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Prolonged Adherence of Human Immunodeficiency Virus-Derived Vector Particles to Hematopoietic Target Cells Leads to Secondary Transduction In Vitro and In Vivo▽

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Human immunodeficiency virus type 1-derived lentivirus vectors bearing the vesicular stomatitis virus G (VSV-G) envelope glycoprotein demonstrate a wide host range and can stably transduce quiescent hematopoietic stem cells. In light of concerns about biosafety and potential germ line transmission, they have been used predominantly for ex vivo strategies, thought to ensure the removal of excess surface-bound particles and prevent in vivo dissemination. Studies presented here instead reveal prolonged particle adherence after ex vivo exposure, despite serial wash procedures, with subsequent transduction of secondary target cells in direct and transwell cocultures. We explored the critical parameters affecting particle retention and transfer and show that attachment to the cell surface selectively protects virus particles from serum complement-mediated inactivation. Moreover, studies with nonmyeloablated murine recipients show that transplantation of vector-exposed, washed hematopoietic cells results in systemic dissemination of functional VSV-G/lentivector particles. We demonstrate genetic marking by inadvertent transfer of vector particles and prolonged expression of transgene product in recipient tissues. Our findings have implications for biosafety, vector design, and cell biology research.

Cellular receptors for retrovirus envelope (Env) glycoproteins participate in initial adherence and virus particle uptake (9, 25). A wide range of Env glycoproteins are available for pseudotyping of recombinant retrovirus vector particles as a means of restricting species and tissue specificity, thereby allowing cellular targeting (40). Cell surface attachment of virus particles to target cells has also been shown to occur without receptor-ligand specificity (36), and most applications rely on serial wash steps after ex vivo exposure to remove bound, noninternalized particles prior to injection in animal models (19, 29).

Human immunodeficiency virus (HIV)-derived recombinant vectors pseudotyped with the vesicular stomatitis virus G (VSV-G) Env glycoprotein provide an extended host range and have proven particularly well suited for the genetic modification of postmitotic tissues and rarely dividing cells (6, 8, 23, 39, 40). Their application for the stable modification of hematopoietic cell populations has permitted the development of ex vivo culture protocols that combine efficient transduction with retention of target stem cell properties (1, 16, 18, 20, 21, 24). Moreover, ex vivo vector exposure permits prior target cell enrichment and avoids systemic vector dissemination, as might be seen after systemic injection of vector particles.

In studies using recombinant retrovirus particles bearing alternate pseudotypes, others have previously demonstrated prolonged binding after ex vivo exposure of cardiomyocytes that was resistant to washing procedures, with resultant tissue transduction from attached particles after local injection in the brain (5). We reasoned that the mechanisms underlying this observation might be more widely applicable and undertook the studies reported here. Specifically, we wished to investigate the potential for prolonged adherence and subsequent "hand-off" of VSV-G/lentivirus particles from vector-exposed hematopoietic target cells. We studied critical parameters governing particle retention and release kinetics in vitro and explored systemic particle dissemination after intravenous injection of vector-exposed—and washed—hematopoietic cells. To bias against overly sensitive detection in vivo, our experimental design with a mouse model deliberately avoided the radiation conditioning of recipients. This was predicted to prevent any substantial engraftment of vector-exposed "carrier" cells and preclude excessive tissue regeneration as a mechanism driving enhanced lentiviral transduction of tissues in vivo (27).

In the results presented here, we show that inadvertent particle transfer to secondary targets occurred across a wide range of experimental conditions. Further, gene transfer to host tissues was seen in nonmyeloablated immunocompetent murine recipients, a finding that may have wide-ranging implications.

MATERIALS AND METHODS

Lentivirus vector production. Vector was produced by transient transfection of HEK 293T kidney fibroblasts (293T cells) seeded at a density of 1.6×10^8 per 15-cm tissue culture dish precoated with 0.01% poly-L-lysine (Sigma), as previously described (18). The lentivirus transfer vector pRRL SIN EF1α cPPT EGFP wpre LoxP (pWPXL-EGFP) was kindly provided by D. Trono, Geneva, Switzerland. Four-plasmid transfection with packaging (pMD-Lg/p-RRE, pRSV-Rev) and envelope (pMD2.G) helper plasmids was carried out, followed by a medium change 16 h later with Dulbecco's modified essential medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin (Pen/Strep) (all from Gibco, Carlsbad CA), and 20 mM HEPES, pH 7.05 (Sigma). Vector supernatant was harvested 24, 36, and 48 h later, filtered through a 0.45-μm-pore-size filter, pooled, and concentrated 100-

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fold by ultracentrifugation. Vector titers were determined as described previously (14) and ranged from 2×10^7 to 5×10^7 293T transducing units/ml. All vector batches were stored at −70°C until use.

Cell culture and retroviral transduction. Fibroblast cell lines (293T and NIH 3T3 cells) were propagated in DMEM supplemented with 10% FBS and 1% Pen/Strep. Whole bone marrow (WBM) cells were prestimulated overnight in Iscove's medium supplemented with 10% FBS, penicillin-streptomycin, and 50 ng/ml murine stem cell factor (Peprotech, Rocky Hill, NJ). Sixteen hours later, 1×10^6 live cells per well were plated in RetroNectin-coated (2 μg/cm^2; Takara Mirus, Madison, WI) non-tissue-culture-treated six-well plates and were exposed to lentivirus vector, pWPXL-EGFP, in the presence of protamine sulfate (4 μg/ml) at various multiplicities of infection (MOI). The final well volume was 1 ml of Iscove-based, mSCF-supplemented medium. Following transduction culture, cells were washed in 1× phosphate-buffered saline (PBS) for 10 min (Gibco) and spun at 1,200 rpm. This wash was repeated once more.

For transwell experiments, 5×10^5 transduced and washed WBM cells were placed on transwell inserts (24 mm diameter, 0.4 μm pore size; Corning) suspended in six-well plates plated with 1×10^5 293T cells. For direct coculture conditions, transduced and washed WBM cells were placed in the well alongside 1×10^5 293T cells (1.5 ml, final volume). Nonadherent cells and media were aspirated 12 to 24 h later (as indicated), and six-well plates were refed with complete DMEM until flow cytometric analysis. For experiments to exclude protein transfer ("pseudotransduction"), vector-exposed, washed WBM cells were cocultured directly or in a transwell setup with 293T cells for 24 h. At the end of coculture, transwells were removed and well media were exchanged. Cells were propagated in culture for 2 weeks, and aliquots were taken at the indicated time points for flow cytometric analysis of green fluorescent protein (GFP) expression. For calculations of infectious virus particles carried over from transduced and washed WBM cells, supernatant was recovered after various time points and various volumes were added directly to 1×10^5 293T cells for titer determination, as described above (14).

Murine plasma complement studies. For in vitro studies involving murine complement, plasma from untreated C57BL/6 mice was collected at sacrifice in anticoagulant-coated microtainers, cleared of cellular components by low-speed centrifugation, and stored at −20°C until use. Experiments involved preincubation of vector particles in neat plasma for 1 h at 37°C with subsequent placement in transduction culture with 293T cells or addition (for 1 h) of plasma to target cells previously exposed to vector followed by subsequent coculture (1 ml, final volume; six-well plates) of 293T cells. In all cases the medium was exchanged 24 h later.

