Lentivirus Vector-Mediated Hematopoietic Stem Cell Gene Transfer of Common Gamma-Chain Cytokine Receptor in Rhesus Macaques

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Nonhuman primate model systems of autologous CD34+ cell transplant are the most effective means to assess the safety and capabilities of lentivirus vectors. Toward this end, we tested the efficiency of marking, gene expression, and transplant of bone marrow and peripheral blood CD34+ cells using a self-inactivating lentivirus vector (CS-Rh-MLV-E) bearing an internal murine leukemia virus long terminal repeat derived from a murine retrovirus adapted to replicate in rhesus macaques. In vitro cytokine stimulation was not required to achieve efficient transduction of CD34+ cells resulting in marking and gene expression of the reporter gene encoding enhanced green fluorescent protein (EGFP) following transplant of the CD34+ cells. Monkeys transplanted with mobilized peripheral blood CD34+ cells resulted in EGFP expression in 1 to 10% of multilineage peripheral blood cells, including red blood cells and platelets, stable for 15 months to date. The relative level of gene expression utilizing this vector is 2- to 10-fold greater than that utilizing a non-self-inactivating lentivirus vector bearing the cytomegalovirus immediate-early promoter. In contrast, animals transplanted with autologous bone marrow CD34+ cells, multilineage EGFP expression was evident initially but diminished over time. We further tested our lentivirus vector system by demonstrating gene transfer of the human common gamma-chain cytokine receptor gene (γc), deficient in X-linked SCID patients and recently successfully used to treat disease. Marking was 0.42 and .001 HIV-1 vector DNA copy per 100 cells in two animals. To date, all EGFP- and γc-transplanted animals are healthy. This system may prove useful for expression of therapeutic genes in human hematopoietic cells.

Lentivirus vectors based on the human immunodeficiency virus (HIV) genome have been proposed as potential vectors for human hematopoietic progenitor cell gene transfer (3, 6, 12, 14, 30, 38, 43, 47). These vectors have a number of advantages over murine retrovirus vectors, in particular the ability to transduce nondividing cells (32), provided they reside or progress through at least the G1b state of the cell cycle (24). Other vectors based on murine retrovirus genomes are unable to establish infection except when cells progress through mitosis (27, 29, 39). The other advantage of lentivirus vectors is that they have evolved to replicate efficiently in human cells. Thus, lentivirus vectors should in theory provide effective transduction of hematopoietic progenitor cells and maintain high levels of gene expression in differentiated cells. However, these potential advantages and their origin also emphasize the need to adequately assess the properties of lentivirus vectors prior to use in humans. Nonhuman primate models represent the ideal model system to test these vectors in regard to efficacy and safety. This rhesus macaque model system of autologous transplant of CD34+ cells has been used effectively to model human hematopoietic progenitor cell human gene therapy (5, 9, 11, 13, 17, 18, 20, 23, 40, 42, 46, 49). We have previously shown that lentivirus vectors can be used for marking and gene expression following transplant of rhesus macaque CD34+ cells, utilizing mobilized peripheral blood (PB) CD34+ cells (3). Thus, this model system is ideal for evaluation of the efficiency of marking and the efficiency and maintenance of gene expression and for initial safety testing regarding introduction of potential human therapeutic genes.

Here we report on the use of a lentivirus vector that combines the best features of murine retrovirus (murine leukemia virus [MLV]) and HIV type 1 (HIV-1) vectors (25). This vector gives long-term expression in rhesus macaque hematopoietic cells following transplant of transduced mobilized CD34+ PB cells in the absence of in vitro cytokine stimulation. The use of this vector demonstrates that marking is more efficient in mobilized PB cells than in bone marrow (BM) cells. Finally, the vector was used to express the human common gamma-chain cytokine receptor gene (γc) in lymphocytes of rhesus macaques.

