Novel lentiviral vectors displaying "early-acting cytokines" selectively promote survival and transduction of NOD/SCID repopulating human hematopoietic stem cells

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A major limitation of current lentiviral vectors (LVs) is their inability to govern efficient gene transfer into quiescent cells, such as human CD34$^+$ cells, that reside in the G_0 phase of the cell cycle and that are highly enriched in hematopoietic stem cells. This hampers their application for gene therapy of hematopoietic cells. Here, we designed novel LVs that overcome this restriction by displaying "early-acting cytokines" on their surface. Display of thrombopoietin, stem cell factor, or both cytokines on the LV surface allowed efficient gene delivery into quiescent cord blood CD34$^+$ cells. Moreover, these surface-engineered LVs preferentially transduced and promoted survival of resting CD34$^+$ cells rather than cycling cells. Finally, and most importantly, these novel LVs allowed superior gene transfer in the most immature CD34$^+$ cells as compared to conventional LVs, even when the latter vectors were used to transduce cells in the presence of recombinant cytokines. This was demonstrated by their capacity to promote selective transduction of CD34$^+$ cell in in vitro derived long-term culture-initiating cell (LTC-IC) colonies and of long-term NOD/SCID repopulating cells (SRCs) in vivo. (Blood. 2005;106:3386-3395)

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Introduction

For the correction of many inherited or acquired defects of the hematopoietic system, the therapeutic gene must be delivered to cells able both to self-renew and to differentiate into all hematopoietic lineages. As such, these gene therapies must be targeted to the "right" cell, the human hematopoietic stem cell (HSC), without modifying its properties. Based on nonobese diabetic/severe combined immunodeficiency (NOD/SCID) repopulating-cell (SRC) assays, defined as a measure for long-term human repopulating cells, HSCs in the G_0 phase represent the most primitive, uncommitted hematopoietic progenitors and, thus, the preferred targets for gene transfer.[1]

Retroviral vectors, in particular the emerging class of lentiviral vectors (LVs), represent the only means to transduce these cells permanently. LVs can integrate into the chromatin of nondividing cells such as neurons, fibroblasts, and hepatocytes,[2-5] whereas resting lymphocytes are refractory to LV-mediated gene transfer.[6-10] We and others have shown that stimulation into the G_{1b} phase of the cell cycle is sufficient to allow productive transduction of quiescent T cells with LVs, whereas these conditions are insufficient for transduction with murine leukemia virus (MLV)–based retroviral vectors.[6-10] Whether HIV or the LVs can integrate into the genome of a truly quiescent cell, in particular an early progenitor HSC in G_0, remains controversial.[9,11-17] Compared to their MLV counterparts, LVs do not need extended cytokine stimulation to transduce HSCs, avoiding strong cell proliferation, which might lead to loss of stem cell potential.[18] Although the LV-mediated transduction of HSCs within the CD34$^+$ cell population seems possible without cytokine stimulation, a clear boosting effect of strong cytokine cocktails on HSCs transduction was demonstrated.[11-14,17,19-21] Additionally, in combination with extended cytokine stimulation, protocols often rely on high vector doses per target cell, multiple vector hits, or an increase in virus/target cell contact by centrifugation or use of fibronectin fragments. Such maneuvers are likely to induce activation and differentiation of stem cells and to promote multiple vector integrations.

To avoid these problems, we refined the surface of LV particles via the display of "early-acting cytokines." We postulated that the new surface-modified LVs would selectively and minimally stimulate HSCs in the CD34$^+$ bulk population during gene transfer with the specific aim to promote high levels of transduction, indispensable for clinical application, in these targets. Thrombopoietin (TPO) and stem cell factor (SCF) were chosen as potent candidates because of their ability to favor the persistence, self-renewal, and even expansion of HSCs in an undifferentiated state.[22-30] Moreover, TPO and SCF act in synergism for these characteristics.[31,32]

Our results demonstrate that the new LVs, surface-engineered to display "early-acting cytokine," markedly outperform the current generation of LVs used in the absence and even in the presence of recombinant cytokines, for transduction of the most immature quiescent CD34$^+$ cells, including the critical HSCs, as defined by their capacity to repopulate NOD/SCID mice.