Flow cytometry. GFP expression in cells was analyzed 48 to 72 h after transduction with a FACSCalibur instrument (BD Biosciences), and data were processed with FlowJo software (Tree Star, Ashland, OR). To exclude bias due to persistence of GFP-expressing hematopoietic cells in coculture conditions, a phycoerythrin (PE)-conjugated anti-CD45-PE antibody was used. All samples were resuspended in PBS–2% FBS containing 1 μg/ml propidium iodide solution to exclude dead cells from the analysis. For serial follow-up studies after transplantation, WBM and peripheral blood underwent hemolysis, and leukocytes were stained with primary anti-CD45.2 (immunoglobulin G [IgG] isotype) antibody at 4°C for 30 minutes and washed twice in 2% FBS-PBS. Cells were then stained with secondary APC-labeled rat anti-mouse IgG antibody at 4°C for an additional 30 minutes and again washed twice (all antibodies were from BD Biosciences Pharmingen, San Diego, CA). GFP emission was detected in FL-1, PE emission was detected in FL-2, and APC emission was detected in FL-4.

PCR assay. DNA was extracted from cells isolated from retro-orbital or saphenous vein bleeds and tissue samples processed with the QiaAmp DNA Mini kit in accordance with the manufacturer's protocol (QIAGEN Inc., Valencia, CA). Real-time PCR probe and primer sequences specific for the GFP transgene—5′-FAM-CCGACAAGCAGAAGAACGGCATCA-TAMRA-3′; sense, 5′-ACTACAACAGCCACAACGTCTATATCA-3′; antisense, 5′-GGCGGATC TTGAAGTTCACC-3′)—were purchased from ABI (Perkin-Elmer Applied Biosystems, Foster City, CA). The GAPDH murine endogenous control 20× primer-probe set was used according to the manufacturer's instructions (Perkin-Elmer Applied Biosystems). DNA from WBM cells isolated from nontransplanted animals was used as a negative control, while DNA from a single-copy clone of NIH 3T3 GFP-transduced cells was used as a positive control. All PCRs were set up in a MicroAmp Optical 96-well reaction plate (Applied Biosystems), and samples were run in duplicate for both GFP and GAPDH amplification. All cycle threshold (C_T) values for the GFP transgene were normalized by the C_T values for the endogenous GAPDH control, and the GFP copy number was corrected relative to the single-copy positive control. Reactions were run with ABI Taqman Universal PCR Mastermix (Applied Biosystems) on the ABI Prism 7300 sequence detection system (Applied Biosystems) under the following thermal cycling conditions: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The spectrum was then analyzed with ABI Sequence Detector, version 1.3. Semiquantitative PCR was performed with GFP sequence-specific primers (sense, 5′-CAA GGG CGA GGA GCT GTT GAC C-3′; antisense, 5′-TGT GGC GGA TCT TGA AGT TCA CC-3′) under the following thermocycler (Gene Amp 9700; Applied Biosystems) conditions: denaturing 95°C for 10 min, followed by 35 cycles of 95°C for 1 min and 58°C for 0.5 min, followed by 68°C for 1.5 min, with a final extension at 68°C for 10 min. Amplified sequences were resolved on a 1% agarose gel and imaged under UV exposure after ethidium bromide staining.

Animal husbandry, transplantation, euthanasia, and organ collection. Mice (C57BL/6 and Boy J-B6.SJL) were group housed and maintained at 23°C on a 12-h light/dark cycle (0700 to 1900 h light). Mice were allowed ad libitum access to standard chow pellets (Purina Laboratory Rodent Diet 5001; Ralston Purina Co., St. Louis, MO). WBM cells were collected by flushing of femurs and tibias from 8- to 12-week-old Boy J mice (CD45.1) with Iscove's modified Dulbecco's medium (IMDM). Cells (0.5×10^6 or 1×10^6) were exposed to lentivirus vector for 1 h (MOI, 5), washed twice in PBS as described, resuspended in 200 to 300 μl of Hanks buffered salts solution, and injected into the tail vein of recipient mice (CD45.2). Following transplantation, retro-orbital eye bleeds were performed at scheduled intervals and white blood cells were analyzed for GFP expression by flow cytometry. Additionally, cell aliquots were resuspended in lysis buffer for DNA extraction (QiaAmp kit; QIAGEN, Valencia, CA). At sacrifice for organ harvest, animals were administered an intraperitoneal injection of standard mouse cocktail (30 mg ketamine, 2.9 mg xylazine, 0.6 mg Ace/1 ml) in an aliquot of 8.5 ml (dose, 0.1 ml intraperitoneal per 25 to 30 g weight). All studies were conducted according to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Oregon Health and Science University.

Immunohistochemistry and hematoxylin-eosin stains. Animals were deeply anesthetized before undergoing transcardial perfusion with ice-cold 0.9% saline/heparin solution, followed by 4% paraformaldehyde (PFM) in 0.01 M PBS. Organs were harvested and then postfixed in 4% PFM solution at 4°C overnight, followed by incubation in 20% (wt/vol) sucrose/PBS solution at 4°C overnight. Following overnight incubation, organs were removed from the solution and stored at −80°C until further processing. Fractions of each organ were harvested, and genomic DNA was extracted with the QiaAmp DNA Mini kit (QIAGEN), according to the manufacturer's protocol for GFP quantification. Sections from perfused organs (25 μm) were cut on a sledge microtome and stored free-floating in 0.01 M PBS containing 0.1% sodium azide. The sections were then incubated for 1 h at room temperature in blocking reagent (5% normal goat serum in 0.01 M PBS containing 0.3% Triton X-100). After the initial blocking step, the sections were incubated in rabbit anti-GFP antibody (Molecular Probes, Carlsbad, CA) diluted 1:5,000 in blocking reagent overnight at 4°C, followed by incubation in goat anti-rabbit Alexa 488 (Molecular Probes) diluted 1:1,000 for 1 h at room temperature. For costains, sections were again prepared in blocking reagent (5% normal donkey serum in 0.01 M PBS containing 0.3% Triton X-100) for 1 h at room temperature and incubated in rat anti-CD45 antibody (BD Biosciences Pharmingen, San Diego, CA) diluted 1:5,000 in blocking reagent overnight at 4°C. This was followed by incubation with donkey anti-rat Alexa 594 antibody (Molecular Probes) diluted 1:1,000 for 1 h at room temperature. Between each stage, the sections were washed thoroughly with 0.01 M PBS. At the end of the incubations, the sections were mounted onto gelatin-coated slides and coverslipped with gel-based fluorescence mounting medium (Vector Laboratories Inc., Burlingame, CA). Images were captured with a Nikon epifluorescence inverted microscope with a SPOT-2 digital charge-coupled-device (CCD) camera (Diagnostic Instruments) or a Zeiss epifluorescence inverted microscope (Carl Zeiss MicroImaging, Inc.) with an ORCA-ER CCD camera (Hamamatsu Corporation). Any adjustments were applied to the whole image.

Enzyme-linked immunosorbent assay for detection of HIV-1 p24 (Gag). Each vector batch used was tested prior to use. In addition, for each transplanted animal, serum was obtained early after SCT and at sacrifice for determination of p24 (Gag) protein to exclude the presence of replication-competent virus (34). Samples were run in duplicate with a commercial assay (Perkin-Elmer, Boston, MA) according to the manufacturer's instructions, with a lower limit of detection at 4.3 pg/ml. All samples tested negative.

Statistical analysis. Numerical results are expressed as averages plus or minus standard deviations (SD). Data were analyzed with the paired two-tailed Student *t* test. *P* values of less than 0.05 were considered significant.