MATERIALS AND METHODS

HIV-1 vector construction and production. Construction of the HIV-1-based vector, pCS-RhMLV-E, was described previously (25). To construct a lentivirus vector carrying the human common γc (pcp-RhMLV-bury), pCS-RhMLV-E was first digested with AgeI and XhoI to remove the enhanced green fluorescent protein (EGFP) cDNA and then blunt ended by Klenow fragment. The resulting vector fragment was ligated to the γc cDNA fragment that was isolated from plasmid SfRaG1 (45) by XhoI digestion followed by Klenow fragment blunt ending. The vesicular stomatitis virus G protein expression plasmid (pHCMV-G) and the packaging plasmid for HIV-1-based vectors (pCMV8.2DVPR) were described previously (3). All virus stocks were prepared by calcium phosphate-mediated, three-plasmid transfection of 293T cells (American Type Culture
Collection, Manassas, Va.) as described previously (2). In brief, 293T cells (20 × 10^6), cultured in Dulbecco's modified Eagle medium with 10% calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml), were transfected with 5 μg of pCMV-G, 12.5 μg of pCMVΔR8.2DVPR, and 12.5 μg of an HIV-1-based vector for HIV-1 ΔR8.2ΔVPR-Cys (pCS-RhMLV-ΔR8.2ΔVPR) virus (6). Viruses were harvested on days 2, 3, and 4 posttransfection, filtered through a 0.22-μm-pore-size filter, and concentrated 100-fold by ultracentrifugation. Virus stocks were titrated by on days 2, 3, and 4 posttransfection, filtered through a 0.22-

c) Virus supernatant was collected of pHCMV-G, 12.5

g vector (pCS-RhMLV-E or pCS-RhMLV-hu

Rhesus macaques (Macaca mulatta) that were serologically negative for simian T-cell lymphotropic virus, simian immunodeficiency virus, simian AIDS-related type D virus, and herpes B virus were used. They were quarantined and housed in accordance with federal guidelines (34) and the policies set by the Veterinary Research Program of the National Institutes of Health. The protocols were evaluated and approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute. Four rhesus ma-

cues were injected subcutaneously with a combination of granulocyte colony-stimulating factor (G-CSF; 10 μg/kg of body weight/day) and stem cell factor (SCF; 200 μg/kg/day) (both kindly provided by Amgen, Inc., Thousand Oaks, Calif.) 4 days before the cell harvest (PB or BM). Mobilized (cytokine-stimulate-
ed) leukapheresis cell product of the PB from two rhesus macaques (95E132 and 96E041) was harvested by a CS3000 Plus blood cell separator (Baxter Health-
care, Fenwal Division, Deerfield, Ill.), using a single small-volume chamber and other modifications made to the fluid path of the CS3000 Plus blood cell sepa-
rator (10). This device allowed leukapheresis procedures to be performed on rhesus macaques weighing less than 5 kg. BM cells were surgically harvested from the femurs and iliac crests of two rhesus macaques (96E041 and 95E131) under anesthesia. After harvest, the PB mononuclear cells (PBMMN) and BM mononuclear cells were isolated by Ficoll-Hypaque (Pharmacia) density centri-

fugation, followed by immunoselection of CD34+ cells. All four animals received a total dose of 10 Gy of total-body irradiation over 2 consecutive days prior to reinfection of the transduced CD34+ cells. PB was collected on the designated days and evaluated by PCR analysis or flow cytometry for EGFP DNA or expression, respectively.

**Immunoselection of nonhuman primate CD34+ cells.** PB- and BM-derived CD34+ cells were isolated according to the manufacturer's instructions by mag-

netic selection using a biotinylated CD34+ antibody (clone 12.8; CellPro, Inc., Bothell, Wash.), streptavidin MicroBeads, and MACS separation columns (Miltenyi Biotec, Inc., Auburn, Calif.). Purity following the immunoselection procedure was routinely >95%, as assessed by surface staining of allopocya-

cin- conjugated anti-CD34 monoclonal antibody (clone 563; a kind gift from Gustav Gaudernack, Institute of Transplantation Immunology, Rikshospitalet, The National Hospital, Oslo, Norway, with allopocycin conjugation per-

formed by Molecular Probes, Inc., Eugene, Oreg.) and analysis in an ELITE flow cytometer (Beckman Coulter Corp., Miami, Fla.).

