripheral blood of HHD mice 8 days after injection of 10⁶ DC, transduced as indicated, and 8 days after boosting of the same mice with 10⁶ NY-ESO-1 vaccinia viruses. Typical mice from a group of three are shown, with the mean response of each group.

Immunization with *ex vivo*-transduced DC

Direct injection of 5×10^5 (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 lentiviral vector transduction, 4×10^7 i.u. were required to infect $\sim 50\%$ of 10⁶ 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 virus (Fig. 3). The boosted response after priming by transduced DC was not substantially higher than boosted responses after direct vector injection.

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 $\sim 90\%$ 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 ⁵¹Cr 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 $\sim 30\%$ of the transduced CD1a⁺ 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 clone isolated by tetramer sorting from peripheral blood of a melanoma patient. Fig. 4C shows that the transduced DC could stimulate IFN- γ secretion by this NY-ESO-1_{157–165}-specific CTL clone 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).

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 resulted in priming of an immune response that could be boosted with vaccinia virus expressing NY-ESO-1. Injection of as few as 5×10^5 (293T i.u.) NY-ESO-1 lentiviruses could prime an NY-ESO-1_{157–165}-specific CD8⁺ T cell response. This response to a relatively low dose is encouraging if vaccination in larger animal models or the clinic is considered, as production of sufficient lentiviral vector would be feasible. A recent study in a similar mouse model, but with different Ags, reported up to 10% Ag-specific CD8⁺ cells after a single immunization of 5×10^7 lentiviruses (23). This stronger response is probably due to the Ags expressed, a human HLA-Cw3 or Cw3 or Melan-A peptides, as injection of 5×10^5 transduced DC expressing these Ags induced up to 5% Ag-specific CD8⁺ cells (23). Our data demonstrate that lentiviruses may also be used in a heterologous prime/boost strategy to elicit CTL responses to weakly antigenic and/or subdominant tumor Ags (35).

Lentiviral vectors are attractive for prime/boost protocols because 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, transduced APC will not express viral proteins that might inhibit priming 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 retroviral vectors (47). However, transduction of nondividing DCs is likely to be less oncogenic than transduction of rapidly proliferating hemopoietic stem cells; targeting vector to DCs may also enhance its safety. Although it is clear from our data that DC transduced *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 transcriptional 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

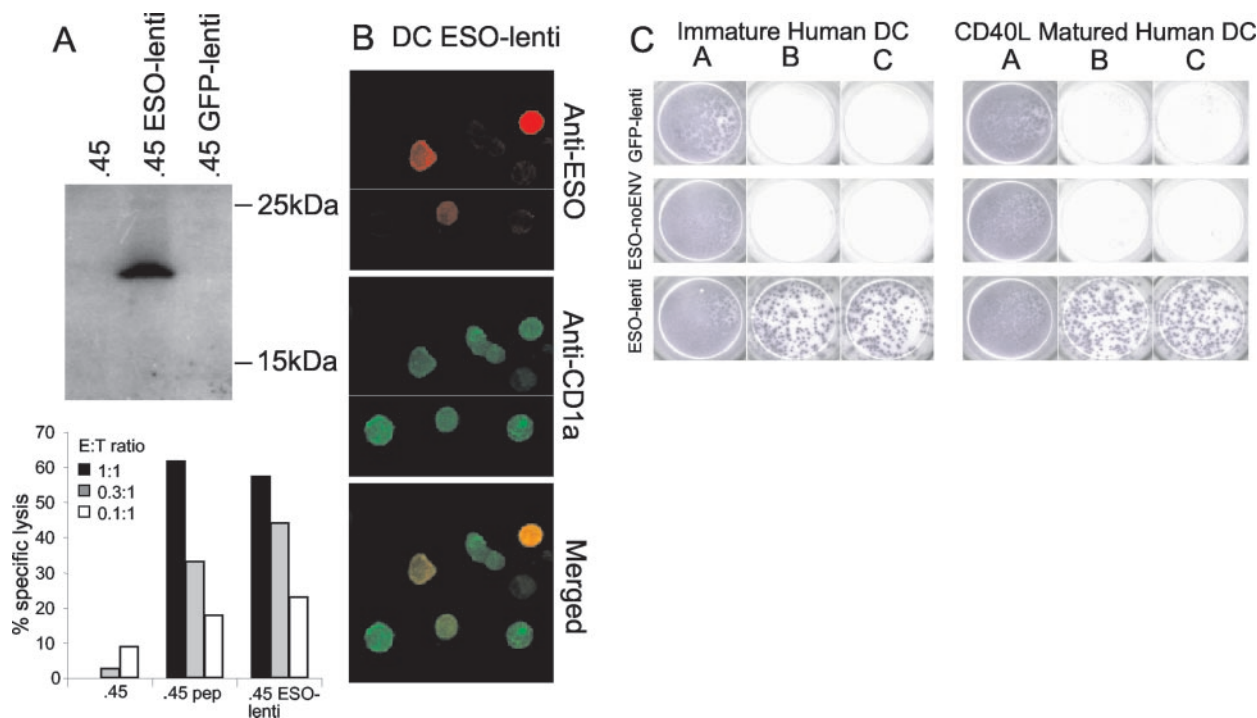


FIGURE 4. A, Lysis of NY-ESO-1-transduced human B cells by an NY-ESO-1_{157–165}-specific CTL clone in a ⁵¹Cr release assay. Expression of NY-ESO-1 in the transduced B cells was detected by immunoblot. B, Expression of NY-ESO-1 in human DC was detected by immunostaining. Stimulation of IFN- γ release by an NY-ESO-1_{157–165}-specific CTL clone by the transduced DC was determined in an ELISPOT assay. Well A, DC plus NY-ESO-1_{157–165} peptide; wells B and C, duplicate wells of transduced DC (C).

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 increase their immune stimulatory potential and focus the immune response on the NY-ESO-1 transgene. Indeed, the coding capacity of the lentiviral vectors will allow us to express potentially immunostimulatory molecules together with the Ag gene in future studies.

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