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Lentivirus-Mediated Gene Transfer and Expression in Established Human Tumor Antigen-Specific Cytotoxic T Cells and Primary Unstimulated T Cells

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ABSTRACT

In this report, we evaluated the efficiency of stable gene transfer into established CD8$^+$ human tumor antigen-specific cytotoxic T cell (CTL) lines and peripheral blood lymphocytes (PBL) by oncoretroviral and lentiviral vectors. In the oncoretroviral vector, the green fluorescent protein (GFP) reporter gene was regulated by the murine stem cell virus (MSCV) promoter. In three human immunodeficiency virus type 1 (HIV-1)–based lentiviral vectors, the GFP transgene was regulated by either a chimeric MSCV/HIV-1 promoter, or cellular promoters from human housekeeping genes PGK and EF1α. We found that several lines of proliferating tumor-specific CTL were poorly (=2%) transduced by the oncoretroviral vector that transduced Jurkat T cell line efficiently (=80%). In contrast, three lentiviral vectors transduced 38–63% of these proliferating CTL. More interestingly, all lentiviral vectors packaged without the HIV-1 accessory proteins transduced human bulk PBL and purified CD4$^+$ and CD8$^+$ lymphocyte subsets without prior stimulation. Detailed analysis indicated that the lentiviral vectors containing the EF1α or PGK ubiquitous promoter can transduce unstimulated PBL and achieve low-level transgene expression in the absence of any T-cell activation. However, T-cell activation subsequent to the transduction of unstimulated PBL is required for high-level transgene expression. Transduced PBL expressing transgene delivered by the lentiviral vectors still preserved resting and naïve cell phenotypes. Taken together, prior T cell stimulation and HIV-1 accessory proteins are dispensable for lentivirus-mediated gene transfer into resting naïve and memory T lymphocytes. These results will have significant implications for the study of T-cell biology and for the improvement of clinical gene therapies of acquired immune deficiency syndrome (AIDS) and cancer.

OVERVIEW SUMMARY

We investigated gene transfer into proliferating human tumor-specific CD8$^+$ cytotoxic T cell (CTL) lines and unstimulated peripheral blood lymphocytes (PBL) by oncoretroviral and lentiviral vectors. We showed that human immunodeficiency virus type 1 (HIV-1)–based lentiviral vectors are superior to the oncoretroviral vectors in gene transduction of proliferating human tumor-specific T cell lines. The transduced tumor-specific CTL lysed target cells and released tumor necrosis factor (TNF)-α in response to the specific antigen as specifically and efficiently as the mock-transduced or transgene-negative counterparts. Therefore, lentivirus-mediated gene transfer and expression at a high level did not alter the specificity and activity of transduced T cells in culture. More interestingly, we achieved detectable level transgene expression in unstimulated primary human PBL with the lentiviral vectors containing EF1α or PGK promoter; and transgene expression can be enhanced by T-cell activation stimuli posttransduction. Lentivirus mediated transgene expression in unstimulated PBL on stimulation after transduction sustained for at least 1 month. The lentiviral-mediated gene transfer into established T-cell lines as well as unstimulated PBL does not require HIV-1 accessory proteins.

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INTRODUCTION

GENETIC MODIFICATION of peripheral blood lymphocytes (PBL) has been shown to be promising in treatment of inherited and acquired immunodeficiencies (Blaese *et al.*, 1995; Bordignon *et al.*, 1995; Kohn *et al.*, 1995, 1998; Fischer *et al.*, 2002), viral infections (Riddell and Greenberg, 1995; Greenberg and Riddell, 1999), cancer (Rosenberg *et al.*, 1990; Hwu *et al.*, 1993; Treisman *et al.*, 1995; Dudley *et al.*, 2002; Sadelain *et al.*, 2003), and transplant complications (Tiberghien *et al.*, 1994). It also holds great potential in the study of T-cell biology (Dahl *et al.*, 2000; Kessels *et al.*, 2000; Maher *et al.*, 2002; Sadelain *et al.*, 2003). Currently, most protocols involving gene transfer into T cells utilize oncoretroviral vectors whose expression requires integration into host chromosomes in dividing cells. Although T lymphocytes can be activated to proliferate *ex vivo* by various means, it has been shown that extended *ex vivo* culture, required by oncoretroviral-mediated gene transfer, may alter biologic properties of target cells. It has been reported that *in vitro* culture and stimulation of T cells altered the $CD4^+/CD8^+$ ratios, T-cell receptor (TCR) repertoires, and cytokine secretion profiles (Kohn *et al.*, 1998; Dietrich *et al.*, 1997; Sedar, 1994). A variety of methods have been used to enhance lymphocyte transduction by oncoretroviral vectors (Mavilio *et al.*, 1994; Bunnell *et al.*, 1995; Lam *et al.*, 1996; Gallardo *et al.*, 1997; Ayuk *et al.*, 1999; Dardalhon *et al.*, 1999, 2000; Wu *et al.*, 1999; Yang *et al.*, 1999; Uckert *et al.*, 2000; Riviere *et al.*, 2000), which include use of fibronectin (Dardalhon *et al.*, 1999), phosphate depletion and low-temperature incubation (Bunnell *et al.*, 1995), utilization of gibbon ape leukemia virus (GALV), and vesicular stomatitis virus G (VSV-G) envelope (Lam *et al.*, 1996; Gallardo *et al.*, 1997). However, all these procedures still require prior T-cell activation and often repeated cycles of transduction with oncoretroviral vectors. Despite this recent progress, transduction efficiencies remained inconsistent and unsatisfactory for proliferating and resting T lymphocytes including freshly isolated PBL.

Recently, attention has been focused on vectors derived from lentiviruses such as HIV-1, which have been shown to transduce a variety of slowly or nondividing cells including unstimulated T lymphocytes (Poznansky *et al.*, 1991; Shimada *et al.*, 1991; Bukrinsky *et al.*, 1992, 1993; Von Schwedler *et al.*, 1994; Naldini *et al.*, 1996a,b, 1998; Reiser *et al.*, 1996; Kafri *et al.*, 1997; Miyoshi *et al.*, 1997, 1998, 1999; Case *et al.*, 1999; Trono, 2000; Zufferey *et al.*, 1997, 1998; Unutmaz *et al.*, 1999; Chinnasamy *et al.*, 2000; Costello *et al.*, 2000; Ducrey-Rundquist *et al.*, 2002; Verhoeyen *et al.*, 2003). Early studies demonstrated that a recombinant HIV-1 vector could efficiently introduce desired genetic elements into cultured human Jurkat leukemic T-cell line and established $CD4^+$ T-cell lines (Poznansky *et al.*, 1991; Shimada *et al.*, 1991; Bukrinsky *et al.*, 1992, 1993; Von Schwedler *et al.*, 1994). Since then, recombinant HIV-1–based lentiviral vectors have been significantly improved to increase safety and potency by splitting viral genome into multiple plasmids and by replacing the HIV-1 envelope gene with another viral envelope gene (such as VSV-G), increasing viral titers and extending their tropism to non-$CD4^+$ cells. With the development of a newer generation of lentiviral vectors (Zufferey *et al.*, 1997; Unutmaz *et al.*, 1999; Chinnasamy *et al.*, 2000; Costello *et al.*, 2000; Maurice *et al.*, 2002), several studies have aimed to transduce freshly isolated PBL. Littman and colleagues first showed that an HIV-1–derived vector was incapable of mediating green fluorescent protein (GFP) marker gene expression in transduced resting T cells unless cytokines are provided in culture (Unutmaz *et al.*, 1999). Chinnasamy and coworkers reported recently that lentiviral vectors failed to integrate into resting lymphocytes in the absence of the HIV-1 Vif, Vpr, Vpu and Nef accessory proteins (APs) (Chinnasamy *et al.*, 2000). However, Costello and colleagues recently reported that the HIV-1 APs were unnecessary for lentiviral vectors to transduce unstimulated PBL under a different transduction condition (Costello *et al.*, 2000). Therefore, more studies are needed to resolve this discrepancy and to achieve consistent and efficient transduction of primary human PBL without altering their biologic properties.

In our effort to modify human CTL lines functionally that were raised specifically against renal cell carcinoma (RCC) and pancreatic cancer, we found that oncoretroviral vectors gave low transduction efficiencies for these proliferating CTL lines, although they transduced Jurkat $CD4^+$ T-cell line efficiently. The oncoretroviral vectors that we have used include the widely used MGIN vector in which transgene expression is regulated by the murine stem cell virus (MCSV) promoter (Cheng *et al.*, 1997). The MSCV promoter located in the U3 region of long-terminal repeat (LTR) has been demonstrated to be active in various human and animal lymphocytes, including those isolated from non-human primates after hematopoietic cell transduction and transplant (Donahue *et al.*, 2000). We therefore used a hybrid lentiviral vector in which the HIV-1 promoter has been replaced by the MSCV promoter, regulating the same transgene cassette as in the MGIN oncoretroviral vector (Gao *et al.*, 2001). We report here the direct comparison of this pair of analogous oncoretroviral and lentiviral vectors in transducing proliferating lymphocytes. In addition, we tested two additional lentiviral vectors in which the transgene is regulated by a cellular promoter of the housekeeping genes such as PGK or EF1α (Dull *et al.*, 1998; Zufferey *et al.*, 1998; Cui *et al.*, 2002, Hass *et al.*, 2000). These different lentiviral vectors have allowed us to evaluate transgene expression as well as gene transfer in proliferating and unstimulated lymphocytes, and the effects of different stimulation schema on transgene expression.

MATERIALS AND METHODS

Vector and plasmid construction

The MSCV-derived oncoretroviral vector MGIN has been described previously (Cheng *et al.*, 1997). In this vector, the enhanced GFP gene is coexpressed with the neomycin resistance (Neo^R) gene, linked by an internal ribosome entry site (IRES). The lentiviral vector GIN-MU3 is a hybrid HIV/MSCV vector provided by Dr. Robert Hawley (American Red Cross Holland Laboratory, Rockville, MD) and described recently (Gao *et al.*, 2001). As in MGIN, the GFP-IRES-Neo^R (GIN) expression cassette in the GIN-MU3 is regulated by the MSCV U3 promoter in transduced cells. A self-inactivating (SIN) HIV-1–based vector (pRLLhPGK.GFP sin-18, abbreviated as PGK.GFP) in which the HIV-1 promoter in the 3′ LTR U3 region is deleted and the reporter GFP expression is solely regu-

lated by the internal human phosphoglycerate kinase (PGK) promoter has been described (Dull *et al*., 1998; Zufferey *et al*., 1998). The EF.GFP vector was recently constructed based on the PGK.GFP backbone, in which the PGK promoter was replaced by the human elongation factor 1α (EF1α) promoter (Cui *et al*., 2002).

The retroviral packaging plasmid pMLVgag/pol expressing murine leukemia virus (MLV) Gag and Pol proteins (Yang *et al*., 1999) was a gift from Dr. Gary Nabel (National Institutes of Health, Bethesda, MD). The lentiviral packaging plasmids pCMVΔR8.2 (Naldini *et al*., 1996b) carrying all the HIV-1 genes except *env*, or pCMVΔR8.91 (Zufferey *et al*., 1997) devoid of all the HIV-1 accessory genes (*vif, vpu, vpr, and nef*) and *env* were used to produce recombinant lentiviruses. The pCMVΔR8.91 was used in most experiments if not otherwise indicated. The plasmid pMD.G was used to produce VSV-G envelope proteins and pseudotype lentiviral vectors (Naldini *et al*., 1996b). The plasmids pRLLhPGK.GFP-sin-18, pCMVΔR8.2, pCMVΔR8.91 and pMD.G were kindly provided by Dr. Didier Trono (University of Geneva, Switzerland).

Viral production

Replication-incompetent oncoretroviral or lentiviral particles pseudotyped with VSV-G were generated by calcium phosphate transfection of 293T cells as described (Gao *et al*., 2001; Cui *et al*., 2002). The transfection medium was replaced after overnight transfection with fresh Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT). Supernatants were collected at 48 and 72 hr after transfection. The virus-containing supernatants were filtered through 0.45 μm filters to remove cells and debris, and frozen in aliquots at -80°C. If necessary, viral supernatants were concentrated using a filtration column (Centricon Plus-20, MW CO 100,000, Millipore, Bedford, MA) by centrifugation at 1800*g* for 30–90 min at 4°C (Cui *et al*., 2002). The viral titers were determined by transduction of 293T cells (2×10^5 cells per well in 6-well plates) with serially diluted virus supernatant in the presence of 8 μg/ml of polybrene (Sigma, St. Louis, MO; Cui *et al*., 2002). Transducing units (TU) were calculated based on numbers of individually transduced GFP$^+$ cells according to the Poisson distribution. The titers of MGIN, GIN-MU3, PGK.GFP, and EF.GFP are typically 0.2×10^6, 0.5×10^6, 1×10^6, and $2–5 \times 10^6$ TU/ml, respectively. A PG13-based (GALV pseudotyped) stable MGIN packaging line (PG13.MGIN) was also used, which produced a viral titer 0.5×10^6 TU/ml by the same assay.