**In vitro lentivirus vector transduction.** Immunoselected PB and BM CD34+ cells were transduced as described previously (3). In brief, the cells were cultured on RetroNectin (BioWhittaker, Walkersville, Md)-coated, non-tissue culture-
treated six-well plates (Becton Dickinson Labware, Franklin Lakes, N.J.) and protamine sulfate (8 μg/ml) according to the manufacturer's instructions. They were transduced with the vesicular stomatitis virus G protein-pseudotyped HIV-1 vector (CS-RhMLV-E or CS-RhMLV-hyhu) for 2 h twice a day for 2 days at a multiplicity of infection (MOI) of approximately 5. The transduced autolo-

gous CD34+ cells were reinfused into the irradiated animals for the evaluation of gene expression and of multilineage and long-term marking in vivo. Rhesus maque PBMMN were isolated from mock-infected rhesus macaque PB by Ficoll-

Hypaque density separation. They were activated by anti-monkey CD3 (1 mg/ml; BioSource International, Camarillo, Calif.) and anti-human CD28 (1 μg/ml; catalog no. P24325M; Biodesign International, Kennebunk, Maine) antibodies and human interleukin-2 (IL-2; 10 U/ml; Amgen) for 2 days as described previ-

ously (3). To test the expression of human HLA class I alleles, PBMMN were collected and Hela cells (5 × 10^5) and activated rhesus PBMMN (5 × 10^5) were infected with CS-RhMLV-hyhu vector at MOI of 100 and for 2 h at 37°C, respectively. The transduced cells were washed with medium and cultured for 3 days before flow cytometric analysis.

**Flow cytometric analysis of rhesus macaque PB hematopoietic cells.** Flow cytometric analysis of rhesus macaque PB hematopoietic cells. Rhesus maque PB was obtained from transplanted macaques with lentivirus vector transduced PB or BM CD34+ cells at various time points posttransplantation. The obtained PB was diluted 100-fold with phosphate-buffered saline (PBS) and analyzed for EGFP expression in red blood cells (RBC) and platelets by flow cytometry. To analyze for EGFP expression in granulocyte, monocyte, and lym-

phocyte populations, the obtained PB was first incubated with red cell lysis buffer 150 mM (ammonium chloride, 10 mM potassium bicharate, 0.1 mM EDTA [pH 7.4]) at 4°C to achieve complete RBC lysis prior to analysis. Each cell populations were gated according to size (forward scatter plot) and granularity (side scatter plot). The cells were analyzed in a FACSscan flow cytometer with the CellQuest software (Becton Dickinson). Fifty thousand events were acquired for analysis.

To analyze surface expression of human γc, transduced cells (5 × 10^4) were first incubated with 50 μl of human AB serum (Omega Scientific, Tarzana, Calif.), 2 μl of Fc Block (PharMingen), and 5 μl of rat immunoglobulin G2b antibodies (Caltag) for 15 min at room temperature to block nonspecific binding. The cells were further incubated on ice for 5 min before the addition of 50 μl of phycoerythrin (PE)-conjugated anti-human γc monoclonal antibodies (di-

luted to 0.004 μg/ml; Tugh4; catalog no. 351945B; PharMingen). The cells were incubated on ice for 20 min, washed with PBS–1% fetal calf serum, and then resuspended in 7AAD buffer (7AAD [1 μg/ml] in PBS, Calbiochem, San Diego, Calif.) for 30 min on ice before analysis. Dead cells were excluded by gating on the 7AAD-negative population by flow cytometric analysis. Cells that were stained with rat immunoglobulin G2b monoclonal antibodies conjugated with PE (Caltag) were used as the isotype control.