Materials and methods

Envelope construction

The TPO C-terminal truncation mutants (163 amino acids and 171 amino acids) were obtained by polymerase chain reaction (PCR) with primers encoding for the *Sfi*I and *Not*I sites at the 5′ and 3′ ends, respectively. The *Sfi*I/*Not*I PCR fragment was fused to the 4070A (amphotrophic) MLV env gene at position +1 of the surface SU subunit using the *Sfi*I/*Not*I backbone fragment of CMVOKT3SU.[7] The resulting chimeric glycoproteins were called TPO163SU and TPO171SU. The *Sfi*I/*Not*I fragment from TPO171SU was fused to the hemagglutinin (HA) env gene using the *Sfi*I/*Not*I backbone fragment of CMVEGFPHA. The SUx mutation,[6,7] that inhibits furin-mediated cleavage of the MLV glycoprotein, was inserted into the SCFSU construct, resulting in a second chimera, named SCFSUx. All chimeric env glycoproteins were expressed in the phCMV-G expression vector backbone.[33]

Production of retroviral vectors

Self-inactivating HIV-1–derived vectors were generated as previously described[7] by transient transfection of 293T cells. For codisplay of vesicular stomatitis virus G (VSV-G), SCFSUx, and TPOHA, 1.5 μg of each envelope plasmid was cotransfected with the Gag-Pol packaging construct 8.91 and the green fluorescent protein (GFP)–encoding HIV-1–derived SIN transfer vector Hloxcppt. Together with G/TPOHA env, a plasmid encoding neuraminidase was transfected to allow efficient release of virus from the producer cell.[34] The plasmid encoding neuraminidase was cotransfected in all other vector preparations as a control (VSV-G, G/TPOHA/SCFSUx, G/SCFSUx).

Cell survival by PI staining

BAF3-Mpl cells, expressing the TPO receptor c-mpl, were a gift from Isabelle Dusanter (Paris, France) and BAF3-cKit cells, expressing the receptor for SCF c-Kit, were a gift from Patrice Dubreuil (Marseille, France). Both cell lines are dependent on interleukin 3 (IL-3) for growth. BAF3-Mpl cells and BAF3-cKit cells were washed twice in phosphate-buffered saline (PBS) to remove IL-3 and make them exclusively dependent on TPO or SCF, respectively. They were plated at 5×10^4 cells/24 wells in RPMI/10% fetal calf serum (FSC) and incubated with decreasing doses of fresh LV supernatant or concentrated over a sucrose cushion (multiplicity of infection [MOI] = 20, 10, 4, 2, or 1) for 72 hours. Cells were stained with propidium iodide (PI; 1 μg/mL) in PBS and the amount of living cells was assessed by fluorescence-activated cell sorting (FACS) analysis.

CD34+ cord blood (CB) cells were seeded at 5×10^4 cells/24 wells in serum-free medium (CellGro; CellGenix, Freiburg, Germany) and incubated for 72 hours with LVs at MOIs of 20 or 4. After 72 hours of incubation the cells were washed and stained with PI in PBS to assess the percentage of living cells by FACS. Control incubations with VSV-G–pseudotyped vectors were performed in the absence or presence of recombinant TPO (rTPO; 10 ng/mL) or recombinant SCF (rSCF; 50 ng/mL).

Western blot analysis was performed as previously described.[7]

Sample collection and isolation of CD34+ cells

Umbilical CB samples from full-term pregnancies were collected in sterile tubes containing anticoagulant. Low-density cells were separated over Ficoll-Hypaque. CD34+ isolation was performed by means of positive selection using magnetic cell separation (Miltenyi MACs) columns according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity of the selected CD34+ fraction was assessed by FACS analysis with a phycoerythrin (PE)–conjugated anti-CD34 antibody (BD PharMingen, Milan, Italy) and exceeded 95% for all experiments.