The absence of replication competent retroviruses (RCR) in lentiviral vector preparations and transduced cells was confirmed. Cell culture supernatants from transduced cells after long-term culture were randomly selected and tested for the presence of the p24 HIV-1 protein by enzyme-linked immunosorbent assay (ELISA; Cui *et al*., 2002). Within the detection limit (5 pg/ml) of the assay, all the samples that we examined were negative for RCR.

Cell culture

The human embryonic kidney cell line 293T and PG13.MGIN producer line were cultured in DMEM supplemented with 10% FBS. The human T cell line Jurkat, leukemia cell line K562, Epstein-Barr virus (EBV)-transformed B cell lines EBV24, and EBV26 were cultured in RPMI-1640 (Gibco-BRL), 10% FBS, 1% nonessential amino acids (NEAA), 1 m*M* sodium pyruvate, 2 m*M* L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. All RCC cell lines RCC24, RCC-Fr, RCC-Lo, and RCC-Sa were grown in RPMI-1640 supplemented with 20% FBS and 10% tryptose phosphate broth (Difco Laboratories, Detroit, MI) in addition to NEAA, sodium pyruvate, L-glutamine, penicillin, and streptomycin.

RCC and pancreatic cancer specific CD8$^+$ T cells were generated *in vitro* by stimulating PBL from patient 24 of RCC and patient 54 of pancreatic cancer with autologous tumor cell lines expressing B7.1 costimulatory molecule. T cells (5×10^5 cells per well) are maintained in 24-well plates by periodically restimulating with irradiated tumor cells (1×10^5 cells per well) as antigens in the presence of recombinant human interleukin (IL)-2 (60 IU/ml, Chiron Corp., Emerville, CA) with 2 ml human T cell medium consisting of RPMI-1640, 10% pooled human serum, 10 m*M* HEPES, 2 m*M* L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. 5B CTL clone was derived from RCC1.24 CTL line by a limiting dilution and recognizes QTACEVLDY derived from a point mutation of KIAA1440 in a HLA-A*0101 restricted manner (Zhou *et al*., manuscript in preparation).

Peripheral blood mononuclear cells (PBMC) were obtained from healthy, HIV-1–seronegative donors through Ficoll-Hypaque (Pharmacia, Sweden). Monocytes were removed by plastic adherence for 2 hr at 37°C. To obtain purified primary CD4$^+$, CD8$^+$ T cells, and CD19$^+$ B cells, PBMC depleted of monocytes were incubated with anti-CD4, anti-CD8, or anti-CD19 conjugated with Dynabeads (Dynal, Norway) at a 1:4 (target/bead) ratio. After 30 min of incubation at 4°C and continuous shaking, the bead-bound cells were removed using a magnet (Dynal). The bead-bound cells were then washed three times to remove unbound cells and the CD4$^+$, CD8$^+$, or CD19$^+$ cells were detached from the beads using Detachbead (Dynal) according to the manufacturer's instructions. The purity of positively selected cells was determined by FACS analysis. To obtain activated T cells, nonadherent PBL were cultured in the presence of 60 IU/ml of IL-2 plus 1 μg/ml of phytohemagglutinin (PHA) (Sigma) or IL-2 plus anti-CD3 and anti-CD28 conjugated beads (anti-CD3/CD28 beads) for 3–5 days. These beads (provided by Drs. Bruce L. Levine and Carl June, University of Pennsylvania, Philadelphia) were used at a 1:3 target/bead ratio. After activation, treated T cells were maintained in human T-cell medium and IL-2 (60 IU/ml).

Gene transduction

Cells (2×10^5) mixed with viral supernatants in the presence of 8 μg/ml of polybrene in a 5-ml tube were centrifuged at 1800*g* for 3 hr 32°C. After overnight culture with viral supernatants, cells were washed and incubated in fresh culture medium. For examination of a long-term transgene expression in tumor-specific T cells, transduced cells were restimulated once every 10 days with autologous tumor cells expressing B7.1. For PBL, cells were cultured with the human T cell medium alone, with IL-2 (60 IU/ml), PHA plus IL-2, or anti-CD3/CD28 beads plus IL-2 for 3–5 days, and then cultured in the presence of IL-2 and stim-

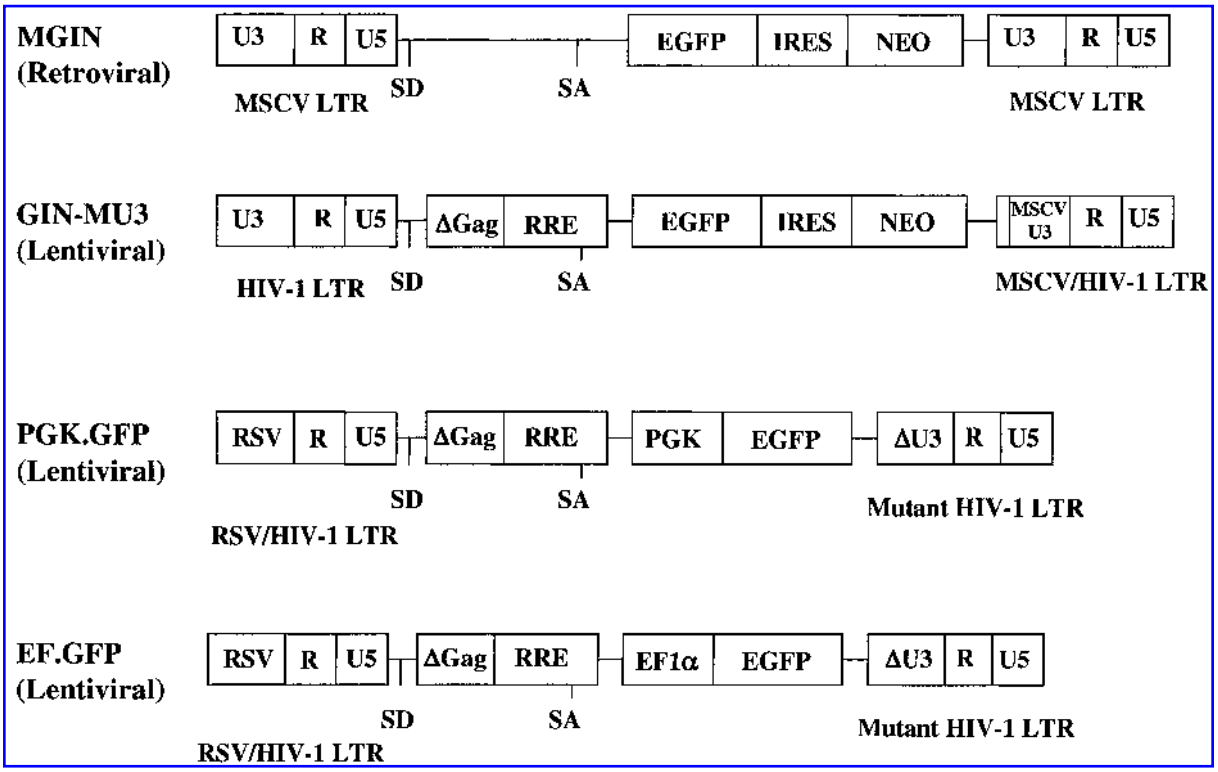

FIG. 1. Schematic representation of viral vectors used in this study. MGIN is a murine stem cell virus (MSCV)-derived retroviral vector in which the reporter gene GFP-IRES-Neo^R expression is regulated by MSCV promoter. GIN-MU3 is a human immunodeficiency virus type 1 (HIV-1)–based lentiviral vector and contains a chimeric MSCV and HIV-1 promoter that drives the reporter gene GFP-IRES-Neo^R expression. PGK.GFP and EF.GFP are HIV-1–based self-inactivating (SIN) vectors containing internal human PGK and EF1α promoters, respectively. SD, splice donor; SA, splice acceptor; ΔGag, deleted Gag region; ΔU3, deleted U3 region; RRE, Rev-responsive element.

TABLE 1. LENTIVIRAL VECTORS ARE MORE EFFICIENT THAN ONCORETROVIRAL VECTOR IN GENE TRANSFER INTO PROLIFERATING LYMPHOCYTES[a]

	MGIN	*GIN-MU3*	*PGK.GFP*	*EF.GFP*
Experiment 1 (MOI = 1)				
Jurkat T cells	20[b] (429)[c]	42 (484)	82 (523)	88 (2674)
Pancreatic CTL line	1 (52)	4 (210)	8 (80)	32 (48)
RCC CTL line	0 (1)	4 (153)	15 (80)	29 (60)
Experiment 2 (MOI = 18)				
Day 5 posttransduction				
Jurkat T cells	80 (651)	98 (2229)	99 (2846)	100 (5278)
RCC CTL line	1 (190)	38 (465)	54 (260)	58 (445)
Day 10 poststimulation				
Jurkat T cells	83 (739)	98 (2091)	100 (3171)	100 (7292)
RCC CTL line	2 (181)	45 (781)	63 (646)	53 (1057)

[a]Jurkat T cells and pancreatic cancer- and RCC-specific CTL lines (day 5 after antigen stimulation) were transduced with VSV-G pseudotyped oncoretroviral viruses (MGIN) or lentiviruses (GIN-MU3, PGK.GFP and EF.GFP). After overnight culture and viral removal, cells were processed as described in Materials and Methods and GFP expression was measured 5 days later. In experiment 2, transduced Jurkat T cells and RCC CTL line were further expanded and stimulated with antigen (for RCC CTL), and GFP expression was reexamined 10 days after stimulation.

[b]Percentage of GFP$^+$ cells.

[c]Mean fluorescence intensity (MFI).

MOI, multiplicity of infection; CTL, cytotoxic T cell lines; RCC, renal cell carcinoma; GFP, green fluorescent protein.

ulated with PHA or anti-CD3/CD28 beads periodically. Cell samples were harvested for flow cytometric analysis of GFP expression.

Flow cytometry

R-phycoerythrin (PE)-conjugated monoclonal antibodies (mAb) for human CD4, CD8, CD19, CD45RA, and CD45RO were purchased from BD PharMingen (San Diego, CA). PE-conjugated mouse immunoglobulin (Ig) G (an isotype control), anti-CD25, and anti-CD69 were purchased from Caltag Laboratories (Burlingame, CA). Stained cells or GFP expression was analyzed on a FACScan using the CellQuest software (Becton Dickinson, Franklin Lakes, NJ).

Cytokine release by activated T cells

Tumor necrosis factor (TNF)-α assay was performed by co-culture of 5×10^4 T cells with 2.5×10^4 target cells per well in 96-well flat-bottom plates in a final volume of 0.2 ml of T cell medium. After 18–24 hr, supernatants were collected for measuring TNF-α production by ELISA kit (R&D Systems, Minneapolis, MN).

Cytotoxicity assay

Cytoxicity assay was performed using a standard 4-hr chromium (^{51}Cr)-release assay. Synthetic peptides were diluted in 96-well plates with RPMI-1640 from 1×10^{-5} *M* to 1×10^{-12} *M*. Fifty microliters of diluted peptides were incubated with 50 μl (5×10^3) target cells per well for 30–60 min prior to addition of effector T cells.