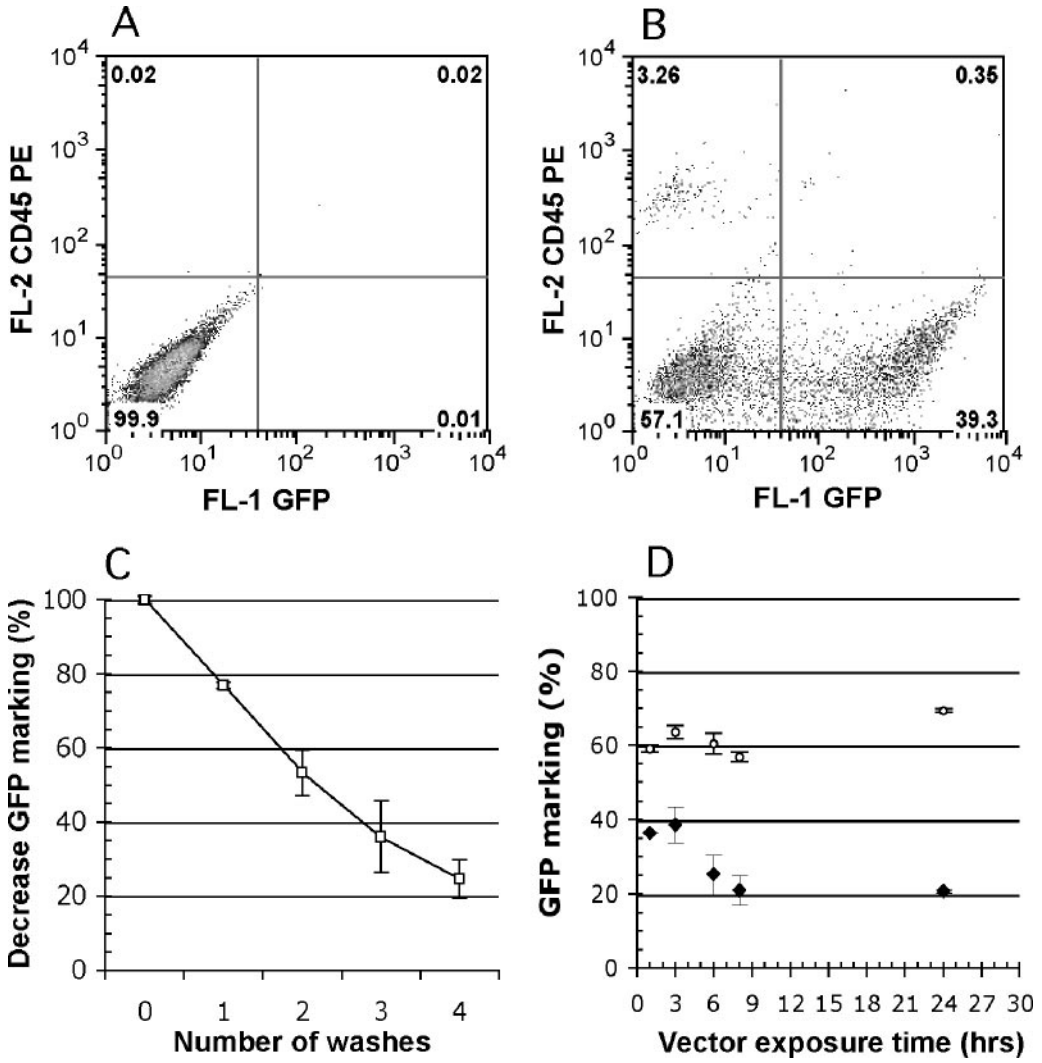

FIG. 1. GFP expression in 293T cells after coculture with lentivirus-exposed murine WBM cells. 293T cells (1×10^5) were cultured without (A) or with (B) VSV-G/lentivector-exposed (1 h; MOI, 5) murine WBM cells (5×10^5). Vector-exposed cells were carefully washed twice in PBS before being placed in direct coculture with 293T cells. Flow cytometric analysis of cocultured cells 72 h later shows GFP expression in the CD45-negative cell population, indicating transfer of lentivector from vector-exposed hematopoietic cells to 293T cells as "secondary targets." Numbers denote the percentage of events in the respective quadrant. (C) Gene transfer to cocultured 293T cells decreases with serial PBS washes of primary targets. Primary cells exposed to lentivector (5×10^5 cells; MOI, 5; 1 h) were sequentially washed, and aliquots were placed in coculture with 293T cells. The figure shows that GFP marking in 293T cells declining with subsequent washes. Gene transfer (*y* axis) is expressed as a percentage of GFP marking in 293T cells cocultured with vector-exposed WBM cells that were not washed. Flow cytometric analysis was performed 72 h after initiation of coculture. (D) The figure shows GFP marking (%) in primary WBM cells (open symbols) cultured in IMDM supplemented with mSCF and 293T cells (after direct coculture with WBM cells; closed symbols) 72 h later. Gene transfer to primary cells and subsequent transfer to secondary targets (293T cells) decline with extended vector incubation time. WBM cells were exposed to lentivector (5×10^5 cells; MOI, 5) for various periods of time, as indicated, followed by washing and coculture over the next 24 h with 293T cells. Averages and SD of multiple determinations are shown. All experiments were repeated twice, with similar results.

RESULTS

Enveloped retrovirus vectors are capable of binding to the target cell surface, not always resulting in particle uptake and successful genomic integration of the provirus. We wished to determine the extent to which prolonged adherence and vector particle transfer to secondary target cells occurred after ex vivo transduction culture of hematopoietic cells with VSV-G-pseudotyped HIV-derived lentivirus vectors.

VSV-G lentivector particles adhere to primary target cells and retain infectivity in vitro. In initial in vitro experiments, we used VSV-G-pseudotyped lentivector at an MOI ranging from 1 to 5 to transduce whole murine bone marrow cells. Following a 1-h exposure, cells were carefully washed twice, resuspended, and placed in coculture alongside 293T cells. Substantial percentages of GFP-expressing events were noted 72 h later in the CD45-negative cell fraction (i.e., among 293T cells) (Fig. 1A and B). Cell culture conditions during coculture did not favor the survival of hematopoietic cells (no cytokine support, base DMEM), and we independently confirmed that this was not the result of loss of CD45