Transduction assays

To determine the infectious titers of HIV-1–derived vectors, serial dilutions of vector preparations were added to HeLa cells. MOIs were determined on proliferating HeLa cells and are indicated in all transduction experiments.

CD34+ CB cells (5×10^4) were transduced with fresh LV supernatant at an MOI of 20 or 4 in serum-free medium (CellGro; CellGenix) in 48-well plates. At 24 hours, transductions were washed and resuspended in serum-free CellGro medium for a further 48 hours before transduction efficiency was determined by flow cytometry.

Cell-cycle fractionation by pyronin Y staining

To distinguish between cells in G_0 or $G_1/S/G_2+M$, RNA staining with pyronin Y (PY) was performed. Briefly, CD34+ cells were resuspended at concentration of 2×10^6 cells/mL in a buffer containing Hanks balanced salt solution, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 1 g/L glucose, and 10% FCS. PY was added at a concentration of 1 μg/mL and cells were incubated for 45 minutes at 37°C. Cells were washed once, resuspended in the same chilled buffer, analyzed, and sorted on a FACstar (Becton Dickinson, Heidelberg, Germany). The living CD34+ cells were gated and in this gate cells in G_0 were identified by their minimal RNA content, whereas cells in $G_1/S/G_2+M$ phase were defined as those with high or maximal PY staining, thus allowing isolation of viable CD34+ cells in G_0 or $G_1/S/G_2+M$. The 2 sorting gates were well separated. After each sorting experiment, the 2 populations were checked for purity by PY (RNA−)/7-amino-actinomycin D (7-AAD; DNA−) staining (Figure 4A)[16,17,19]

Clonogenic cell assays

Assays for colony-forming cells (CFCs) and long-term culture-initiating cells (LTC-ICs) were performed as described elsewhere.[16] After 14 days of culture in methylcellulose, GFP+ colonies were identified.

In vitro differentiation assays

We tested lymphoid and myeloid potentials of transduced CD34+ CB cells by culture on MS5 cells in Iscove medium containing 5% FCS or by culture in RPMI on MS5 cells in the presence of recombinant human IL-2 (rhuIL-2; 5 ng/mL), rhuIL-15 (10 ng/mL), and rhuSCF (50 ng/mL), 10% human AB serum, and 5% FCS for 15 days. The latter cultures were continued for 5 days in the presence of IL-3 (10 ng/mL) and erythropoietin (Epo; 2 U/mL) to assess erythroid maturation.

NOD/SCID repopulating assays

After a 72-hour (MOI of 20) or 24-hour (MOI of 4) transduction with LVs, CD34+ CB cells were injected by tail vein injection into sublethally irradiated (3.5 Gy) NOD/SCID mice without in vivo administration of cytokines. Six to 8 weeks after transplantation, the bone marrow (BM) from femurs was harvested and assessed for levels of GFP-expressing human cells. Cells were prepared for 3-color flow cytometry to detect the percentage of enhanced GFP (EGFP+) lineage-positive cells as described (see "Immunophenotyping by flow cytometry").

Immunophenotyping by flow cytometry

EGFP expression in the CD34+ CB cell population was analyzed 72 hours after transduction by flow cytometry after immunolabeling with an anti–CD34-PE (BD PharMingen, Milan, Italy) antibody. In vitro differentiated CD34+ cells were analyzed for expression of both GFP and differentiation markers, using the following mouse monoclonal antibodies: hCD19-PE, hCD15-PE, hCD14-PE, hGpA-PE (BD PharMingen, Milan, Italy), hCD56- and hCD34-PE-cyanin 5 (Cy5; Immunotech, Marseille, France). Three-color flow cytometry was used to detect GFP+ human cells of various lineages in NOD/SCID BM using anti–hCD45-cychrome and anti–hCD19-PE, anti–hCD14-PE, anti–hCD13-PE, and anti–hCD34-PE antibodies. In all cases, corresponding PE-conjugated mouse IgG controls were used.