**Quantitative PCR assay.** Each PCR amplification was performed as described elsewhere (50). In brief, to detect HIV-1 vector sequences, one of the oligonu-

cleotide primers for each pair used was end labeled with 3P, and 25 ng was included in 10^6 to 10^7 copies) from rhesus macaque PBMN.

The second oligonu-
cleotide primer was not labeled, and 50 ng was incorporated into each reaction. Each reaction mixture contained 0.25 mM each of the four deoxynucleoside triphosphates, 50 mM NaCl, 25 mM Tris-HCl (pH 8.0), 5 mM MgCl2, 100 μg of bovine serum albumin per ml, and 1.25 U of Taq DNA polymerase (Promega, Madison, Wis.). The reaction mixture was overlaid with 25 μl of mineral oil and then subjected to 25 cycles of denaturation for 1 min at 94°C and polymerization for 2 min at 65°C. The reaction was performed on a Perkin-Elmer thermocycler. Amplified products resulting from the PCR were analyzed by electrophoresis on 6% nondenaturing polyacrylamide gels and visualized by direct autoradiography of the dried gels. Quantitative analysis of the amplified products was performed with a Phosphorimagere (Molecular Dynamics, Sunnyvale, Calif.), and data were analyzed with the ImageQuaNT program (Molecular Dynamics). The nucleotide sequences of the oligonucleotide primers (M667 and AA55) used for pCS-

RhMLV-E DNA detection were derived from the nucleotide sequence of the HIV-1 long terminal repeat (LTR) as previously described (50). A pair of oligonucleotide primers complementary to the first exon of the human β-globin gene (LAI and LAZ) (50) was used in each reaction mixture in PCR analyses to normalize the total amount of rhesus macaque cellular DNA present. During PCR amplification, labeled β-globin-specific oligonucleotides were incorporated into the reaction at 5 × 10^5 to 1 × 10^5 copies.

HIV-1 vector DNA was quantitated during PCR amplifications by analyzing a standard curve of dilution of pHRCMVEGFP plasmid DNA digested with (HpaI), a restriction enzyme which does not cleave the vector sequence. This DNA was diluted in 0.01 μg of rhesus macaque PBMMN DNA per ml. The copy number for the HIV-1 vector included in the standard curve was (50). Standard curves for HIV-1 vector DNA were obtained by amplifica-

tion of 0.001 to 0.03 μg of rhesus macaque cellular DNA (10 to 3,000 cell equivalents) from rhesus macaque PBMMN.

**RESULTS**

Transduction of immunoselected mobilized PB- and BM-derived CD34+ cells with the CS-Rh-MLV-E lentivirus vector. We previously established a nonhuman primate rhesus maque transfection model for the evaluation of lentivirus transduction of CD34+ cells in vitro (3). We used an HIV-1 vector (HR/CMVEGFP) (33) bearing an internal cytomegalovirus (CMV) immediate-early promoter to express EGFP as a reporter gene for assessment of marking efficiencies (3). To date, multilineage PB hematopoietic cells in transplanted rhes-

macaques expressed EGFP stably for 2 years. However, gene expression from the HR/CMVEGFP vector was low in rhesus macaque hematopoietic cells. We developed a self-
inactivating HIV vector, CS-Rh-MLV-E, which bears an LTR (Rh-MLV) derived from the MLV replication-competent ret-