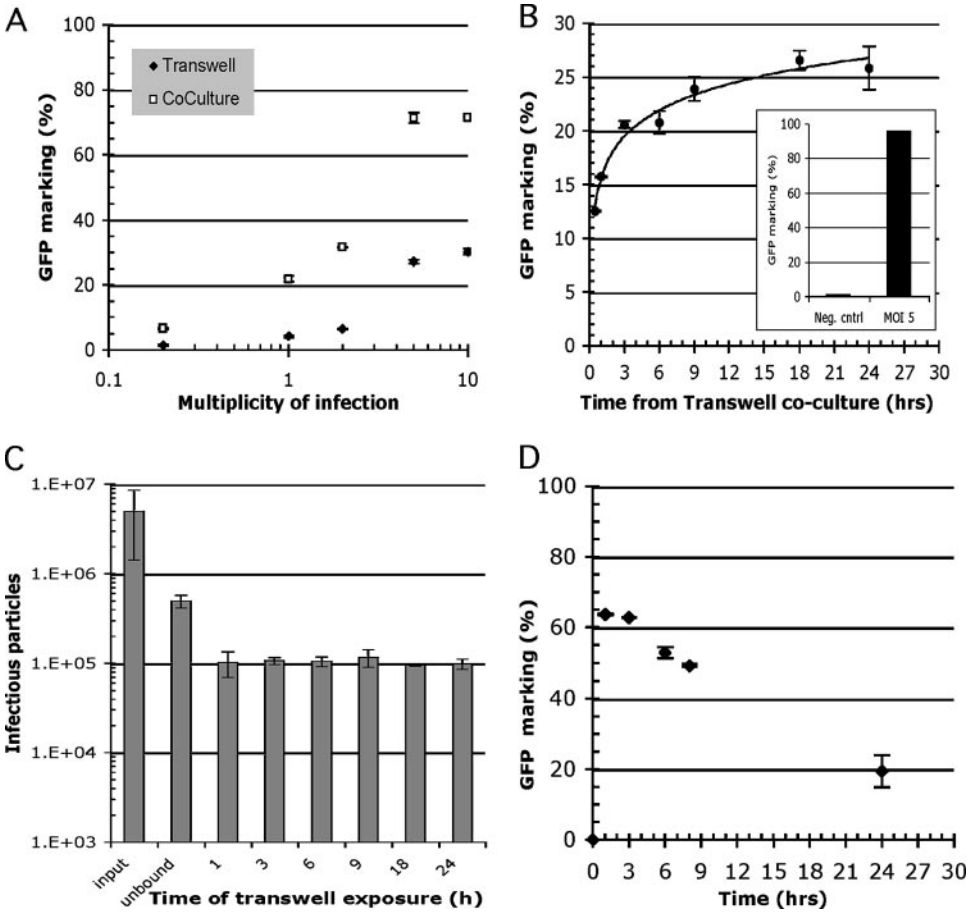

FIG. 2. Transfer of infectious lentivector to secondary target cells does not require cell-cell contact and increases with MOI and coculture time. (A) Primary WBM cells were exposed to escalating vector particle numbers of VSV-G/lentivector (5×10^5 cells, 1 h), washed twice in PBS, and cocultured with 293T secondary target cells (six-well plate; 1.5 ml final volume/well) for 24 h, either in direct contact (open symbols) or separated by a transwell membrane (0.4 μm) (closed symbols). (B) Virus particle and thereby gene transfer via transwell coculture occurs in an exponential manner over time and with saturating kinetics. Following a single vector exposure of WBM cells (5×10^5 cells; 1 h; MOI, 5), cells were washed twice, placed on transwell inserts, and kept in culture with 293T secondary target cells for various lengths of time. The panel shows GFP marking in 293T cells 72 h later. The panel insert shows the negative control (nontransduced) and gene transfer rate for direct exposure of 293T cells to lentivector at MOI of 5. (C) Average numbers of recovered particles preexposure (input), postexposure in cell-free medium, and after removal of transwell at the indicated serial time points after initiation of coculture. (D) Decreasing infectivity of vector particles kept at 37°C in complete DMEM prior to placement in culture for transduction of 293T cells (MOI, 3; 24-h particle-target cell exposure). Averages and SD of multiple determinations are shown. Experiments were repeated at least once with similar results.

expression among hematopoietic cells (data not shown). Further experiments illustrated the systematic decline in gene transfer to secondary targets with subsequent washes of the hematopoietic ("carrier") cells and confirmed that infectious particles remained cell associated and could be transferred even after four sequential washes in PBS (Fig. 1C). While a decrease in particle transfer (or rather, in successful secondary transduction) was noted with extended vector exposure times, transfer in direct cell-cell coculture was clearly not limited to short vector exposure times. Over a range of vector exposure times, gene transfer (Fig. 1D, open symbols) remained relatively stable early on and increased after 24-h vector exposure time, while hand-off and secondary transduction (closed symbols) initially declined and subsequently stabilized between the 9- and 24-h time points.

Transfer of infectious particles occurs without direct cell-cell contact. To determine whether particle transfer requires direct cell-cell contact, we used a transwell insert that places carrier cells on a support grid with a pore size restricting any cell movement across while permitting passage of vector particles to cells cultured on the well bottom. Results showed that vector particles that remain attached to primary target cells after sequential washes were released into the medium and did not require direct cell-cell contact to transduce secondary target cells (Fig. 2A). Across a range of MOI, the transduction frequency in secondary targets increased but was relatively reduced after transwell culture compared with direct coculture at the same MOI. Overall, particle transfer in transwell culture showed first-order exponential kinetics approaching saturation after ~24 h, perhaps reflecting an evolving equilibrium between the ongoing

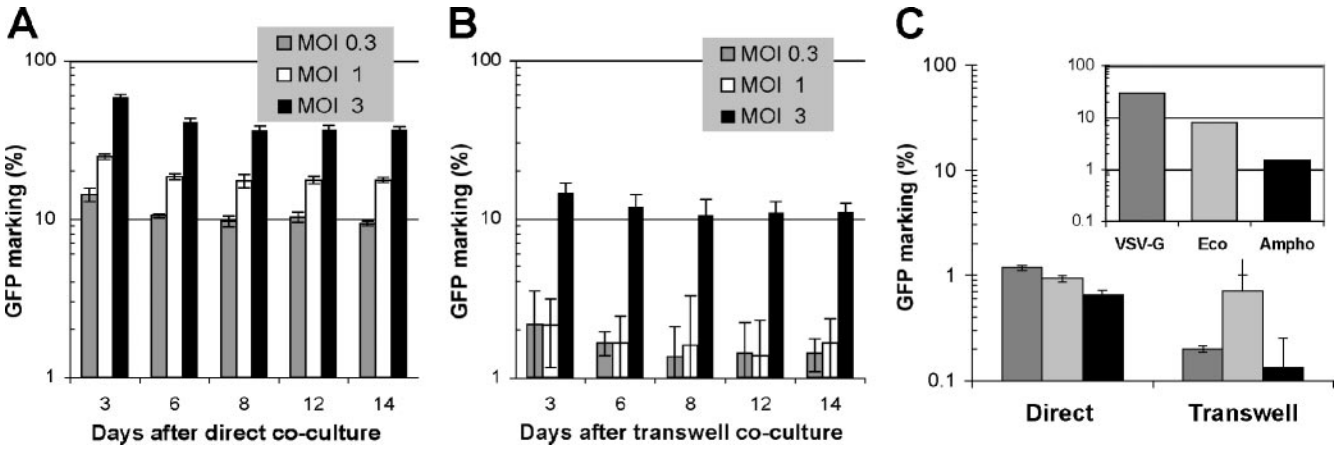

FIG. 3. Vector particle transfer and secondary transduction do not represent pseudotransduction and occur independently of pseudotype. (A) WBM cells were exposed to concentrated VSV-G lentivector at the indicated MOI for 1 h, washed, and placed in direct coculture with 293T cells followed by a medium change after 24 h. Expression of GFP in 293T cells was assessed by flow cytometry at the indicated intervals up to 2 weeks after transduction. (B) Vector exposure (as in panel A) was followed by transwell coculture for 24 h, transwell removal, medium change, propagation for 2 weeks, and interval flow cytometric analysis. (C) WBM cells were exposed to unconcentrated lentivector particles pseudotyped with amphotropic (Ampho), ecotropic (Eco), and VSV-G envelope (MOI, 1; six-well plate final volume, 1 ml) and washed twice in PBS prior to direct or transwell coculture, and they transduced secondary target cells (NIH 3T3 cells) with similar efficiency. All vector stock for this experiment was unconcentrated, with effective particle titers ranging from 0.5×10^5 to 1×10^5. The graphs show averages from multiple samples. The inset shows gene transfer after direct transduction of NIH 3T3 cells with identical vector particle numbers.

release of particles from primary targets (carrier cells) and particle decay kinetics in culture (Fig. 2B). Remarkably, enumeration of infectious particles recovered after the indicated intervals from media in transwell cocultures show a relatively stable number over time (Fig. 2C). This is in conflict with the half-life of 18 h we determined for neat vector particles in culture at 37°C (Fig. 2D). Notably, the observed retention and release were not specific to WBM cells but extended to lineage-depleted bone marrow progenitor cells and SupT-1 cells (data not shown).