RESULTS

Lentiviral vectors are more efficient than oncoretroviral vector in transducing proliferating T cells

To achieve a fair comparison between oncoretroviral and lentiviral vectors in gene transfer into proliferating tumor-specific T cells, we used a MSCV-based oncoretroviral vector MGIN and a hybrid MSCV/HIV-1 lentiviral vector GIN-MU3 using the same MSCV LTR promoter (Gao *et al.*, 2001). In addition, improved self-inactivating (SIN) lentiviral vectors containing either the human PGK or EF1α promoter were also used (Dull *et al.*, 1998; Zufferey *et al.*, 1998; Cui *et al.*, 2002) (Fig. 1). All the vectors were pseudotyped with the VSV-G envelope and produced by transient transfection of 293T cells. For lentiviral vectors, we used a packaging construct pCMVΔR8.91 lacking all the HIV-1 accessory genes (*vif, vpr, vpu,* and *nef*). RCC- and pancreatic cancer-specific CD8$^+$ T cells were transduced with the oncoretroviral MGIN or the lentiviral (GIN-MU3, PGK.GFP, or EF.GFP) vectors. At the same low viral concentration (multiplicity of infection [MOI] =1, shown in Table 1, experiment 1), the EF.GFP vector mediated the best transduction of both RCC- and pancreatic cancer-specific T cells as well as human Jurkat T cell line, followed by the PGK.GFP vector. The oncoretroviral MGIN vector was the poorest. Next, we repeated the experiment with a higher viral concentration measured as MOI. Viral supernatants derived from the oncoretroviral MGIN and the lentiviral GIN-MU3 vectors (their titers were lower, typically $1–5 \times 10^5$ TU/ml) were concentrated, and a higher viral concentration (MOI =18) was used for all the four vectors to transduce RCC-specific CTL and Jurkat T cells (Table 1, experiment 2). Although all the vectors transduced Jurkat cells efficiently (80–100%), the oncoretroviral MGIN vector transduced RCC-specific CTL poorly. Monitored at days 5 and 10 posttransduction, the three lentiviral vectors (GIN-MU3, PGK.GFP, and EF.GFP) were superior to the MGIN oncoretroviral vector (38–63% vs. 1–2%). The MGIN vector pseudotyped with the GALV envelope from a PG13-based producer line gave similar results as those pseudotyped with VSV-

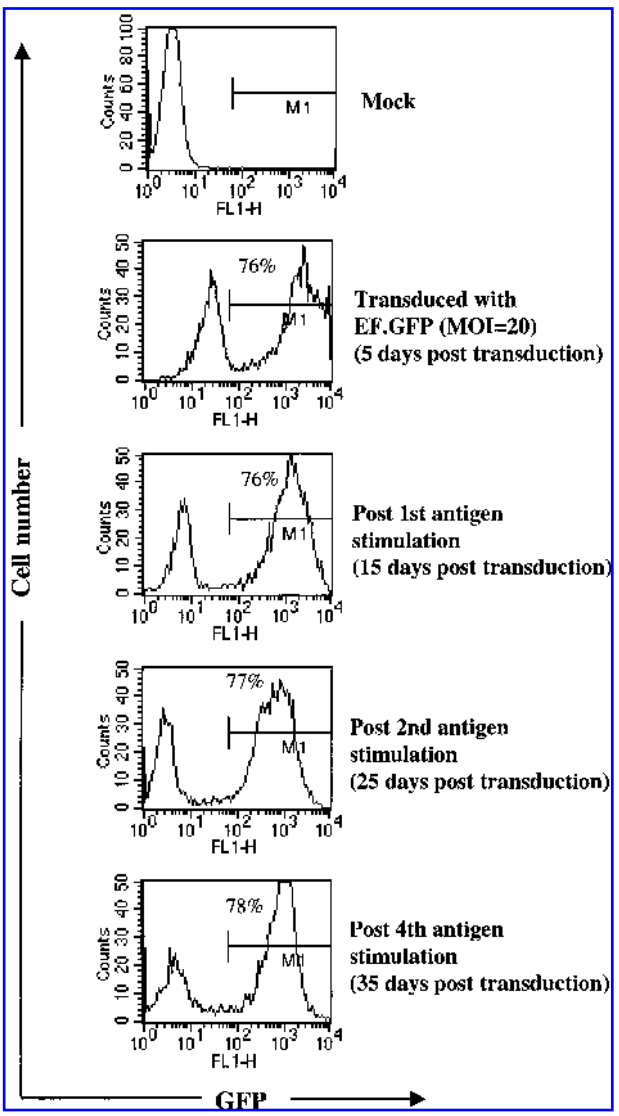

FIG. 2. Kinetics of transgene expression in lentiviral transduced renal cell carcinoma (RCC)-specific T cells posttransduction. RCC-specific cytotoxic T cell (CTL) line was transduced with EF.GFP (multiplicity of infection [MOI] = 20) overnight, and maintained in culture by restimulation with tumor cells once every 10 days. Green fluorescent protein (GFP) expression was determined by fluorescence-activated cell sorter (FACS) analysis.

G (data not shown). Therefore, we conclude that lentiviral vectors are much more efficient to transduce the proliferating CTL lines. However, we should point out that our T cell transduction conditions by retroviral vector in this study may not be optimal because it has been reported that use of either fibronectin (Dardalhon *et al.*, 1999) or phosphate depletion plus low-temperature incubation (Bunnell *et al.*, 1995) can significantly enhance retroviral-mediated lymphocyte gene transfer. Because viral titers of PGK.GFP and EF.GFP vectors that we obtained were 2- to 10-fold higher than those of GIN-MU3 lentiviral vectors, we used PGK.GFP and EF.GFP vectors in the subsequent experiments.

Kinetics of transgene expression in cultured tumor-specific T cells

To assess sustained transgene expression in transduced tumor-specific T cells, RCC-specific $CD8^+$ T cells were transduced with the lentiviral EF.GFP vector (MOI = 20). Transduced cells were maintained in culture for 35 days by serial restimulation with antigens from an autologous tumor cell line once every 10 days. No difference in T-cell expansion between transduced and untransduced T cells was observed (data not shown). Five days after overnight transduction, all the transduced cells appeared GFP^+, but in two distinct populations displaying different levels of GFP expression. After one round of antigen stimulation and 10-day cell proliferation, the GFP^{low} population (25% of the total cells) disappeared and a GFP^- population (25% of the total cells) emerged. The GFP^{low} cells population observed at earlier time points post transduction is likely because of transgene expression from nonintegrated lentiviral vectors or some form of protein carry-over or both as observed previously with other cell types (Liu *et al.*, 1996; Gallardo *et al.*, 1997; Haas *et al.*, 2000). Importantly, the majority of cells (approximately 75%) expressing the high-level GFP remained. The high level of transgene expression was stable even after 4 rounds of antigen stimulation (Fig. 2). Therefore, the high level of stable gene transfer into CTL by lentiviral vectors is feasible, and stable transgene expression can be achieved.

Unaltered antigen specificity of transduced tumor-specific T cells

One important aspect in genetic modification of T cells is to preserve their antigen specificity after manipulations. Thus, we examined functional parameters of transduced RCC-specific T cells such as specific cytokine (TNF-α) release on antigen stimulation, compared to their mock-transduced counterparts. As shown in Figure 3, transduced (76% GFP^+) 1.24 CTL recognized autologous RCC24 tumor cells as well as $HLA\text{-}A2^+$ allogeneic RCC lines such as RCC-Fr and RCC-Sa (to a less degree) but not $HLA\text{-}A2^-$ RCC-Lo and released TNF-α quantitatively similar to mock-transduced 1.24 CTL. Control cell lines such as K562 (a human natural killer cell-sensitive target cell line), EBV-transformed autologous B cells EBV24, and allogeneic EBV26 did not stimulate either transduced or mock-transduced T cells to release TNF-α (Fig. 3).

Next, we examined the effect of transgene expression in an RCC-specific CTL clone on their functionality. A 5B CTL clone derived from RCC-specific 1.24 CTL line was transduced with EF.GFP lentiviral supernatant. The GFP^+ and GFP^- 5B CTL

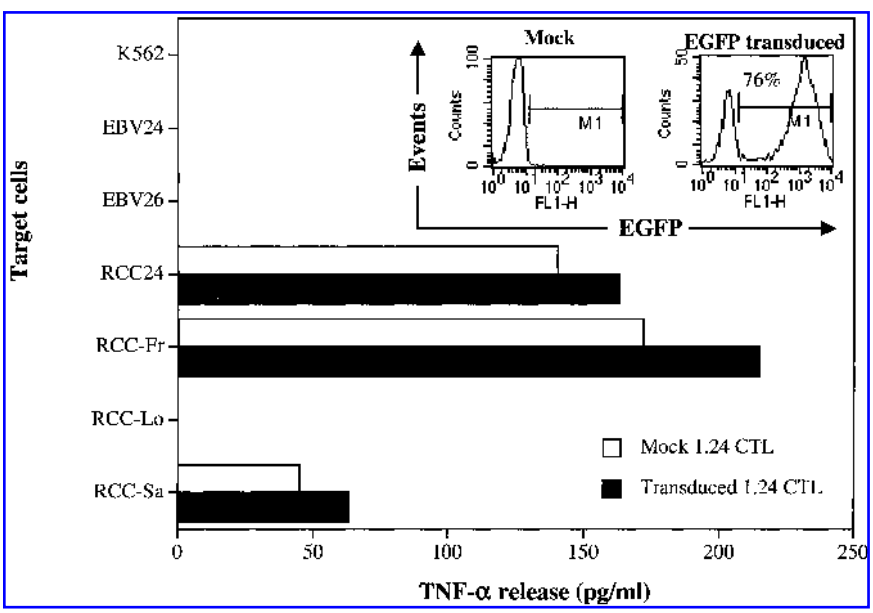

FIG. 3. Tumor necrosis factor (TNF)-α release by lentiviral transduced renal cell carcinoma (RCC)-specific cytotoxic T cell (CTL) line. RCC-specific CTL line was transduced as shown in Figure 2. Before the TNF-α release assay, transduced cells were reexamined for green fluorescent protein (GFP) expression by fluorescence-activated cell sorter (FACS) and results were shown in the insert. Transduced and mock transduced 1.24 RCC-specific CTL were mixed with different types of target cells including autologous RCC24 line ($HLA\text{-}A*0201^+$) derived from the same patient. TNF-α release by antigen activated CTL was measured by enzyme-linked immunosorbent assay (ELISA). Other target cells: K562, human NK sensitive target cells; EBV24 ($HLA\text{-}A*0201^+$) and EBV26 ($HLA\text{-}*0205^+$), autologous and allogeneic Epstein-Barr virus (EBV)-transformed B cells; RCC-Fr ($HLA\text{-}A*0201^+$), RCC-Lo ($HLA\text{-}A*0201^-$), and RCC-Sa ($HLA\text{-}A*0201^+$), allogeneic RCC lines.

from the same transduced populations were sorted out and expanded separately in culture. The purity (nearly 100%) of FACS sorted GFP^+ and GFP^- 5B CTL populations was confirmed after cell expansion (data not shown). The GFP^+ 5B CTL specifically lysed RCC tumor cells and responded to the peptide antigen QTACEVLDY (Zhou *et al.*, manuscript in preparation) as efficiently as the GFP^- 5B CTL, whereas both GFP^+ and GFP^- 5B CTL did not recognize the wild-type peptide QTTCEVLDY and the melanoma peptide RSDQQQARY (Fig. 4A and 4B). Therefore, we concluded that no adverse effects as a result of the high level of gene transfer and expression mediated by lentiviral vectors were observed, based on cell growth (data not shown), cytokine release assays (Fig. 3), and antigen-specific cytotoxic T-cell activity (Fig. 4A and 4B).

Gene transfer into unstimulated PBL by lentiviral vectors: HIV-1 APs are not required

We next examined if freshly isolated or cyropreserved PBL can be transduced by the SIN lentiviral vectors in the absence of prior T cell stimulation. Two different SIN vectors (EF.GFP and PGK.GFP) were produced either in the absence of any HIV-1 APs using the pCMVΔR8.91 packaging plasmid as before, or in the presence of all the four APs (Vif, Vpu, Vpr, and Nef) using the pCMVΔR8.2 plasmid. The viral titer from EF.GFP packaged with pCMVΔR8.2 (designated EF.GFP/8.2) was similar to that packaged with pCMVΔR8.91 (designated EF.GFP/8.91, data not shown). Similar titers were obtained among the PGK.GFP/8.91 and PGK.GFP/8.2 viruses (data not shown). As we did with proliferating CTL, unstimulated PBL were transduced with both types of EF.GFP and PGK.GFP vectors at the same MOI of 5 by centrifugal infection (spinoculation) for 3 hr followed by overnight incubation without added stimuli. After removal of viral supernatants (containing 10% FBS), cells were washed and further cultured with the T-cell medium in the presence of IL-2 plus PHA for 5 days. We found that 12–24% of PBL expressed GFP (Fig. 5A). Both EF.GFP and PGK.GFP vectors without APs transduced unstimulated PBL. These results were contrary to a recent report that the transduction of unstimulated PBL failed by a lentiviral vector packaged in the absence