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rovirus found in the sera of one rhesus macaque monkey that developed T-cell lymphoma (25). We previously showed that CS-Rh-MLV-E had 5- to 10-fold-higher EGFP expression in human T cells than a CMV immediate-early promoter-based self-inactivating HIV vector (25). We therefore evaluated the CS-Rh-MLV-E vector in rhesus macaques in the transplantation model for gene expression and for multilineage marking, and long-term reconstitution. We compared immunoselected mobilized PB and BM CD34+ cells of the rhesus macaques that were treated with SCF and G-CSF for their efficiency of lentivirus transduction and reconstitution of rhesus macaque hematopoietic system in vivo. Nonhuman primate immunoselected PB and BM CD34+ cells of four animals were transduced with CS-RhMLV-E twice a day for 2 days in the absence of further cytokine stimulation ex vivo at an MOI of approximately 5 and reinfused into autologous animals as described in Materials and Methods. Two animals (96E041 and 95E131) received autologous transplants with transduced PB CD34+ cells. These animals had received 10 Gy of total-body irradiation as a means of reconstitution. We also examined the presence of vector DNA in PB of animals 95E132 and 96E035 by quantitative DNA PCR analysis at 22 weeks posttransplantation (Fig. 2). The highest EGFP-marked animal (95E132) had highest amount of vector DNA (2.2 copies per 100 cells).

EGFP expression from CS-Rh-MLV E was higher than that from HR’CMVEGFP in rhesus macaque PB. Previously, we showed that the HIV vector bearing the Rh-MLV LTR promoter has 5- to 10-fold-higher EGFP expression in human T lymphocytes than the HR’CMVEGFP vector, bearing the CMV immediate-early promoter in vitro (25). We therefore examined the fluorescence intensity of EGFP expression in multiple lineages of rhesus hematopoietic cells and compared their mean fluorescence intensities (MFI) of EGFP expression to that of the previously described animal (RC505) that was transplanted with CD34+ cells transduced by the CMV promoter-bearing HIV-1 vector (HR’CMVEGFP) (3). The MFI of EGFP expression in the transduced hematopoietic cells of animal RC505 has been stably maintained since week 13 posttransplantation and remains unchanged for 127 weeks to date. We found that the CS-Rh-MLV-E vector consistently gave 2- to 10-fold-greater levels of gene expression than the CMV promoter in granulocyte, monocyte, lymphocyte, RBC, and platelet populations (Fig. 3). To date, this higher level of EGFP expression has been stably maintained for 65 weeks. It should also be noted that similar MFI of EGFP were observed in both animals 95E132 and 96E035, despite a lower percentage of EGFP+ cells in monkey 96E035. Taken together, we confirm that CS-RhMLV-E allows a higher level of EGFP expression in multiple lineages of rhesus macaque hematopoietic cells than HR’CMVEGFP. Since these vectors differ in both self-inactivation and promoter, we cannot differentiate which of these properties is responsible for greater expression, although in vitro, it is attributed primarily to the promoter (25).

Long-term marking was not achieved in rhesus macaques transplanted with CS-Rh-MLV-E-transduced BM CD34+ cells. Two animals (96E041 and 95E131) received autologous transplants with CS-RhMLV-E-transduced BM CD34+ cells. These animals had received 10 Gy of total-body γ irradiation as a 5-Gy fractionated dose given on 2 consecutive days before transplantation (days −1 and 0, with day 0 being the date of reinfusion). Leukocyte counts recovered to 1,000 cells/μl by day 18, with platelet counts recovering to greater than 50,000/μl by day 32 (Table 1).

The two macaques transplanted with autologous BM CD34+ cells showed a different pattern of reconstitution and marking. No granulocyte and monocyte populations (marked and unmarked) were found at 2 and 3 weeks posttransplantation (Fig. 1). After 3 weeks, some hematopoietic lineages were reconstituted in both animals. Low percentages of EGFP+ cells were initially detected in PBC of both animals and were found to diminish gradually over time. In one animal (96E041), the