Transgene expression in secondary targets requires provirus integration. Passive transfer of protein from producer cells along with vector particles (i.e., pseudotransduction) can result in transient expression in target cells. In an effort to confirm that the observed GFP expression in secondary 293T target cells resulted from transfer of intact vector particles and genomic integration of provirus, we investigated the stability of GFP expression in target cells over time. Vector-exposed murine bone marrow cells were cocultured with 293T cells for 24 h, followed by a medium exchange and subsequent passage for repeat interval analyses of GFP expression over time. Results show that gene transfer rates after direct (Fig. 3A) or transwell (Fig. 3B) coculture and at various MOI are largely stable when assayed at intervals over the course of 2 weeks following transduction. There is an apparent decline (~30%) in the percentage of GFP-expressing cells between 3 and 6 days after vector exposure at higher MOI and direct coculture. This may indicate a partial contribution of protein transfer to overall GFP expression under these conditions. By contrast, gene transfer over time at multiple MOI was stable after transwell coculture, suggesting that GFP expression predominantly relied on provirus integration and arguing against GFP expression in cocultured secondary target cells merely on the basis of protein transfer.

Vector particle attachment and transfer are not pseudotype dependent. We generated additional lentivector particles with both amphotropic and ecotropic Env pseudotypes to investigate whether attachment of lentivirus particles and hand-off to secondary targets were VSV-G pseudotype specific. Experiments showed that after a 1-h incubation with hematopoietic cells (and two sequential wash steps), both amphotropic and ecotropic particles could be transferred and transduced secondary targets (NIH 3T3 cells) in direct coculture or with transwells (Fig. 3C). Transduction efficiency was predictably lower than in experiments using highly concentrated VSV-G vector, reflecting the low titer of the unconcentrated vector preparations.

Infectivity is maintained after direct and indirect mouse plasma exposure. The biodistribution and efficiency of any potential secondary tissue transduction in vivo are limited by specific and nonspecific immune responses in the recipient. Serum complement has been shown to contribute to VSV-G vector particle inactivation (12). We performed a series of experiments to determine infectivity after vector preincubation in mouse plasma (obtained by removing cellular components from blood collected in anticoagulant-coated microtainers) or by the addition of plasma to transduction cultures. Results confirmed that VSV-G/lentivector particles are largely inactivated after plasma preincubation for 1 h at 37°C, although some residual infectivity was maintained across a range of MOI (Fig. 4A). Intriguingly, plasma incubation had no significant impact on the infectivity of vector particles attached to hematopoietic cells. In fact, subsequent secondary transduction of cocultured 293T cells was essentially unaffected by a 1-h incubation of particle-loaded cells with plasma (Fig. 4B). This suggests selective protection of cell-bound vector particles from plasma inactivation.

In sum, these in vitro experiments demonstrate attachment

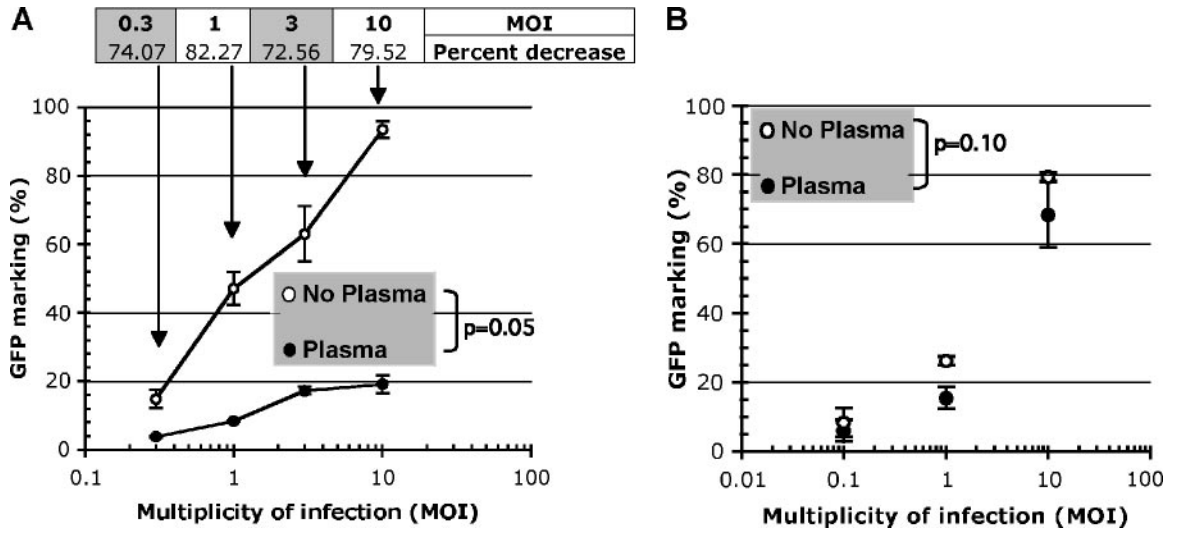

FIG. 4. Vector particles attached to target cells, but not those in suspension, are largely protected from mouse plasma complement inactivation. (A) The curves depict infectivity in comparing transduction of 293T cells at identical MOI in the presence (closed symbols) or absence (open symbols) of prior plasma preincubation of vector particles. The figure shows gene transfer rates determined by flow cytometric detection of GFP in 293T cells. Increasing vector particle numbers (expressed as MOI based on the number of 293T cells in transduction culture) were incubated with a stable volume of undiluted mouse plasma, resulting in a twofold maximum final dilution of serum (i.e., identical serum and vector volumes at maximum MOI). Particles were incubated for 1 h at 37°C before transfer to 293T cells cultured in six-well plates (1 ml of medium containing protamine sulfate at 4 μg/ml). Also noted is the percent decrease in infectivity at a given MOI. (B) Murine WBM cells were exposed to vector for 12 h at escalating MOI. Cells were then placed in undiluted plasma (closed symbols) for 1 h, followed by transfer to direct coculture with 293T cells in DMEM for 24 h. Control cells (open symbols) were treated identically but without plasma exposure. Gene transfer was measured by flow cytometric determination of GFP expression 72 h later. Plasma was obtained from routine blood draws by separation of plasma from peripheral blood cells by low-speed centrifugation and stored frozen at −20°C until use. Error bars denote SD from averages, based on multiple determinations. The experiment was repeated with similar results.

and transfer of infectious lentivector particles and secondary transduction across a range of vector exposure times, vector concentrations, and after direct incubation of vector-loaded cells with plasma.