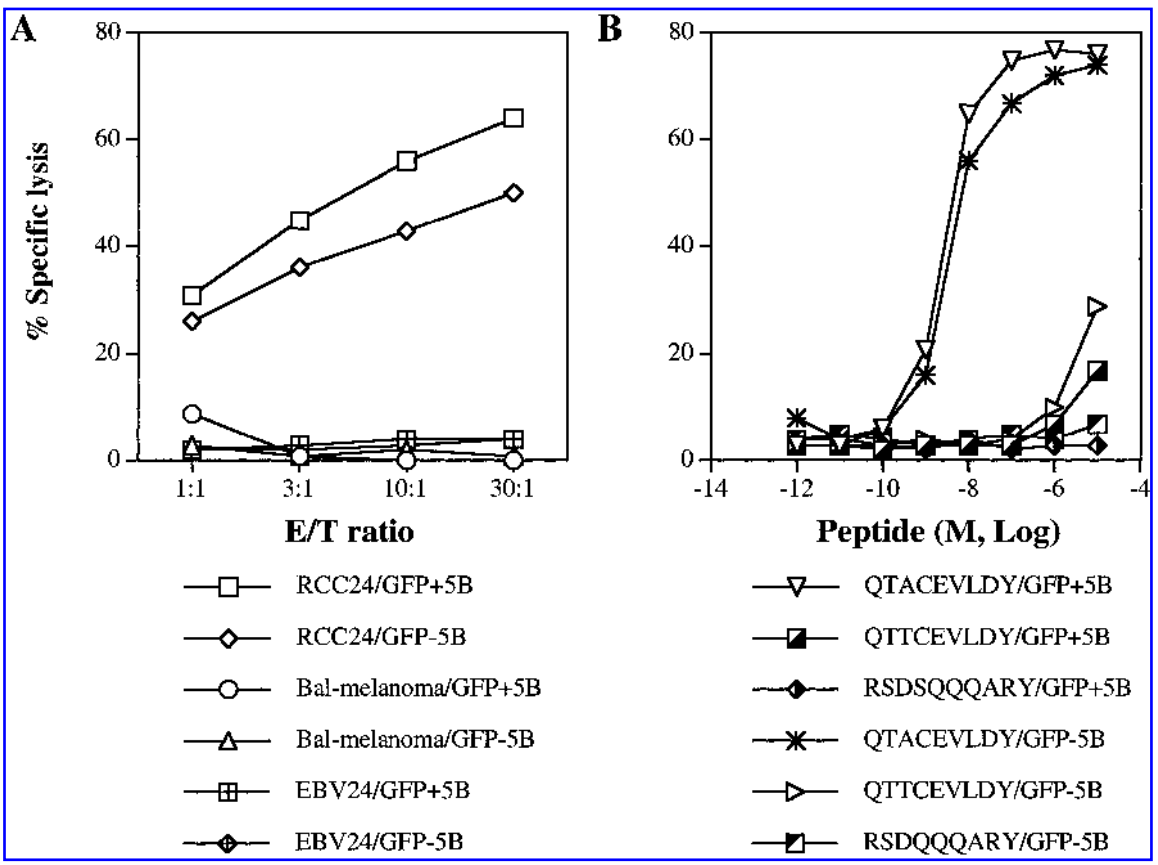

FIG. 4. Cytotoxicity by the lentiviral transduced renal cell carcinoma (RCC)-specific cytotoxic T cell (CTL) clone. **A**: The RCC-specific CTL clone 5B was transduced with EF.GFP (multiplicity of infection [MOI] =10) and GFP^+ and GFP^- 5B CTL from the same transduced populations were sorted out and expanded *in vitro*. A standard chromium (^{51}Cr) release assay was performed to test their antigen specificity of GFP^+ and GFP^- 5B CTL clone against autologous RCC24 tumor cells, allogeneic melanoma cells (HLA-A*0101$^+$) and autologous Epstein-Barr virus (EBV)-transformed B cells EBV24 since 5B CTL clone has been demonstrated to only recognize autologous tumor cells in a HLA-A*0101 restricted fashion (Zhou *et al.*, manuscript in preparation). **B**: Cytoxicity of GFP^+ and GFP^- 5B CTL against the cognate peptide QTACEVLDY and wild-type peptide QTTCEVLDY, and an irrelevant melanoma peptide RSDQQQARY (Harada *et al.*, 2001) loaded on EBV24 cells at effector:target ratio of 50:1.

of APs, under a different transduction protocol (Chinnasamy *et al.*, 2000).

To make a direct comparison, we next performed the transduction of unstimulated PBL under the condition described by the report (Chinnasamy *et al.*, 2000). Unstimulated PBL were transduced in fibronectin-coated plates. After 30 min of centrifugation, transduction was allowed to proceed for additional 48 hr as described (Chinnasamy *et al.*, 2000). Then transduced cells were stimulated with PHA plus IL-2 for 5 days before being analyzed by FACS (Fig. 5B). We did not observe the requirement of APs for the transduction of unstimulated PBL by either PGK.GFP or EF.GFP vector, although the overall transduction efficiencies were lower for both types of vectors (with or without APs) under this transduction protocol. Our data are consistent with other reports that fibronectin had little effect on transduction by VSV-G–pseudotyped lentiviral vectors, although it enhanced other types of retroviral vectors, and that spinoculation can enhance VSV-G–pseudotyped lentiviral transduction of diverse cell types including unstimulated PBL (Costello *et al.*, 2000; Haas *et al.*, 2000). Thus, we used this simple and more

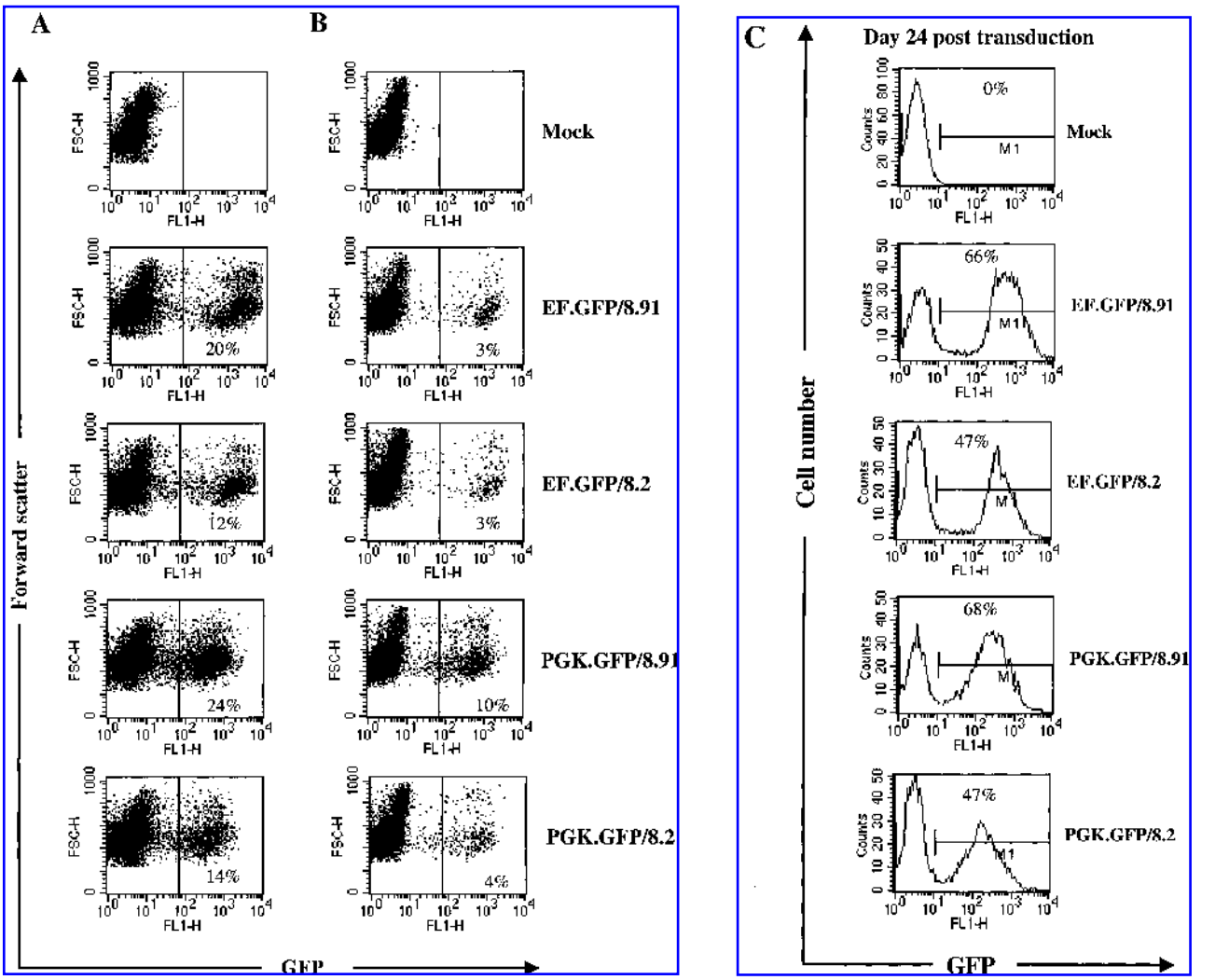

FIG. 5. Direct comparison of gene transfer into resting peripheral blood lymphocytes (PBL) by lentiviral vectors with or without human immunodeficiency virus (HIV) accessory proteins (APs). Thawed PBL were transduced with EF.GFP or PGK.GFP lentiviruses (multiplicity of infection [MOI] = 5) produced in the absence (EF.GFP/8.91 and PGK.GFP/8.91) or presence (EF.GFP/8.2 and PGK.GFP/8.2) of all HIV APs. Transduction was preformed in parallel with two different protocols. **A**: Unstimulated PBL were mixed with lentiviruses, spun for 3 hr, and cultured overnight. After removal of viruses, cells were washed and then cultured with the T-cell medium containing phytohemagglutinin (PHA; 1 μg/ml) and interleukin (IL)-2. Gene transfer efficiency was determined by fluorescence-activated cell sorter (FACS) analysis after 5 days. **B**: Gene transduction after a previously published protocol (Chinnasamy *et al.*, 2000). Unstimulated PBL were plated onto fibronectin-coated plates, spun for 30 min, and then allowed to proceed for 48 hr as described (Chinnasamy *et al.*, 2000). Transduced cells were then washed and cultured in the same medium containing PHA (5 μg/ml) and IL-2. Five days later, transduced cells were analyzed as in (A). **C**: Unstimulated PBL were transduced overnight and then cultured with T-cell stimuli anti-CD3/CD28 beads and IL-2 for 5 days. Transduced cells were maintained in culture in the presence of IL-2 and analyzed by FACS 24 days posttransduction. Data shown are representative of three independent experiments.

efficient protocol (3-hr spinoculation followed by overnight incubation) for the subsequent experiments.

To confirm our observation further that HIV APs are not essential for lentiviral-mediated gene transfer into unstimulated PBL, we examined GFP expression 24 days posttransduction by EF.GFP and PGK.GFP with or without APs. Unstimulated PBL were transduced overnight and then activated with a TCR activator (the anti-CD3/CD28 beads) for 5 days. After removing the beads, transduced and activated PBL were maintained with IL-2 for 24 days. Vectors EF.GFP/8.91 and PGK.GFP/8.91 (without APs) could mediate stable gene transfer into unstimulated PBL as efficiently as EF.GFP/8.2 and PGK.GFP/8.2 (with all APs), as indicated by stable transgene expression (Fig. 5C).

We next evaluated the importance of T-cell stimulation subsequent to the transduction of unstimulated PBL on gene transfer in the absence of APs. After overnight transduction by either EF.GFP or PGK.GFP vector, transduced PBL were cultured in the T cell medium in the presence or absence of any T-cell stimuli for 5 days. We observed that fractions of cells expressed low levels of GFP even in the absence of any stimulation (Fig. 6, top row). Addition of IL-2 alone posttransduction did not enhance transduction efficiency by either vector (second row). However, addition of T-cell activation stimuli such as PHA significantly elevated transgene expression level. In addition, subsequent stimulation by the TCR activator (anti-CD3/CD28 beads) also led to the elevation of transgene expression. Thus, lentiviral vectors used in this study without all the HIV APs mediated efficient gene transfer into freshly isolated or cyropreserved PBL without prior stimulation. High levels of transgene expression were observed when transduced PBL were subsequently stimulated.