<table>
<thead>
<tr>
<th>Animal (CD34+ cell origin)</th>
<th>No. of CD34+ cells reinfused (10⁵)</th>
<th>% EGFP in reinfused CD34+ cells</th>
<th>Day of white blood cell count &gt;1,000/μl</th>
<th>Day of platelet count &gt;50,000/μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>96E035 (PB)</td>
<td>9</td>
<td>15</td>
<td>16</td>
<td>42</td>
</tr>
<tr>
<td>95E132 (PB)</td>
<td>36</td>
<td>20</td>
<td>8</td>
<td>0</td>
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<tr>
<td>96E041 (BM)</td>
<td>36</td>
<td>52</td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td>95E131 (BM)</td>
<td>10</td>
<td>53</td>
<td>18</td>
<td>32</td>
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a Nonhuman primate immunoselected CD34+ cells of four animals were transduced with CS-RhMLV-E twice a day for 2 days in the absence of further cytokine stimulation ex vivo at an MOI of approximately 5 and reinfused into autologous animals as described in Materials and Methods.

b CS-RhMLV-E-transduced CD34+ cells were analyzed for EGFP expression by flow cytometry 12 h postinfection.

"Never fell below 50,000."
EGFP marking in all lineages was lost at 32 weeks posttransplantation. In the second animal (95E131), EGFP marking was lost in granulocytes, monocytes, platelets, and RBC at 26 weeks posttransplantation. Marking was observed only in the lymphocyte population at 26 weeks posttransplantation. To date, EGFP marking in the lymphocyte population was stable for 61 weeks.

Gene transfer of human $\gamma_c$. We further tested the potential of our lentiviral vector system by modeling gene transfer of human common $\gamma_c$. Mutations of the common $\gamma_c$ have been identified in X-linked SCID patients and have been shown to contribute to the impaired lymphocyte development in these patients (35). Transplantation of the CD34$^+$ cells that were transduced with retrovirus vector carrying the $\gamma_c$ cDNA has been shown to restore normal lymphocyte development and functions in X-linked SCID patients and animal models (7, 28). We inserted human $\gamma_c$ cDNA in the place of EGFP cDNA in the CS-Rh-MLV-E vector. Human common $\gamma_c$ expression from the vector was confirmed in HeLa cells and rhesus macaque primary PBMC in vitro (Fig. 4). Rhesus macaque immunoselected PB CD34$^+$ cells were transduced twice a day for 2 days with the lentivector bearing human common $\gamma_c$ on non-tissue-coated six well plates treated with the recombinant fibronectin fragment CH-296 (RetroNectin) without further cytokine stimulation ex vivo. Two animals were transplanted with autologous PB CD34$^+$ cells transduced with the HIV-1 vector bearing human common $\gamma_c$. The gene transfer of $\gamma_c$ was determined by quantitative DNA PCR analysis to be 0.42 and 0.001 copies/100 cells (Fig. 5). Cell surface expression of $\gamma_c$ was determined by flow cytometric analysis on the rhesus macaque lymphocyte population (Fig. 6). Expression of the $\gamma_c$ has been stable for 27 weeks.