Transplantation with ex vivo vector-exposed WBM cells leads to transduction of recipient bone marrow cells and transgene expression. To determine whether the observed prolonged adherence and subsequent "hand-off" of infectious lentivector particles leads to vector particle transfer in vivo, we next performed a series of transplantation experiments. The experimental design involved CD45 isotype-mismatched donor (CD45.1) and recipient (CD45.2) animals to positively identify transduction of recipient blood and marrow cells. We chose nonmyeloablated recipients to maintain immune surveillance and to avoid an overestimate of tissue transduction based on avid tissue regeneration and cell turnover after radiation injury. To maintain "carrier" cell numbers and particle load while minimizing the engraftment of primary target cells, we used whole bone marrow containing a limited number of potentially engrafting stem and progenitor cells. WBM target cells (1×10^6) were exposed in culture to vector at an MOI of 5 for 1 h, followed by two sequential 10-min washes, resuspension, and intravenous injection. In total, 15 animals in two independent experiments received injections of vector-exposed, washed WBM cells. Animals were monitored with serial blood draws beginning 3 to 4 weeks after cell injection until the time of sacrifice and organ harvest at 9, 16, or 22 weeks (experiment 1) and 18 weeks (experiment 2) following cell injection, respectively. Results show a small but persistent population of CD45.2-positive, host-derived, GFP-expressing cells in the peripheral blood that persists and slightly increases over time (Fig. 5). The GFP fluorescence intensity appeared low but remained stable over time. We therefore analyzed median fluorescence intensities (MFI) in leukocyte subsets of 10 animals at three time points after transplantation and in vector dose escalation studies in vitro. Results indicate that GFP fluorescence from the EF1α-GFP expression cassette is vector dose responsive in vitro and persists in vivo in leukocytes from all animals over time (data not shown). Further, to exclude fusion of vector-loaded donor and host hematopoietic cells, we evaluated bone marrow from a subset of animals ($n = 4$) at sacrifice 22 weeks after transplantation along with an untreated control. Results show a small fraction (<1%) of CD45.1- and CD45.2-staining, double-positive events in the experimental cohort and the untreated control animal. None of these events in treated animals expressed GFP, suggesting low-level nonspecific antibody binding rather than cell fusion (data not shown). To further determine the potential transduction of cells present in the bone marrow of recipients, we obtained marrow cells from animals at sacrifice to analyze for GFP marking in bone marrow hematopoietic (CFU-C) and mesenchymal (CFU-F) cell progenitors. Results show GFP-expressing methylcellulose progenitor colonies from one animal harvested 16 weeks after injection of vector-exposed (and washed) cells that are representative of those from other animals (Fig. 6A and B). We also found GFP-expressing CFU-F derived from multiple animals, indicating transduction of nonhematopoietic (mesenchymal) progenitor cells in the recipient bone marrow (data not shown).

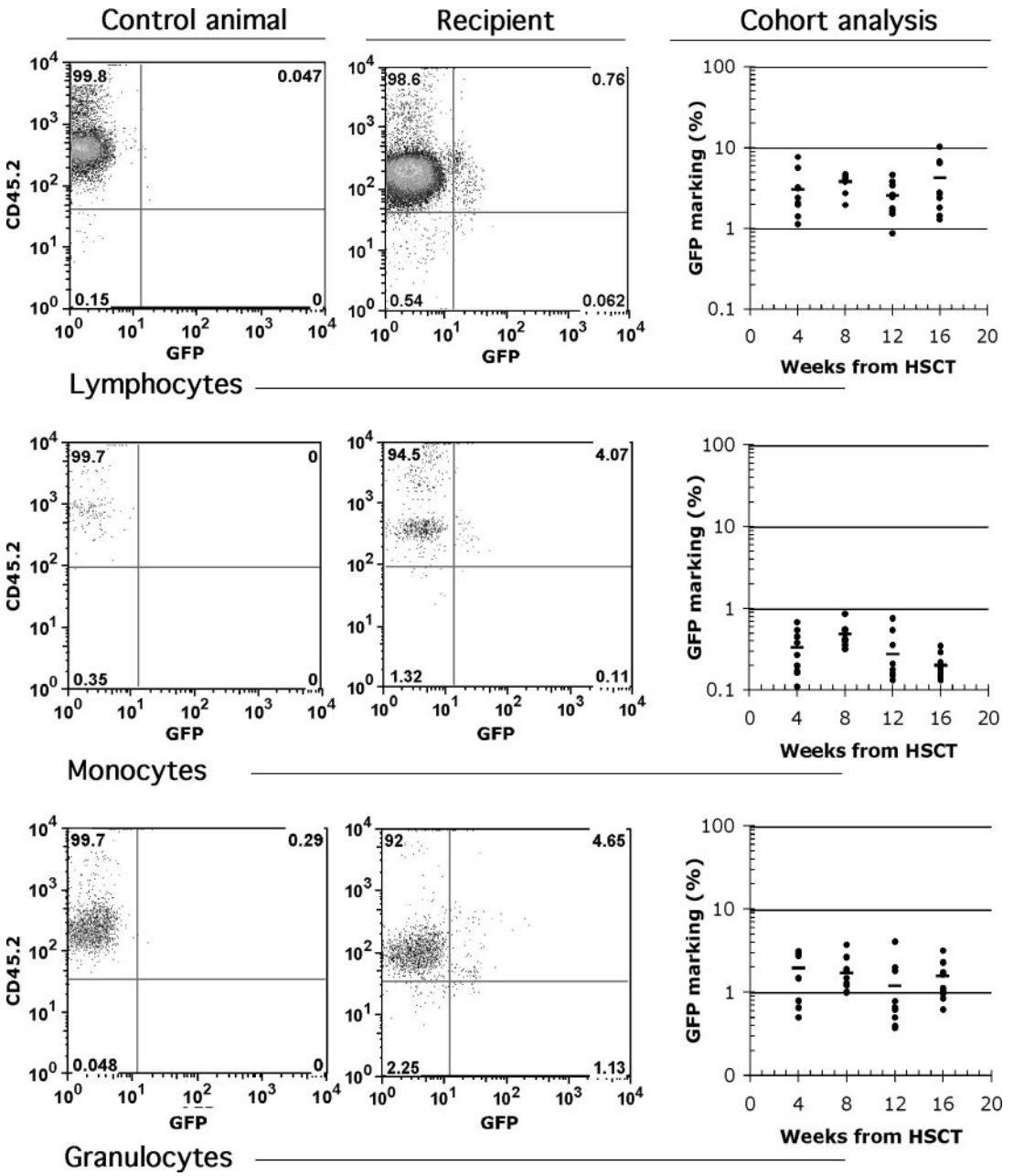

FIG. 5. GFP expression in host CD45.2 genotype peripheral blood leukocyte subsets in a representative animal 22 weeks after injection of lentivector-transduced WBM cells. Panels show flow cytometry dot plots from an untreated control animal (left-hand column), a treated animal (middle column), and the combined serial analysis in a cohort of five treated animals (right-hand column). Marking was analyzed separately for forward and side scatter (i.e., size)-gated events in granulocytes, lymphocytes, and monocytes. Between 0.5×10^5 and 2×10^5 events were collected for each file. Specific frequencies of GFP events per quadrant are noted in the corner of each panel quadrant. Crossbars in graphs of the right-hand column indicate average GFP marking frequency for the cohort.

Detection of GFP expression in spleen and liver by IHC. We next studied vector particle dissemination and host tissue transduction by immunohistochemistry (IHC) of spleen and liver sections. In an effort to minimize contamination by peripheral blood cells, animals underwent extended in vivo perfusion with heparinized saline and paraformaldehyde for fixation. To enhance microscopic detection of GFP expression in tissues and to avoid bias from GFP-expressing marrow-derived hematopoietic cells, we costained tissues with antibodies against GFP and CD45 (secondary antibody Alexa Fluor 594 labeled and individually detected in the red spectrum). Representative images show GFP expression in the spleen and liver with characteristic splenic stromal elements and liver parenchymal morphology, respectively (Fig. 6C to E). Not surprisingly, some GFP-expressing events (green) in the spleen costain with CD45 (overlay with the red spectrum, resulting in orange), indicating their hematopoietic origin. These IHC stains are representative of those from organs of multiple an-