Sustained transgene expression post gene transduction

A critical issue for the genetic modification of T cells is the duration of transgene expression. Thus, maintenance of GFP gene

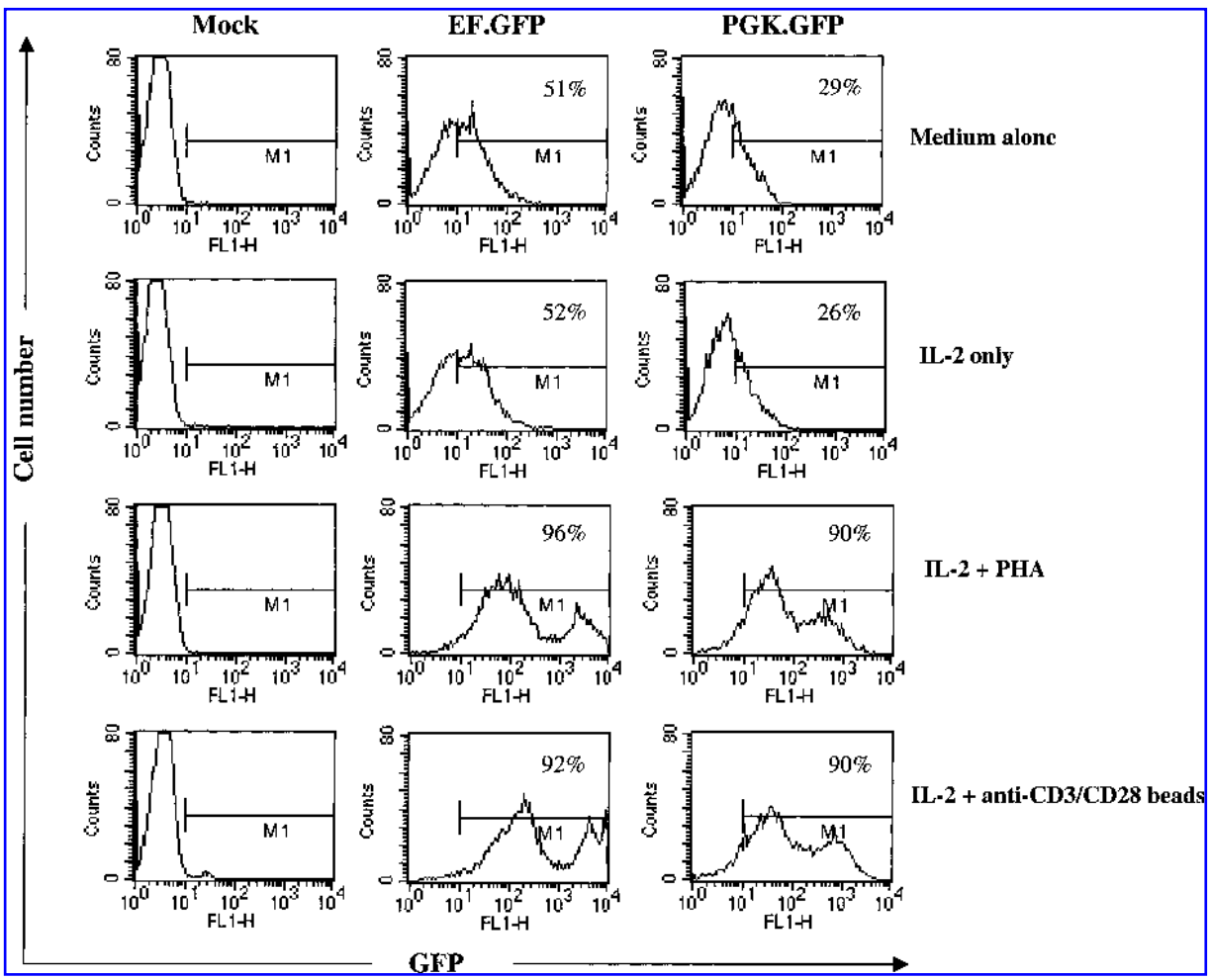

FIG. 6. Gene transfer and expression in unstimulated peripheral blood lymphocytes (PBL) by lentiviral vectors. Thawed PBL were transduced with EF.GFP or PGK.GFP (multiplicity of infection [MOI] = 15). After overnight transduction, cells were washed to remove viruses and cultured in 24-well plates with human T-cell medium for 5 days. Either interleukin (IL)-2 only or phytohemagglutinin (PHA) plus IL-2 or anti-CD3/CD28 beads plus IL-2 were also added. After removing PHA or anti-CD3/CD28 beads and IL-2, cells were analyzed for green fluorescent protein (GFP) expression by fluorescence-activated cell sorter (FACS). Data shown are representative of similar results obtained from two independent experiments.

expression in transduced resting PBL was assessed by passing the stably transduced PBL for an extended period of time and periodically analyzing GFP fluorescence (Fig. 5C and Table 2). As before, unstimulated PBL were transduced overnight by either EF.GFP or PGK.GFP vector, and then activated with PHA or anti-CD3/CD28 beads for 5 days. After removing PHA and anti-CD3/28 beads, cells were maintained in culture with IL-2 and restimulated when necessary. As shown in Table 2, there was no significant decrease in numbers of GFP^+ cells in transduced PBL by both EF.GFP and PGK.GFP in the absence of APs within 34 days monitored. In addition, stimulation by anti-CD3/CD28 beads subsequent to transduction resulted in higher percentages of GFP^+ cells and often stronger GFP transgene signals. Taken together, these results indicate that the lentiviral EF.GFP and PGK.GFP vectors can introduce transgene stably into unstimulated PBL, resulting in persistent transgene expression after multiple rounds of T cell activation.

Subsequent T-cell stimulation significantly affects transgene expression

To distinguish whether the posttransduction stimulation was only acting on gene transfer ultimately leading to stable integration or also acting on transgene expression post-gene transfer, we performed experiments shown in Figure 7. After overnight transduction of unstimulated PBL with two types of vectors (MOI = 12), transduced cells were continuously cultured in the T-cell basal medium for 5 more days. Then aliquots of cells were either analyzed by FACS at day 6 or stimulated with IL-2 and anti-CD3/CD28 beads for 5 additional days. Transgene expression was reexamined poststimulation (day 13 posttransduction). GFP expression at day 13 was higher after stimulation as compared to that at day 6 after being maintained in the basal T cell medium. If the stimulation was applied immediately after transduction (at day 1, instead of day 6), levels of transgene expression were ~2 fold higher than those observed at day 13 (data not shown). This result indicated that a substantial fraction of transduced resting PBL contained transgene after overnight lentiviral transduction. Low levels of the transgene were expressed under the unstimulated condition. In addition to enhancing gene transfer presumably by facilitating vector integration into host chromosomes, the subsequent T-cell stimulation had profound effects on stimulating transgene expression from either the EF1α or PGK promoter. We have observed that the intensity of transgene expression mediated by the EF1α promoter was always 2- to 4-fold higher than by the PGK promoter either in unstimulated or stimulated PBL (Figs. 5–7). The latter observation was in support of recent reports that EF1α promoter-containing lentiviral vectors gave improved transgene expression in various human cells including PHA-activated human primary lymphocytes (Ramezani *et al.*, 2000; Salmon *et al.*, 2000; Cui *et al.*, 2002). Without T-cell stimulation, however, transduced PBL can be maintained only without cell proliferation; low levels of transgene expression (lasting at least for several days) were achieved with lentiviral vectors using either EF.GFP or PGK.GFP promoter. The transgene expression regulated even by these housekeeping gene promoters was influenced greatly by T-cell activation status.

The second approach to prove gene transfer into unstimulated PBL by lentiviral vectors is to demonstrate the majority of GFP^+ transduced cells retained their resting phenotypes. FACS analysis was used to assess T-cell activation markers such as CD25 and CD69 after transduction of unstimulated PBL. The results shown in Figure 8 indicate that both EF.GFP- and PGK.GFP-transduced resting lymphocytes were negative for the expression of CD25 and CD69 activation markers. The results suggest that either viral supernatants (containing 10% FBS) or transduction procedure itself did not significantly induce cellular activation. Transduced PBL expressing GFP without T-cell stimulation remained their unstimulated phenotypes.

TABLE 2. SUSTAINED TRANSGENE EXPRESSION IN PRIMARY T CELLS POSTTRANSDUCTION OF UNSTIMULATED PBL[a]

	% of GFP^+ PBL after culture (MFI)		
	10 days	*23 days*	*34 days*
EF.GFP			
PHA	32[b] (1729)[c]	30 (1008)	32 (1665)
anti-CD3/CD28 beads	60 (2237)	49 (1064)	58 (3007)
PGK.GFP			
PHA	27 (644)	26 (450)	24 (654)
anti-CD3/CD28 beads	49 (772)	33 (491)	52 (828)

[a]Thawed PBL without any prestimulation were transduced with EF.GFP or PGK.GFP without HIV APs (MOI = 15). Following virus removal, PHA or anti-CD3/CD28 beads were added to culture in the presence of IL-2 for 5 days. After removing PHA or anti-CD3/CD28 beads, cells were cultured with IL-2, and GFP expression was measured on day 10 and day 23 posttransduction. Cells were restimulated on day 24 with PHA or anti-CD3/CD28 beads for 5 days and GFP expression was reexamined on day 34.

[b]Percentage of GFP^+ cells.

[c]Mean fluorescence intensity (MFI).

PBL, peripheral blood lymphocytes; GFP, green fluorescent protein; HIV, human immunodeficiency virus; APs, accessory proteins; MOI, multiplicity of infection; PHA, phytohemagglutinin; IL-2, interleukin-2.

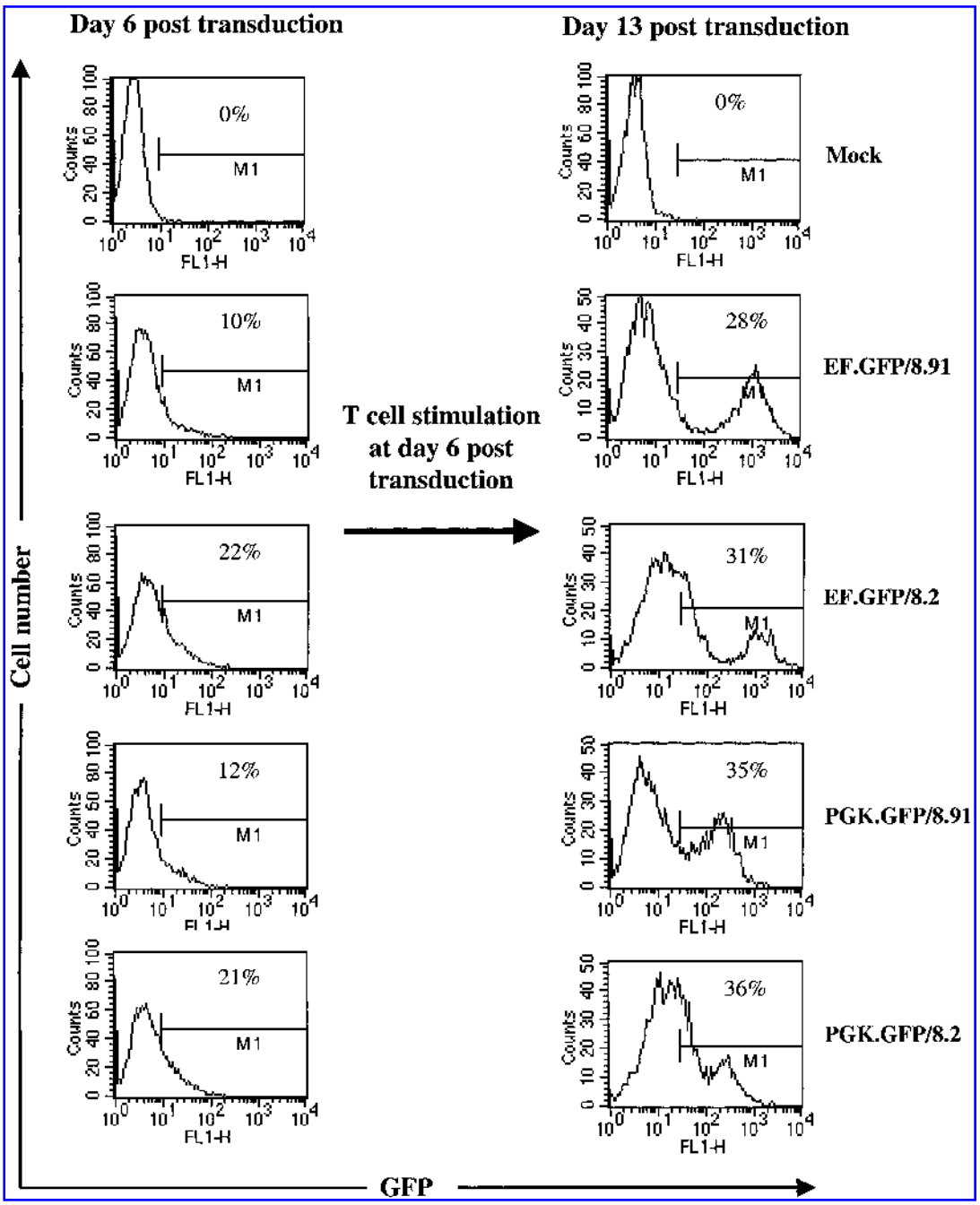

FIG. 7. Effects of T-cell activation posttransduction on gene transfer and transgene expression. Unstimulated peripheral blood lymphocytes (PBL) were transduced with EF.GFP or PGK.GFP lentiviruses (multiplicity of infection [MOI] = 12) as described in Figure 5A. After removing viral supernatants, cells were cultured with the T-cell medium alone for 6 days without any added cytokines or T-cell stimuli. Green fluorescent protein (GFP) expression was measured on day 6 posttransduction. Then, transduced PBL were activated by anti-CD3/CD28 beads plus interleukin (IL)-2 for 5 days. GFP expression was reexamined at day 13 posttransduction.