Results

Functional display of SCF and TPO on HIV-1–derived vectors

To improve gene transfer into HSCs, we displayed 2 "early-acting cytokines," TPO and SCF, on the surface of lentiviral particles. We fused either of 2 TPO truncated forms (163 and 171 amino acids long), previously shown to have a 3-fold higher biologic activity than wild-type TPO,[35,36] to the N-terminus of the MLV envelope glycoprotein. These env chimeras are called TPO163SU and TPO171SU, respectively. Additionally, we fused the TPO171 form to the influenza hemagglutinin (HA) glycoprotein (TPOHA). Both fusion partners, the MLV and HA glycoproteins incorporate efficiently on LVs.[6,7,34] The second cytokine, SCF, was fused to the MLV env glycoprotein (SCFSUx).[37]

We then tested LVs displaying TPO163SU, TPO171SU, or TPOHA for functional display of TPO by incubation with BAF3-Mpl cells, which are dependent on rTPO for growth and survival in the absence of IL-3. LVs pseudotyped with either TPO-MLV env chimera (G/163SU and G/171SU) sustained BAF3-Mpl survival (Figure 1A). Survival was significantly reduced only at low doses of virus/cell. In contrast, we observed that TPO presentation on LVs, by fusion to the HA glycoprotein, ensured high BAF3-Mpl survival even at extremely low vector input. This emphasized the functional display of TPO by TPOHA equivalent to high doses of rTPO (Figure 1A). Thus, in all further experiments, the TPOHA chimera was used as the envelope of choice for surface display on LVs. SCF-displaying LVs (G/SCFSUx) efficiently promoted survival of BAF3-cKit cells at high MOIs and a bit less at low vector doses (Figure 1B). To evaluate a potential synergistic effect between SCF and TPO, we produced TPO/SCF-codisplaying vectors using the combination of VSV-G/TPOHA/SCFSUx glycoproteins. Clearly, the TPO/SCF-displaying vectors sustained survival of BAF3-Mpl and BAF3-cKit cells to the same extent as their corresponding single cytokine-displaying vectors. As expected, control incubations with VSV-G–displaying vectors in the absence of cytokines resulted in rapid cell death of both cell lines (Figure 1A-B).

Efficient incorporation of TPOHA and SCFSUx chimera on LV particles was demonstrated by immunoblot detection of highly purified viral pellets with antibodies against MLV glycoprotein and HA glycoprotein (Figure 1C). Because LVs displaying these chimeric envelope glycoproteins alone showed reduced infectivity, the chimera were incorporated together with the VSV-G protein in all experiments described. This did not affect the incorporation of the TPOHA chimeric envelopes on LV particles and only slightly reduced SCFSUx incorporation. Furthermore, through Western blot analysis of vector particles purified on sucrose-density gradients, we verified that the cytokine-displaying glycoproteins were detected in the fraction containing the viral particles and that such association to the viral surface was not disrupted on coexpression of VSV-G (data not shown). Additionally, G/TPOHA and G/SCFSUx LVs concentrated and purified by ultracentrifugation over a sucrose cushion could also promote the survival of BAF3-Mpl and BAF3-cKit cells, respectively (G/TPOHA-conc in Figure 1A; G/SCFSUx-conc in Figure 1B). Altogether, these results indicated that the biologic activity of the displayed cytokines was tightly linked to the viral particles.

Finally, we evaluated specific ligand-receptor interactions on the primary targets, immature CD34+ progenitor cells. Of utmost importance was that TPO-, SCF-, or TPO/SCF-displaying LVs efficiently protected CB CD34+ cells against apoptosis and this to
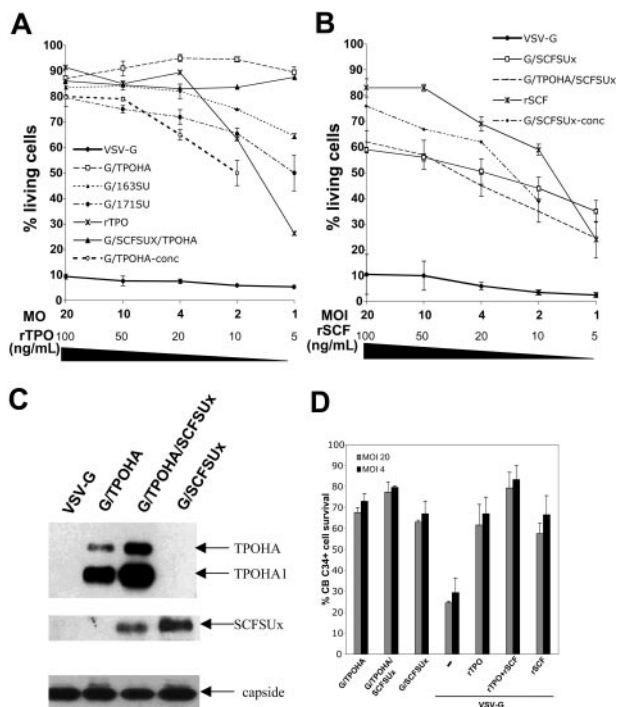