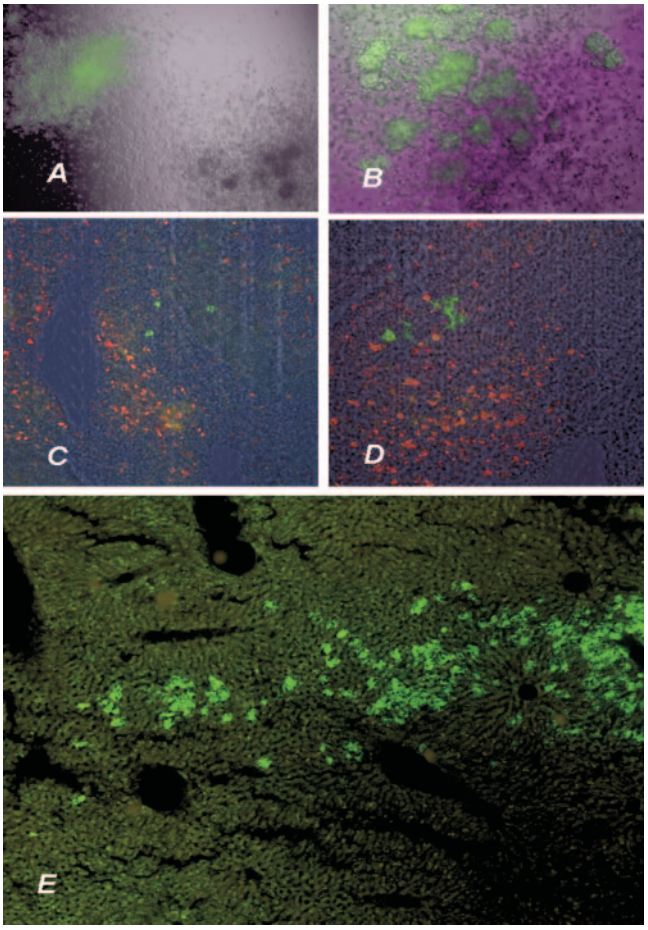

FIG. 6. GFP expression in murine tissues from animals 15 weeks after injection of GFP lentivector-modified hematopoietic cells in the absence of myeloablation. (A and B) Overlay microscope images (bright-field and GFP layer) of bone marrow progenitor colonies grown in methylcellulose. (C and D) Overlay images of immunohistochemical stains of spleen tissue (bright-field GFP, green; CD45, red; GFP plus CD45, orange). Images demonstrate GFP expression in hematopoietic and stromal elements of the spleen. (E) Overlay image (bright-field and GFP layers) of a liver section showing GFP expression in parenchymal cells. Images are representative of those obtained from multiple animals and tissue slices.

imals and consistent with transduction of host tissues by vector transmitted from injected lentivirus-exposed WBM donor cells.

Detection of provirus sequence in diverse tissues. Aliquots of peripheral blood, whole bone marrow, and multiple organs were processed for DNA extraction and subjected to PCR to detect integrated GFP provirus. Using semiquantitative PCR, we amplified GFP sequence in samples from the bone marrow, peripheral blood, and spleen of five animals sacrificed 22 weeks after injection of vector-exposed, washed cells (Fig. 7A). We also performed real-time PCR analysis on DNA extracted from various organs of three additional animals sacrificed at 16 weeks after injection of cells (Fig. 7B). Consistent with IHC images (Fig. 6C to D), there is relatively prominent transgene amplification in DNA from spleen samples. While we took care to perfuse animals with heparinized saline and formaldehyde prior to organ harvest, contamination with residual blood cells cannot be excluded and may have contributed to the detection of GFP in these samples. We next performed real-time PCR for serial analysis of GFP provirus in peripheral blood leukocytes in an additional cohort of five animals at monthly intervals over 3 months following injection of vector-exposed, washed cells. Results demonstrate persistent low-level proviral amplification over time (Fig. 7C). Finally, DNA was also amplified from pooled MSC progenitor colonies (i.e., CFU-F) and individual CFU-C colonies grown in methylcellulose and picked 14 days after plating (data not shown).

Exclusion of replication competency in recipients. In an effort to exclude gain of replication competency underlying the observed vector marking in recipients, we performed prior p24 (HIV Gag) testing on all vector lots by standard methods (34). In addition, we tested recipient peripheral blood samples from all animals involved in this study in duplicate assay samples and on two separate occasions, early after transplantation and within 2 weeks of sacrifice. No p24 (Gag) was detected in any of the samples analyzed.

DISCUSSION

HIV-derived lentivirus vectors provide a means for efficient transduction and heritable genomic integration in largely nondividing, quiescent stem cells. As a result of recent advances in vector design and by using VSV-G pseudotyping, hematopoietic stem cells can be readily transduced ex vivo and engraft following subsequent injection. However, not all binding after ex vivo exposure leads to cell entry, nuclear translocation, and proviral integration. Attachment of virus particles to the target cell surface can be nonspecific, without the requisite binding to a specific receptor or cellular uptake (30, 31, 35, 36). Such nonspecific binding is of considerable magnitude in vitro and likely contributes to dilution effects and limited target cell transduction efficiency after in vivo particle delivery (31). Indeed, in our own studies we recovered only ~10% infectious particles in the non-cell-associated fraction after a 1-h exposure of vector to primary hematopoietic cells, consistent with results by Cole and colleagues (10). Moreover, retention of particles on the cell surface can result in localized secondary transduction in recipients after direct injection of lentivector-exposed cardiomyocytes into brain and muscle tissue (5). However, direct extrapolation of those findings to protocols involving ex vivo gene transfer to and subsequent intravenous injection of washed hematopoietic cells is difficult, and the quality and quantity of any potential inadvertent transfer and secondary transduction in vivo under these circumstances is unclear.

Our initial studies herein confirmed inadvertent cell-cell transfer of particles from vector-exposed WBM cells to fibroblasts during subsequent coculture despite routine wash procedures. These studies, along with PCR results in transplantation studies, indicate that vector particle transfer results in proviral integration and that mere protein transfer (i.e., pseudotransduction) accounts for only a minor portion of GFP expression in recipient fibroblasts. We also found that particle transfer diminishes with additional posttransduction washes of primary cells but is not completely abolished, consistent with studies by Blomer and coworkers (5). Importantly, particles shed from primary targets (i.e., hematopoietic cells) remain