Transduction of isolated resting lymphocyte subsets

We next assessed the ability of the EF.GFP vector to transduce isolated subsets of resting lymphocytes. Purified resting $CD4^+$ and $CD8^+$ T cells from PBL were exposed to EF.GFP viruses without HIV APs. Five days posttransduction, gene transfer efficiency in unstimulated lymphocyte subsets was determined by analyzing GFP^+ cells in $CD4^+$, $CD8^+$, $CD45RA^+$ or $CD45RO^+$ cell populations. The results demonstrated that nearly 50% of purified both $CD4^+$ and $CD8^+$ T cell subsets expressed GFP (Fig. 9A and 9B). Transduction efficiency (11%) in $CD19^+$ resting B cells was less compared to those in

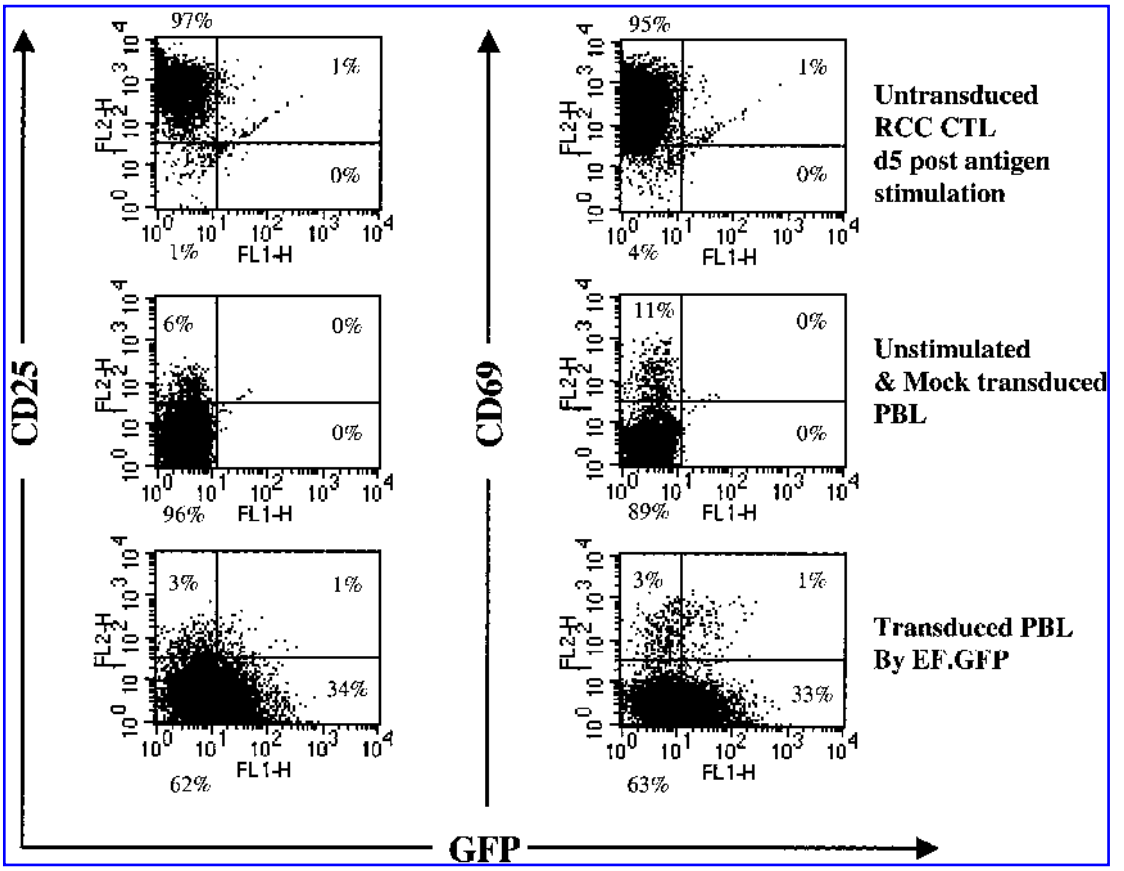

FIG. 8. Lentiviral-mediated transduction of resting lymphocytes preserves naïve phenotypes. Unstimulated peripheral blood lymphocytes (PBL) were transduced by EF.GFP viruses without lentiviral accessory proteins (APs; multiplicity of infection [MOI] = 30) as described in Figure 5A. Transduced or untransduced cells were maintained in the T-cell medium alone without any added cytokines or T-cell stimuli for 5 days. Transduced PBL were analyzed for the presence of GFP^+ transduced cells expressing or lacking CD25 and CD69 activation markers. Proliferating renal cell carcinoma (RCC)-specific cytotoxic T cell lines (CTL) activated by antigen for 5 days were used as a positive control (top row). Note that majority of transduced (GFP^+) cells lacked the expression of CD25 or CD69 activation markers. Data shown are representative of two independent experiments.

$CD4^+$ and $CD8^+$ T cells (data not shown). Importantly, $CD45RA^+$ (naïve) and $CD45RO^+$ (memory) resting $CD4^+$ and $CD8^+$ T cells were both efficiently transduced (Fig. 9A and 9B). These results further support our previous conclusion that the lentiviral vectors can transduce unstimulated T cells.

DISCUSSION

In this study, we used lentiviral and oncoretroviral vectors to transduce two types of lymphocytes: established cultured CTL lines and unstimulated primary PBL. It is not surprising that with improved lentiviral vectors we can transduce unstimulated PBL, which are in the G_0/G_1 phase of the cell cycle and are refractory to oncoretroviral vectors without prior stimulation. However, we observed that lentiviral vectors were more efficient at transducing proliferating antigen-specific CTL than oncoretroviral vectors at the same MOI. With an MOI of 10–20, we can readily transduce all or the majority of CTL with one round of transduction by lentiviral vectors, but poorly (= 2%) by the oncoretroviral vector. The much higher efficiencies by lentiviral vectors to transduce some proliferating cells that are still refractory to oncoretroviral vectors have been reported previously with other human cell lines (Mascarenhas *et al.*, 1998). For example, the human REH B cell leukemic line can be transduced nearly 100% by lentiviral vectors but was completely refractory to multiple types of oncoretroviral vectors (Mascarenhas *et al.*, 1998). It is unclear currently why lentiviral vectors are more efficient to transduce these oncoretroviral-refractory proliferating cells but the cell cycle status or the level of virus surface receptor of host cells was not the rate-limiting step. Possible mechanisms include differences in reverse transcription and integration steps. The much higher stability of the preintegrated lentiviral complex may be a key factor (Amado and Chen, 1999).

The second important aspect of this study is our ability to efficiently transduce unstimulated PBL in the absence of lentiviral APs. The presence of the four HIV-1 APs imposes a safety concern (even for SIN lentiviral vectors that we used) and may also complicate biological functions of target cells. Although recent data clearly indicated the HIV APs are unnecessary for many types of cells including hematopoietic stem cells (Miyoshi *et al.*, 1999; Gao *et al.*, 2001; Cui *et al.*, 2002), it remains con-

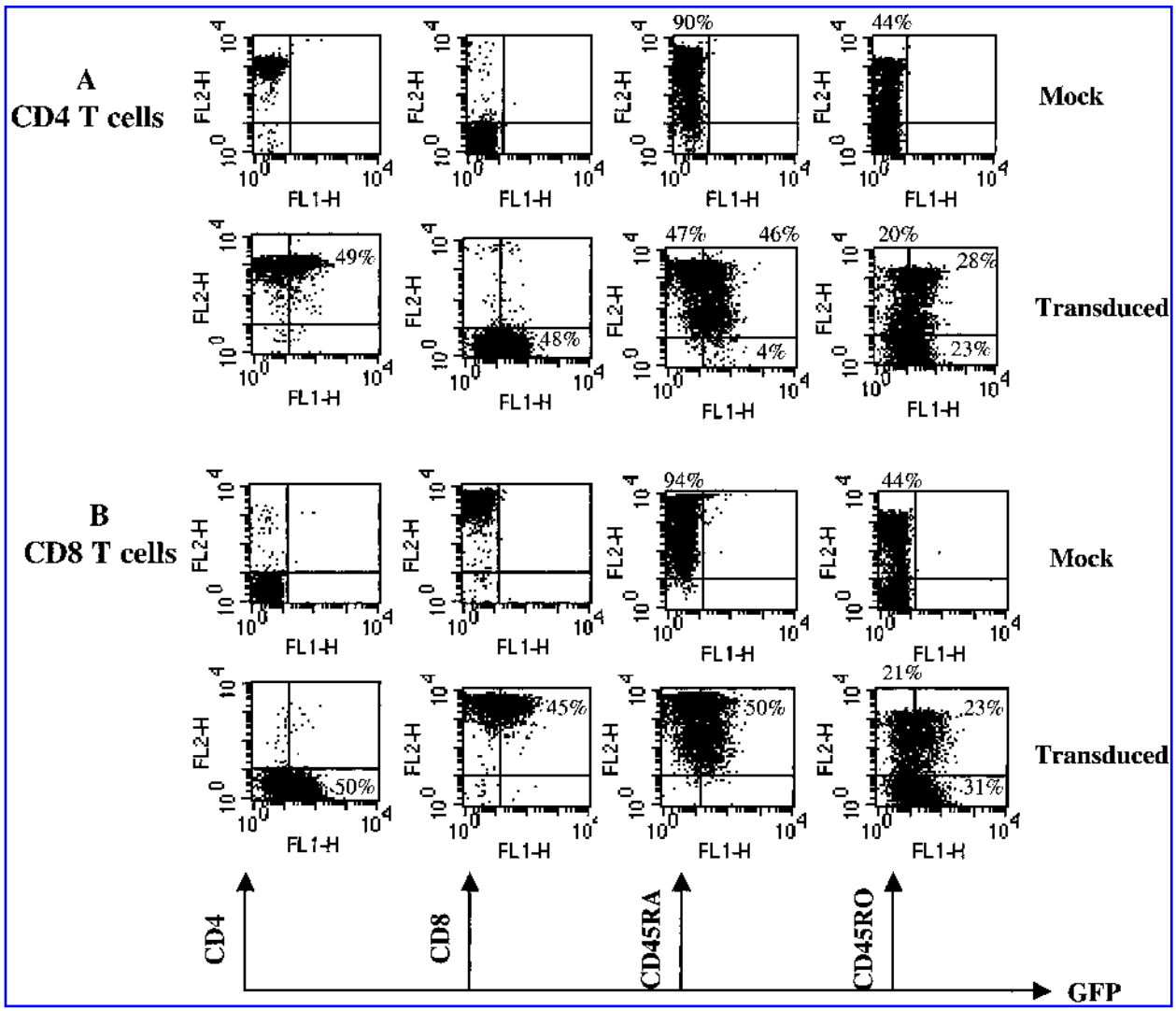

FIG. 9. Efficient gene transfer into purified lymphocyte subsets by lentiviral vectors. Purified $CD4^+$ and $CD8^+$ T cells were transduced with EF.GFP (multiplicity of infection [MOI] = 30) as described in Figure 5A for total unstimulated peripheral blood lymphocytes (PBL). After 5-day culture with medium without any stimulation, expression of green fluorescent protein (GFP) and various surface markers was analyzed by fluorescence-activated cell sorter (FACS). CD45RA, a marker for human naïve lymphocytes; CD45RO, a marker for human memory lymphocytes. Data shown are representative of two independent experiments.

troversial whether these APs are required for transducing unstimulated or resting lymphocytes. In support of the study by Costello and colleagues (Costello *et al.*, 2000), our ability to transduce these cells efficiently indicated that the HIV APs are unnecessary for improved lentiviral vectors such as those we used to transduce unstimulated PBL. It is unclear at present, however, what factors contribute to the apparent discrepancy between Costello and colleagues (Costello *et al.*, 2000) and this study versus Chinnasamy and colleagues (Chinnasamy *et al.*, 2000). These factors include the difference in the lentiviral vectors and transduction protocols that we and Costello and colleagues used. For example, we found a simple overnight transduction protocol (including 3-hr spinoculation) gave a higher transduction efficiency than that using fibronectin and a 48-hr transduction as used by Chinnasamy and colleagues (Chinnasamy *et al.*, 2000).

Several lines of evidence confirm that we have transduced resting or naïve lymphocytes using our lentiviral vectors in the absence of APs and cell stimulation. First, we did not stimulate PBL by T-cell stimuli such as PHA or anti-CD3/CD28 beads either prior to or during viral transduction. Transgene was observed in these transduced cells that were maintained under survival conditions (i.e., IL-2 only) as long as 6 days before a proliferative signal (PHA or a TCR activator) was provided (Fig. 7). Although T-cell activation is required for propagating transduced T cells for extended periods of time and for high-level transgene expression, it is not a prerequisite for gene transfer. Second, we observed GFP^+ cells after transduction of unstimulated PBL, which were lacking activation marker (CD25 and CD69) expression (Fig. 8). Taken together, our data and others (Costello *et al.*, 2000) clearly indicated that APs are unnecessary to transduce resting as well as proliferating lymphocytes, albeit our inability to resolve the controversy with a previous report (Chinnasamy *et al.*, 2000).