DISCUSSION

The modeling of gene therapy vectors in nonhuman primate model systems is critical for evaluation of potential efficacy in the clinical setting. In addition, the recent development of lentivirus and retrovirus vectors has led to safety concerns that can best be addressed in nonhuman primate and murine animal model systems including SCID-NOD (4, 8, 15, 16, 26, 30, 31, 37, 41) SCID-hu (1, 3), rhesus macaque (3, 5, 9, 11, 17, 18, 19, 20, 23, 40, 42, 46, 48, 49), and baboon (21, 22).
internal CMV promoter to demonstrate multilineage marking in rhesus macaques. Those animals were transplanted with ex vivo cytokine-stimulated CD34+ cells transduced with the lentivirus vector. Marking of multiple hematopoietic lineages was achieved in up to 3% of the cells. To date, those animals are stably transduced, nearly 28 months following transplant, and are healthy. Here, we use a self-inactivating lentivirus vector bearing an MLV-related promoter to demonstrate marking in rhesus macaques. Circulating leukocytes from rhesus macaques transduced with CS-RhMLV-E or HR’CMVEGFP were evaluated for fluorescent intensity of EGFP expression by flow cytometry. Granulocyte, monocyte, lymphocyte, RBC, and platelet populations were identified and gated according to size (forward scatter) and granularity (side scatter) and analyzed for EGFP expression. Representative results from CS-RhMLV-E-transduced rhesus macaque 95E132 (top, 18 weeks posttransplant) and HR’CMVEGFP vector-transduced rhesus macaque RC505 (bottom, 82 weeks posttransplant) are shown. The x axis represents logarithmic fluorescent intensity of EGFP; the y axis represents the forward scatter. Fifty thousand events were acquired for flow cytometric analysis. Samples were analyzed with a FACSCalibur machine (Becton Dickinson) under identical settings.
multiple lineages of hematopoietic cells. The CD34+ cells were not stimulated in vitro prior to transduction. The levels of gene expression are significantly higher than that of the non-self-inactivating lentivirus vector utilizing an internal CMV promoter. The level of marking is stable for approximately 15 months to date for those animals transplanted with mobilized PB CD34+ cells. All animals are healthy. Thus, this study represents the first demonstration of the use of lentivirus vectors to transduce non-ex vivo cytokine-stimulated CD34+ cells in a primate.

The results of this study and our previous study (3) indicate considerable variability in the extent of marking between different animals. Such results are consistent with those observed by other investigators using other model systems where the extent of long-term marking with different vectors varies considerably but in most cases is less than 10% (5, 9, 11, 17, 18, 20, 23, 40, 42, 46, 49), with a few exceptions (21, 48, 49). The MOIs of CD34+ cells used here and in our previous study are estimated to be approximately 5. Therefore, it is likely that the extent of marking reflects the relative extent to which progenitor cells are transduced. Given the differences in methodologies for transplant and transduction and use of different animal systems, it is difficult to compare our results with those of other groups. However, we can contrast our results with one other study (by Donohue et al. [11]) and others, where an oncoretrovirus vector expressing EGFP was used for rhesus macaque transplant under similar transplant conditions and in the same facility as that described here, except that the CD34+ cells were stimulated with IL-6, SCF, and fit-3 before transduction. In that study, a transient peak of marking was observed within a few weeks following transplant, most markedly in monocyte and granulocyte lineages, up to as high as 55% marking. The levels of marking in these cells then decreased significantly to

![FIG. 4. In vitro transduction of CS-RhMLV-huγc vector. Normal rhesus macaque PBMN (10^6/ml) were stimulated with immobilized anti-monkey CD3 antibodies, human IL-2, and human CD28 for 2 days as described in Materials and Methods. HeLa cells (5 × 10^5) or the stimulated PBMN (5 × 10^7) were infected with CS-RhMLV-huγc vector at MOIs 100 and 10, respectively, as determined by infection of HeLa cells. Three days postinfection, cells were stained with anti-human γc antibodies conjugated with PE as described in Materials and Methods. Ten thousand events were collected for flow cytometric analysis. The percentage of γc-positive lymphocyte populations is indicated in the upper right of each panel. The x axis represents log fluorescent intensity of γc expression; the y axis represents the forward scatter.](image1)

![FIG. 5. PCR analysis of human γc-transduced rhesus macaque hematopoietic cells following transplantation. DNA from rhesus macaque PBC was analyzed for the presence of HIV-1 vector DNA by PCR at 7 and 5 weeks after reconstitution of rhesus macaques 90E068 and 95E025, respectively. HIV-1 vector DNA-specific signal was compared to that of the amplified β-globin DNA signal to determine the number of vector copies per 100 cells (HIV-1 vector DNA copies/100 cells, calculated as number of HIV-1 vector DNA copies/number of cell equivalents × 1/10 × 100). For PCR amplification, 10-fold less DNA was used for β-globin DNA standards (std) in order to obtain quantitative β-globin DNA signals. Quantitative HIV-1 vector DNA and β-globin DNA standards were assayed in parallel with DNA from a nontransduced rhesus sample (Mock); no HIV-1 vector signals were detected for the latter.](image2)
cells do not have genomic DNA, the EGFP must be sufficiently
marking was also observed in RBC and platelets. Since these
lineages or differential silencing in some lineages. Interestingly,
relate to greater transduction of progenitors for granulocytic
HIV-1-based vectors. The reasons for this are unclear but may
moter and therefore appears to be a consistent property of
study using the HIV-1-based vector bearing the CMV pro-
two animals in this study and for four animals in a previous
lymphocyte populations is indicated in the upper right of each panel. The x axis is log fluorescent intensity of \( \gamma_c \) expression; the y axis represents the forward scatter.