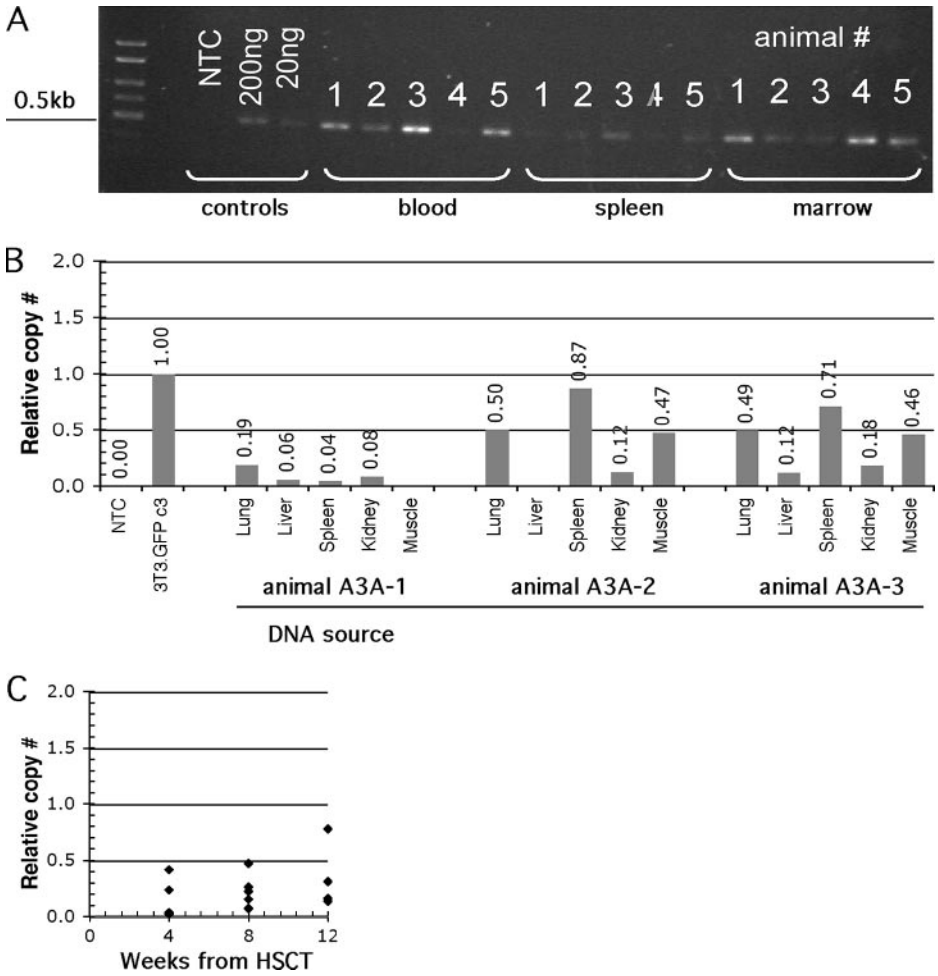

FIG. 7. Persistence of proviral sequence in host tissues. (A) Amplification of GFP sequence by semiquantitative PCR from hematopoietic tissues of five recipients sacrificed at week 22. Input template was adjusted to parity for all samples. Controls represent dilutions (20 ng, 200 ng) of genomic DNA extracted from a clonal NIH 3T3 cell line with a confirmed single GFP copy. NTC, nontemplate control. (B) Real-time PCR results for amplification of GFP sequence from genomic DNA extracted from tissues of three treated animals sacrificed at week 16. (C) Serial analysis of proviral marking by real-time PCR in a cohort of five recipients during the first 12 weeks following injection of vector-exposed, washed cells. A reference sample for real-time PCR is also single GFP copy clonal NIH 3T3 cell line. GAPDH amplification was used as an internal control for differential loading (nonmultiplexed).

fully infectious, and the transfer efficiency to secondary targets is a function of vector particle loading (i.e., MOI). Our observations further show that particle release after prolonged retention does not seem to require direct cell-cell contact, in contrast to results published by Cole. This is perhaps a reflection of alternate pseudotyping (amphotropic versus VSV-G) and primary target cell identity (OT-1 cells versus murine WBM cells). The data also show rapid release of vector particles from primary targets, reaching saturation over 24 h of culture. These kinetics suggest that the transduction efficiency among secondary target cells is the result of an evolving equilibrium between sustained release by carrier cells and the half-life of particles in culture at 37°C. In agreement with the literature, particle retention and release are not Env pseudotype restricted, and we show similar magnitudes of transfer and secondary transduction for amphotropic, ecotropic, and VSV-G pseudotype vector particles, adjusted for vector titer (30, 31). We also confirmed that the transduction of secondary target cells is only moderately sensitive to vector exposure duration and is not limited to the relatively brief exposure (1 h) in our experimental protocol, again consistent with studies by Cole (10). Serum components are known to rapidly inactivate retrovirus vector particles after intravenous injection in a complement-mediated and vector producer cell-specific manner (11, 12, 15, 33, 38). Indeed, the present studies demonstrate that inactivation of vector particles in vitro occurs rapidly (albeit incompletely) upon direct culture in mouse plasma at 37°C. Our in vitro data, however, also suggest that vector particles retained on primary target cells are largely protected from inactivation by plasma complement components. Taken together, these in vitro studies suggested that there was considerable potential for in vivo transduction of recipient tissues after intravenous injection of VSV-G/lentivector-exposed cells, even after standard post-vector-exposure washing procedures.

In designing our in vivo studies, we made a number of deliberate choices to bias the model against excessive sensitiv-

ity and in favor of specificity. These included (i) the use of CD45 isotype-mismatched donor cells (CD45.1) to document vector "hand-off" to host (CD45.2) hematopoietic cells. (ii) For transduction and transplantation, we used a relatively low number of 1×10^6 cells, (iii) not enriched for stem or progenitor phenotype, to minimize the absolute number of stem cells in the inoculum and thereby reduce the probability of long-term engraftment of transduced donor cells. Further, (iv) we did not irradiate recipients, to avoid tissue damage as a mechanism for promoting more avid viral transduction in nonhematopoietic tissue, as well as to minimize engraftment of injected cells in the marrow space. Finally, (v) we used a relatively low MOI of 5 to limit total vector particle numbers and carried out thorough serial wash procedures in PBS prior to injection.

These measures notwithstanding, results from flow cytometric and PCR analyses after transplantation consistently demonstrated low levels of circulating, vector-transduced blood cells in injected animals. Flow cytometric analysis revealed GFP-expressing CD45.2 (host isotype) events in blood, bone marrow, and spleens. While we could not categorically exclude rare events of donor-host cell fusion, this clearly did not account for the great majority of GFP-marked flow cytometric events. We noted relatively low levels of GFP fluorescence intensity in cells from these animals, likely related to known EF1α promoter performance characteristics (22) or perhaps as a result of selective immune elimination of cells with higher expression levels (32). Analysis of fluorescence intensities over time in leukocyte subsets of a large cohort of recipients indicated that fluorescence levels remained low but clearly persisted. MFI levels were consistent with values seen in vitro after transduction at low MOI. Clearly, transduction events in our in vivo model would be rare and would likely occur under conditions of a low vector particle number/target cell ratio. Consistent with FACS analysis, the presence of integrated provirus was confirmed by real-time PCR in DNA from multiple tissues, including serial analysis of peripheral blood leukocytes. Moreover, bone marrow harvested from these animals and plated in methylcellulose showed GFP expression from colonies with granulocytic and monocytic appearance, indicating progenitor cell transduction. Mesenchymal lineage progenitor colonies (CFU-F) were also established and showed rare, but distinct, GFP-positive colonies derived from multiple animals. Together, these studies demonstrate transduction of host marrow-derived hematopoietic and stromal elements after injection of ex vivo vector-exposed hematopoietic cells. Further, we demonstrated the detection of GFP by IHC and PCR in liver and spleen. Overall, the distribution of particles and resultant marking in these animals appear to be different from and less diffuse than those after IV injection of particles reported in other studies (7, 13, 26, 28).

Taken together, the experiments presented herein provide multiple lines of evidence for the inadvertent cell-cell particle transfer and transduction of secondary target cells after injection of ex vivo VSV-G/lentivector-exposed hematopoietic cells, in spite of serial wash procedures. The magnitude of vector particle attachment is likely related to the primary target cell identity, and prolonged retention and release may therefore vary substantially. However, while attachment to the primary target (i.e., carrier) cell is not pseudotype restricted, the susceptibility of secondary tissue targets remains subject to the specific interaction of vector envelope protein with its cognate cell surface receptor. Thus, for in vivo applications of vector-exposed cells, the combination of extended VSV-G tissue tropism and the ability of lentivirus vectors to transduce nondividing cells may lead to off-target transduction (3, 22, 37). Recent promising Env-based targeting approaches conferring tissue-restricted entry may be suitable in reducing off-target transduction in secondary tissues (41). Our experimental transduction culture and transplantation design is representative of other investigators' strategies for stem cell gene therapy, and the implications of our study go beyond issues of biosafety or the potential for vector transmission to the germ line. The disseminated, albeit mostly low-level, transduction of host tissues should be considered in studies using lentivirus to investigate cell tracking and fate, as well as those employing nonmyeloablative conditioning strategies in the context of stem cell gene therapy (2, 4, 17). Expression of the reporter gene in permissive tissues in vivo may not necessarily imply engraftment or a cell fate switch in such cases.

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