The third important aspect of this report is the elucidation of the impact of T-cell activation on vector-mediated transgene expression as well as on gene transfer. It has been well documented that in T cells, the function of viral LTR promoters (used in the majority of oncoretroviral vectors) is greatly influenced by the T-cell activation status (Plavec *et al.*, 1997; Auten *et al.*, 1999; Parkman *et al.*, 2000). In the past several years, it has been recognized that either MLV/MSCV or HIV-1 LTR is essentially inactive as a promoter in resting T cells (Tong-Starksen *et al.*, 1990; Plavec *et al.*, 1997; Auten *et al.*, 1999; Parkman *et al.*, 2000). Bearing in mind these results, we have purposely included in this study SIN lentiviral vectors in which a housekeeping gene promoter is used to regulate transgene expression. We used two different lentiviral vectors in which transgene expression is regulated by either the EF1α or PGK promoter. Although the EF1α promoter is consistently 2- to 4-fold stronger than the PGK promoter, we obtained the same results with either vector: T-cell activation profoundly activated transgene expression by either promoter after transduction. Nonetheless, low levels of transgene expression can be achieved with the SIN lentiviral vector containing the EF1α promoter in the absence of any T-cell stimulation. Therefore, for the first time we can transduce and express a transgene in resting T lymphocytes without altering T-cell activation status. Recently, it was reported that a *cis* element, which is present in the wild-type of HIV-1 genome but absent in the lentiviral vectors that we used, can further enhance gene transfer and/or expression in a variety of human cells including proliferating and minimally stimulated T cells (Follenzi *et al.*, 2000; Zennou *et al.*, 2000; Dardalhon *et al.*, 2001). The inclusion of this 99-bp element (often called the central polypurine tract or cPPT) enhanced both levels (percentages) and intensities of transgene expression in transduced cells (Follenzi *et al.*, 2000; Zennou *et al.*, 2000; Dardalhon *et al.*, 2001). By adding the cPPT to the vectors we used, however, we may further increase the level and intensity of transgene expression in transduced human T cells after either minimal or complete T-cell stimulation.

We established in this report an efficient method to transduce primary blood lymphocytes (particularly T cells) by lentiviral vectors without lentiviral APs. Either total PBL or isolated T lymphocyte subsets can be directly transduced by an overnight transduction protocol without any prior stimulation. Transduced cells can be expanded in the presence of proper T-cell stimuli. Post-T–cell transduction and stimulation, high-level transgene expression will be achieved using the promoter EF1α. For studies that require that transduced cells remain unstimulated, however, transgene expression under the EF1α promoter may not be high enough. With the flexibility of SIN lentiviral vectors to accommodate any internal promoter, it is likely that in the near future we will find another housekeeping gene promoter or engineer one that is highly active in both unstimulated and stimulated T cells.

The T-cell transduction approach by lentiviral vectors reported here and similarly by others (Chinnasamy *et al.*, 2000; Costello *et al.*, 2000; Haas *et al.*, 2000; Salmon *et al.*, 2000; Dardalhon *et al.*, 2001) has many potential applications. First, anti-HIV gene therapy by expressing an intracellular HIV inhibitor (ribozyme, anti-sense, siRNA, etc.) may benefit from the fact that our lentiviral vectors containing a constitutive promoter can express a transgene in resting lymphocytes that can be HIV reservoirs (Pierson *et al.*, 2000). Because HIV-1 will not be able to synthesize more viral RNA from the HIV-LTR and replicate in resting lymphocytes, the abundant anti-HIV transgene may win the competition with residual HIV-1 RNA, and ultimately lead to the elimination of HIV-infected lymphocytes at the resting stage. Second, the ability to transduce genes to T cells with high and consistent efficiencies should facilitate the study of T cell development and biology (Dahl *et al.*, 2000; Kessels *et al.*, 2000; Maher *et al.*, 2002; Sadelain *et al.*, 2003). Third, it should be possible to deliver growth promoting genes to antigen-committed T cells thereby facilitating antigen-specific T-cell adoptive immunotherapy (Rosenberg *et al.*, 1990; Hwu *et al.*, 1993; Treisman *et al.*, 1995; Dudley *et al.*, 2002; Maher *et al.*, 2002; Sadelain *et al.*, 2003). Such studies utilizing this technology for these applications are underway.

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REFERENCES

AMADO. R.G., and CHEN, I.S. (1999). Lentiviral vectors: The promise of gene therapy within reach? Science **285,** 674–676.

AUTEN, J., AGARWAL, M., CHEN, J., SUTTON, R., and PLAVEC, I. (1999). Effect of scaffold attachment region on transgene expression in retrovirus vector-transduced primary T cells and macrophages. Hum. Gene Ther. **10,** 1389–1399.

AYUK, F.A., LI, Z., KÜHLCKE, K., LINDEMANN, C., SCHADE, U.M., ECKERT, H.-G., ZANDER, A.R., and FEHSE, B. (1999). Establishment of an optimised gene transfer protocol for human primary T lymphocytes according to clinical requirements. Gene Ther. **6,** 1788–1792.

BLAESE, R.M, CULVER, K.W., MILLER, A.D., CARTER, C.S., FLEISCHER, T., CLERICI, M., SHEARER, G., CHANG, L., CHIANG, Y., TOLSTOSHEV, P., GREENBLATT, J.J., ROSENBERG, S.A., KLEIN, H., BERGER, M., MULLEN, C.A., EAMSEY, W.J., MUUL, L., MORGAN, R.A., and ANDERSON, F.A. (1995). T lymphocyte-directed gene therapy for ADA-SCID: Initial trial results after 4 years. Science **270,** 475–480.

BORDIGNON, C., NOTARANGELO, L.D., NOBILI, N., FERRARI, G., CASORATI, G., PANINA, P., UGAZIO, A.G., and MAVILIO, F. (1995). Gene therapy in peripheral blood lymphocytes and bone marrow for ADA-immunodeficient patients. Science **270,** 470–475.

BUKRINSKY, M.I., SHAROVA, N., DEMPSEY, M.P., STANWICK, T.L., BUKRINSKAYA, A.G., HAGGERTY, S., and STEVENSON, M. (1992). Active nuclear import of human immunodeficiency virus

type 1 preintegration complexes. Proc. Natl. Acad. Sci. U.S.A. **89,** 6580–6584.

BUKRINSKY, M.I., HAGGERTY, S., DEMPESEY, M.P., SHAROVA, N., ADZHUBEL, A., SPITZ, L. LEWIS, P., GOLDFARB, D., EMERMAN, M., and STEVENSON, M. (1993). A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. Nature **365,** 666–669.

BUNNELL, B.A., MUUL, L.M., DONAHUE, R.E., BLAESE, R.M., and MORGAN, R.A. (1995). High-efficiency retroviral-mediated gene transfer into human and nonhuman primate peripheral blood lymphocytes. Proc. Natl. Acad. Sci. U.S.A. **92,** 7739–7743.

CASE, S.S., PRICE, M.A., JORDAN, C.T., YU, X.J., WANG, L., BAUER, G. HASS, D.L., XU, D., STRIPECKE, R., NALDINI, L., KOHN, D.B., and CROOKS, G. M. (1999). Stable transduction of quiescent $CD34^+CD38^-$ human hematopoietic cells by HIV-1–based lentiviral vectors. Proc. Natl. Acad. Sci. U.S.A. **96,** 2988–2993.

CHENG, L., DU, C., MURRAY, D., TONG, T., ZHANG, Y.A., CHEN, B.P., and HAWLEY, R.G. (1997). A GFP reporter system to assess gene transfer and expression in viable human hematopoietic progenitor cells. Gene Ther. **4,** 1013–1022.

CHINNASAMY D., CHINNASAMY N., ENRIQUEZ M.J., OTSU, M., MORGAN, R.A., and CANDOTTI, F. (2000). Lentiviral-mediated gene transfer into human lymphocytes: role of HIV-1 accessory proteins. Blood **96,** 1309–1316.

COSTELLO, E., MUNOZ, M., BUETTI, E., MEYLAN, P.R.A., DIGGELMANN, H., and THALI, M. (2000). Gene transfer into stimulated and unstimulated T lymphocytes by HIV-1–derived lentiviral vectors. Gene Ther. **7,** 596–604.

CUI, Y., GOLOB, J., KELLEHER, E., PARDOLL, D., and CHENG, L. (2002). Targeting transgene expression to antigen presenting cells derived from lentivirus transduced human hematopoietic stem/progenitor cells. Blood **15,** 399–408.

DAHL, A.M., KLEIN, C., ANDRES, P.G., LONDON, C.A., LODGE, M.P., MULLIGAN, R.C., and ABBAS, A. (2000). Expression of Bcl-X_L restores cell survival, but not proliferation and effector differentiation in CD28-deficient T lymphocytes. J. Exp. Med. **191,** 2031–2037.

DARDALHON, V., NORAZ, N., POLLOK, K., REBOUISSOU, C., BOYER, M., BAKKER, A.Q., SPITS, H., and TAYLOR, N. (1999). Green fluorescence protein as a selectable marker of fibronectin-facilitated retroviral gene transfer in primary human T lymphocytes. Hum. Gene Ther. **10,** 5–14.

DARDALHON, V., JALECO, S., REBOUISSOU, C., FERRAND, C., SKANDER, N., SWAINSON, L., TIBERGHIEN, P., SPITS, H., NORAZ, N., and TAYLOR, N. (2000). Highly efficient gene transfer in naïve human T cells with a murine leukemia virus-based vector. Blood **96,** 885–893.

DARDALHON, V., HERPERS, B., NORAZ, N., PFLUMIO, F., GUETARD, D., LEVEAU, C., DUBART-KUPPERSCHMITT, A., CHARNEAU, P., and TAYLOR, N. (2001). Lentivirus-mediated gene transfer in primary T cells is enhanced by a central DNA flap. Gene Ther. **8,** 190–198.

DIETRICH, P.Y., WALKER, P.R., SCHNURIGER, V., SAAS, P., PERRIN, G., GUILLARD, M., GAUDIN, C., and CAIGNARD, A. (1997). TCR analysis reveals significant repertoire selection during in vitro lymphocyte culture. Int. Immunol. **9,** 1073–1083.

DONAHUE, R.E., WERSTO, R.P., ALLAY, J.A, AGRICOLA, B. A., METZGER, M.E., NIENHUIS, A.W., PERSONS, D.A., and SORRENTINO, B.P. (2000). High levels of lymphoid expression of enhanced green fluorescent protein in nonhuman primates transplanted with cytokine-mobilized peripheral blood $CD34^+$ cells. Blood **95,** 445–452.

DUCREY-RUNDQUIST, O., GUYADER, M., and TRONO, D. (2002). Modalities of interleukin-7–induced human immunodeficiency virus permissiveness in quiescent T lymphocytes. J. Virol. **76,** 9103–9111.

DUDLEY, M.E., WUNDERLICH, J.R., ROBBINS, P.F., YANG, J.C., HWU, P., SCHWARTZENTRUBER, D.J., TOPALIAN, S.L., SHERRY, R., RESTIFO, N.P., HUBICKI, A.M., ROBINSON, M.R., RAFFELD, M., DURAY, P., SEIPP, C.A., ROGERS-FREEZER, L., MORTON, K.E., MAVROUKAKIS, S.A., WHITE, D.E., and ROSENBERG, S.A. (2002). Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. Science **298,** 850–854.

DULL, T., ZUFFEREY, R., KELLY, M., MANDEL, R.J., NGUYEN, M., TRONO, D., and NALDINI, L. (1998). A third-generation lentiviral vector with a conditional packaging system. J. Virol. **72,** 8463–8471.

FISCHER, A., HACEIN-BEY, S., and Cavazzana-Calvo, M. (2002). Gene therapy of severe combined immunodeficiencies. Nat. Rev. Immunol. **2,** 615–621.

FOLLENZI, A., AILLES, L.E., BAKOVIC, S., GEUNA, M., and NALDINI, L. (2000). Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences. Nat. Genet. **25,** 217–222.

GALLARDO, H.F., TAN, C., ORY, D., and SADELAIN, M. (1997). Recombinant retroviruses pseudotyped with the vesicular stomatitis virus G glycoprotein mediate both stable gene transfer and pseudotransduction in human peripheral blood lymphocytes. Blood **90,** 952–957.

GAO, Z., GOLOB, J., TANAVDE, V.M, CIVIN, C.I., HAWLEY, R.G., and CHENG, L. (2001). High levels of transgene expression following long-term NOD/SCID-repopulating human cells with a modified lentiviral vector. Stem Cells **19,** 247–259.

GREENBERG, P.D., and RIDDELL, S.R. (1999). Deficient cellular immunity-finding and fixing the defects. Science **285,** 546–551.

HAAS, D.L., CASE, S.S., CROOKS, G.M., and KOHN, D.B. (2000). Critical factors influencing stable transduction of human $CD34^+$ cells with HIV-1–derived lentiviral vectors. Mol. Ther. **2,** 71–80.