less than 0.1%, with long-term maintenance observed only in
the lymphocyte subpopulation by several months following
transplant. Although transient marking was observed in RBC
early following transplant, no significant long-term marking
was observed. This kinetics of marking with the oncoretrovirus
vector contrasts with that observed with the lentivirus vectors.
Both here and in our previous study (3), we did not observe any
early transient increase followed by a decline in marking.
Rather, the level of EGFP marking rose steadily, peaking
within 4 to 5 weeks following transplant and maintained over
time. Donohue et al. (11) hypothesized that the early transient
marking was the result of infection of committed progenitor
cells with less self-renewal capacity. It is possible that the
differences observed may reflect the ability of lentivirus vectors
to more effectively transduce pluripotent hematopoietic stem
cells, thought to be in a more quiescent state.

In contrast to mobilized PB, transduction and transplant of
mobilized BM cells did not result in efficient marking. Indeed,
in the two animals, marking gradually declined, with loss of
marking in most lineages by 26 (95E131) and 32 (96E041)
weeks after transplant. There was approximately a 3-week time
difference in the rate of reconstitution utilizing PB CD34+
cells compared to BM CD34+ cells. Thus, these results may be
due to more efficient reconstitution utilizing PB CD34+ cells or
alternatively mobilization of CD34+ target cells from BM to
the PB. Our results are consistent with those observed in hu-
mn clinical studies (36).

The level of marking observed by EGFP expression is con-
sistently higher in the granulocyte lineages followed by mon-
cytes and lymphocytes. This pattern is observed both for
the two animals in this study and for four animals in a previous
study using the HIV-1-based vector bearing the CMV pro-
moter and therefore appears to be a consistent property of
HIV-1-based vectors. The reasons for this are unclear but may
relate to greater transduction of progenitors for granulocytic
lineages or differential silencing in some lineages. Interestingly,
marking was also observed in RBC and platelets. Since these
cells do not have genomic DNA, the EGFP must be sufficiently
stable to be retained in these cells for detection. The capability
to express proteins in RBC and platelets raises a number of
potential therapeutic strategies for potential correction of de-
ficiencies in these hematopoietic lineages.

Having developed conditions for transplant and marking, we
tested those strategies with a potential human therapeutic
gene. Recently, Cavazzana-Calvo et al. demonstrated therapeu-
tic benefit in humans following transplant of \( \gamma_c \) into X-
linked SCID patients, using a murine retrovirus vector (7). We
therefore used that gene to model potential human therapeutic
gene transfer strategies utilizing the lentivirus gene transfer
approach described here. We achieved marking and expression
of \( \gamma_c \) using the lentivirus vector in lymphocytes of the rhesus
macaque, utilizing non-cytokine-stimulated CD34+ cells. Al-
though the levels are relatively low, we anticipate that similar
to the transplant into X-linked SCID patients with an oncoret-
rovirus vector (7), under conditions of selective pressure, the
\( \gamma_c \) transduced cells would be expanded. Thus, these studies in
nonhuman primate animals provide model strategies and the
basis for the future application of lentivirus vectors to poten-
tially treat human diseases.

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The first two authors contributed equally to this work.
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