HARADA, M., LI, Y.F., EL-GAMIL M., OHNMACHT G.A., ROSENBERG, S.A., and ROBBINS, P.F. (2001). Melanoma-reactive CD8+ T cells recognize a novel tumor antigen expressed in a wide variety of tumor types. J. Immunother. **24,** 323–333.

HWU, P., SHAFER, G.E., TREISMAN, J., SCHINDLER, D.G., GROSS, G., COWHERD, R., ROSENBERG, S.A., and ESHHAR, Z. (1993). Lysis of ovarian cancer cells by human lymphocytes redirected with a chimeric gene composed of an antibody variable region and the Fc receptor γ chain. J. Exp. Med. **178,** 361–366.

KAFRI, T., BLOMER, U., PETERSON, D.A., GAGE, F.H., and VERMA, I.M. (1997). Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. Nat. Genet. **17,** 314–317.

KESSELS, H.W.H.G., VAN DEN BOOM, M.D., SPITS, H., HOOIJBERG, E., and SCHUMACHER, T.N.M. (2000). Changing T cell specificity by retroviral T cell receptor display. Proc. Natl. Acad. Sci. U.S.A. **97,** 14578–14583.

KOHN, D.B., WEINBERG, K.I., NOLTA, J.A., HEISS, L.N., LENARSKY, C., CROOKS, G.M., HANLEY, M. E., ANNETT, G., BROOKS, J.S., EL-KHOUREIY, A., LAWRENCE, K., WELLS, S., MOEN, R.C., BASTIAN, J., WILLIAMS-HERMAN, D.E., ELDER, M., WARA, D., BOWEN, T., HERSHFIELD, M.S., MULLEN, C.A., BLAESE, R.M., and PARKMAN, R. (1995). Engraftment of gene-modified umbilical cord blood cells in neonates with adenosine deaminase deficiency. Nat. Med. **1,** 1017–1023.

KOHN, D.B., HERSHFIELD, M.S., CARBONARO, D., SHIGEOKA, A., BROOKS, J., SMOGORZEWSKA, E.M., BARSKY, L.W., CHAN, R., BUROTTO, F., ANNETT, G., NOLTA, J.A., CROOKS, G., KAPOOR, N., ELDER, M., WARA, D., BOWEN, T., MADSEN, E., SNYDER, F.F., BASTIAN, J., MUUL, L., BLAESE, R.M., WEINBERG, K., and PARKMAN, R. (1998). T lymphocytes with a normal ADA gene accumulate after transplantation of transduced autologous umbilical cord blood $CD34^+$ cells in ADA-deficient SCID neonates. Nat. Med. **4,** 775–780.

LAM, J.S., REEVES, M.E., COWHERD, R., ROSENBERG, S.A., and

HWU, P. (1996). Improved gene transfer into human lymphocytes using retroviruses with the gibbon ape leukemia virus envelope. Hum. Gene Ther. **7,** 1415–1422.

LIU, M.-L., WINTHER, B.L., and KAY, M.A. (1996). Pseudotransduction of hepatocytes by using concentrated pseudotyped vesicular stomatitis virus G glycoprotein (VSV-G)-Moloney murine leukemia virus-derived retrovirus vectors: Comparison of VSV-G and amphotropic vectors for hepatic gene transfer. J. Virol. **70,** 2497–2502.

MAHER, J., BRENTJENS, R.J., GUNSET, G., RIVIERE, I., and SADELAIN, M. (2002). Human T-lymphocyte cytotoxicity and proliferation directed by a single chimeric TCRζ/CD28 receptor. Nat. Biotechnol. **20,** 70–75.

MASCARENHAS, L., STRIPECKE, R., CASE. S.S., XU, D., WEINBERG, K.I., and KOHN, D.B. (1998). Gene delivery to human B-precursor acute lymphoblastic leukemia cells. Blood. **92,** 3537–3545.

MAURICE, M., VERHOEYEN, E., SALMON, P., TRONO, D., RUSSELL, S.J, and COSSET, F.L. (2002). Efficient gene transfer into human primary blood lymphocytes by surface-engineered lentiviral vectors that display a T cell-activating polypeptide. Blood **99,** 2342–2350.

MAVILIO, F., FERRARI, G., ROSSINI, S., NOBILI, N., BONINI, C., CASORATI, G., TRAVERSARi, C., and BORDIGNON, C. (1994). Peripheral blood lymphocytes as target cells of retroviral vector-mediated gene transfer. Blood **83,** 1988–1997.

MIYOSHI, H., TAKAHASHI, M., GAGE, F.H., and VERMA, I.M. (1997). Stable and efficient gene transfer into the retina using a HIV-1–based lentiviral vector. Proc. Natl. Acad. Sci. U.S.A. **94,** 10319–10323.

MIYOSHI, H., BLÖMER, U., TAKAHASHI, M., GAGE, F.H., and VERMA, I.M. (1998). Development of a self-inactivating lentivirus vector. J. Virol. **72,** 8150–8157.

MIYOSHI, H., SMITH, K.A., MOSIER, D., VERMA, I.M., and TORBETT, B.E. (1999). Transduction of human CD34+ cells that mediate long-term engraftment of NOD/SCID mice by HIV vectors. Science **283,** 682–686.

NALDINI, L., BLÖMER, U., GALLAY, P., ORY, D., MULLIGAN, R., GAGE, F.H., VERMA, I.M., and TRONO, D. (1996a). In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science **272,** 263–267.

NALDINI, L., BLÖMER, U., GAGE, F.H., TRONO, D., and VERMA, I.M. (1996b). Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. Proc. Natl. Acad. Sci. U.S.A. **93,** 11382–11388.

NALDINI, L. (1998). Lentiviruses as gene transfer agents for delivery to non-dividing cells. Curr. Opin. Biotechnol. **9,** 457–463.

PARKMAN, R., WEINBERG, K., CROOKS, G., NOLTA, J., KAPOOR, N., and KOHN, D. (2000). Gene therapy for adenosine deaminase deficiency. Annu. Rev. Med. **51,** 33–47.

PIERSON, T., MCARTHUR, J., and SILICIANO, R.F. (2000). Reservoirs for HIV-1: Mechanisms for viral persistence in the presence of antiviral immune responses and antiretroviral therapy. Annu. Rev. Immunol. **18,** 665–708.

PLAVEC, I., AGARWAL, M., HO, K.E., PINEDA, M., AUTEN, J., BAKER, J., MATSUZAKI, H., ESCAICH, S., BONYHADI, M., and BOHNLEIN, E. (1997). High transdominant RevM10 protein levels are required to inhibit HIV-1 replication in cell lines and primary T cells: Implication for gene therapy of AIDS. Gene Ther. **4,** 128–139.

POZNANSKY, M., LEVER, A., BERGERON, L., HASELTINE, W., and SODROSKI, J. (1991). Gene transfer into human lymphocytes by a defective human immunideficiency virus type 1 vector. J. Virol. **65,** 532–536.

RAMEZANI, A., HAWLEY, T.S., and HAWLEY, R.G. (2000). Lentiviral vectors for enhanced gene expression in human hematopoietic cells. Mol. Ther. **2,** 458–469.

REISER, J., HARMISON, G., KLUEPFEL-STAHL, S., BRADY, R.O., KARLSSON, S., and SCHUBERT, M. (1996). Transduction of nondividing cells using pseudotyped defective high-titer HIV type 1 particles. Proc. Natl. Acad. Sci. U.S.A. **93,** 15266–15277.

RIDDELL, S.R., and GREENBERG, P.D. (1995). Principles for adoptive T cell therapy of human viral diseases. Annu. Rev. Immunol. **13,** 545–586.

RIVIERE, I., GALLARDO, H.F., HAGANI, A.B., and SADELAIN, M. (2000). Retroviral-mediated gene transfer in primary murine and human T-lymphocytes. Mol. Biotechnol. **15,** 133–142.

ROSENBERG, S.A., AEBERSOLD, P., CORNETTA, K., KASID, A., MORGAN, R., MOEN, R., KARSON, E., LOTZE, M., YANG, J., TOPALIAN, S., MERINO, M., CULVER, K., MILLER, A.D., BLAESE, R.M., and ANDERSON, W.F. (1990). Gene transfer into humans—immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. N. Engl. J. Med. **323,** 570–578.

SADELAIN, M., RIVIERE, I., and BRENTJENS, R. (2003). Targeting tumors with genetically enhanced T lymphocytes. Nat. Rev. Cancer **3,** 35–45.

SALMON, P., KINDLER, V., DUCREY, O., CHAPUIS, B., ZUBLER, R.H., and TRONO, D. (2000). High-level transgene expression in human hematopoietic progenitors and differentiated blood lineages after transduction with improved lentiviral vectors. Blood **96,** 3392–3398.

SEDAR, R.A. (1994). Acquisition of lymphokine-producing phenotype by CD4+ T cells. J. Allergy Clin. Immunol. **94,** 1195–1202.

SHIMADA, T., FUJII, H., MITSUYA, H., and NIENHUIS, A.W. (1991). Targeted and highly efficient gene transfer into $CD4^+$ cells by a recombinant human immunodeficiency virus retroviral vector. J. Clin. Invest. **88,** 1043–1047.

TIBERGHIEN, P., REYNOLDS, C.W., KELLER, J., SPENCE, S., DESCHASEAUX, M., CERTOUX, J.-M., CONTASSOT, E., MURPHY, W.J., LYONS, R., CHIANG, Y., HERVE, P., LONGO, D.L., and RUSCETTI, F.W. (1994). Ganciclovir treatment of herpes simplex thymidine kinase-transduced primary T lymphocytes: An approach for specific in vivo donor T-cell depletion after bone marrow transplantation. Blood **84,** 1333–1341.

TONG-STARKSEN, S.E., WELSH, T.M., and PETERLIN, B.M. (1990). Differences in transcriptional enhancers of HIV-1 and HIV-2. Response to T cell activation signals. J. Immunol. **145,** 4348–4354.

TREISMAN, J., HWU, P., MINAMOTO, S., SHAFER, G.E., COWHERD, R., MORGAN, R.A., and ROSENBERG, S.A. (1995). Interleukin-2-transduced lymphocytes grow in an autocrine fashion and remain responsive to antigen. Blood **85,** 139–145.

TRONO, D. (2000). Lentiviral vectors: turning a deadly foe into a therapeutic agent. Gene Ther. **7,** 20–23.

UCKERT, W., BECKER, C., GLADOW, M., KLEIN, D., KAMMERTOENS, T., PEDERSEN, L., and BLANKENSTEIN, T. (2000). Efficient gene transfer into human $CD8^+$ T lymphocytes by MuLV-10A1 retrovirus pseudotype. Hum. Gene Ther. **11,** 1005–1014.

UNUTMAZ, D., KEWALRAMANI, V.N., MARMON, S., LITTMAN, D.R. (1999). Cytokine signals are sufficient for HIV-1 infection of resting human T lymphocytes. J. Exp. Med. **189,** 1735–1746.

VERHOEYEN, E., DARDALHON, V., DUCREY-RUNDQUIST, O., TRONO, D., TAYLOR, N., and COSSET, F.L. (2003). IL-7 surface-engineered lentiviral vectors promote survival and efficient gene transfer in resting primary T lymphocytes. Blood **101,** 2167–2174.

VON SCHWEDLER, U., KORNBLUTH, R.S., and TRONO, D. (1994). The nuclear localization signal of the matrix protein of human immunodeficiency virus type 1 allows the establishment of infection in macrophages and quiescent T lymphocytes. Proc. Natl. Acad. Sci. U.S.A. **91,** 6992–6996.

WU, A.G., LIU, X., MAZUMDER, A., BELLANTI, J.A., and MEEHAN, K.R. (1999). Improvement of gene transduction efficiency in T lymphocytes using retroviral vectors. Hum. Gene Ther. **10,** 977–982.

YANG, S., DELGADO, R., KING, S.R., WOFFENDIN, C., BARKER, C.S., YANG, Z.-Y., XU, L., NOLAN, G.P., and NABEL, G.J. (1999). Generation of retroviral vector for clinical studies using transient transfection. Hum. Gene Ther. **10,** 123–132.

ZENNOU, V., PETIT, C., GUETARD, D., NERHBASS, U., MONTAGNIER, L., and CHARNEAU, P. (2000). HIV-1 genome nuclear import is mediated by a central DNA flap. Cell **101,** 173–185.

ZUFFEREY, R., NAGY, D., MANDEL, R.J., NALDINI, L., and TRONO, D. (1997). Multiple attenuated lentiviral vector achieves efficient gene delivery in vivo. Nat. Biotechnol. **15,** 871–875.

ZUFFEREY, R., DULL, T., MANDEL, R.J., BUKOVSKY, A., QUIROZ, D., NALDINI, L., and TRONO, D. (1998). Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. J. Virol. **72,** 9873–9880.

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