Figure 1. Functional display of TPO and SCF on HIV-derived vectors. (A) BAF3-Mpl cells expressing the TPO receptor were incubated with LVs presenting either of 2 different truncated forms of TPO at their surface. The TPO constructs were obtained by fusing the first 163 or 171 amino acids of TPO to the N-terminus of the amphotropic MLV glycoprotein (G/163SU and G/171SU) or by fusing the first 163 amino acids of TPO to the N-terminus of the influenza HA glycoprotein (G/TPOHA). The SCF construct was generated by fusing SCF to the N-terminus of the amphotropic MLV glycoprotein (SCFSUx). Incubations were also performed with vectors codisplaying TPO and SCF. After a 72-hour incubation with decreasing doses of vector (MOI ranging from 20 to 1), cell survival was determined and compared to that observed following incubation with VSV-G–displaying vectors alone or with rTPO (100 ng/mL to 5 ng/mL). G/TPOHA vectors were concentrated (G/TPOHA-conc) by ultracentrifugation and incubations were performed under the same conditions as for nonconcentrated TPO-displaying vectors. (B) BAF3-cKit cells expressing the SCF receptor were incubated with LVs presenting SCF at their surface by fusion to the amphotropic MLV glycoprotein (G/SCFSUx) or copresenting TPO and SCF at their surface. After a 72-hour incubation with decreasing doses of virus (MOI ranging from 20 to 1), cell survival was determined and compared to that observed following incubation with VSV-G–displaying vectors alone or with rSCF (100 ng/mL to 5 ng/mL). G/SCFSUx vectors were concentrated (G/SCFSUx-conc) by ultracentrifugation and incubations were performed under the same conditions as for nonconcentrated SCF-displaying vectors. (C) Immunoblots of LV particles displaying TPOHA or SCFSUx or both chimeric glycoproteins at their surface. Virions were purified over a sucrose cushion by ultracentrifugation. The upper part of the membrane was stained with antibodies against the influenza HA glycoprotein, the middle section with antibodies against MLV-SU to detect the TPOHA and SCFSUx envelopes, respectively. The lower part of the membrane was stained with antibodies against HIV-1 capsid to assess equivalent loading of purified vectors. The positions of the chimeric precursor protein (TPOHA), its processed isoform (TPOHA1), the SCFSUx protein, and the HIV capsid are indicated. (D) CD34+ CB cells were incubated for 72 hours with the indicated TPO-, SCF-, or TPO/SCF-displaying vectors at MOIs of 20 or 4. Survival of the cells was determined by PI staining. As control, CD34+ cells were incubated with vectors displaying VSV-G in the absence (−) or in the presence of cytokines (rTPO = 10 ng/mL; rSCF = 50 ng/mL). Data are shown as means ± SD, n = 4.

an extent equivalent to that observed following incubation with high levels of their recombinant cytokine counterparts (Figure 1D).

TPO/SCF-displaying LVs promote high-level gene transfer in CB CD34+ cells

We then assessed whether coupling of cytokine stimulation to transduction of CD34+ CB cells by TPO-, SCF-, or SCF/TPO-displaying LVs would be sufficient to promote efficient transduction of a marker gene encoding GFP. A single exposure to either
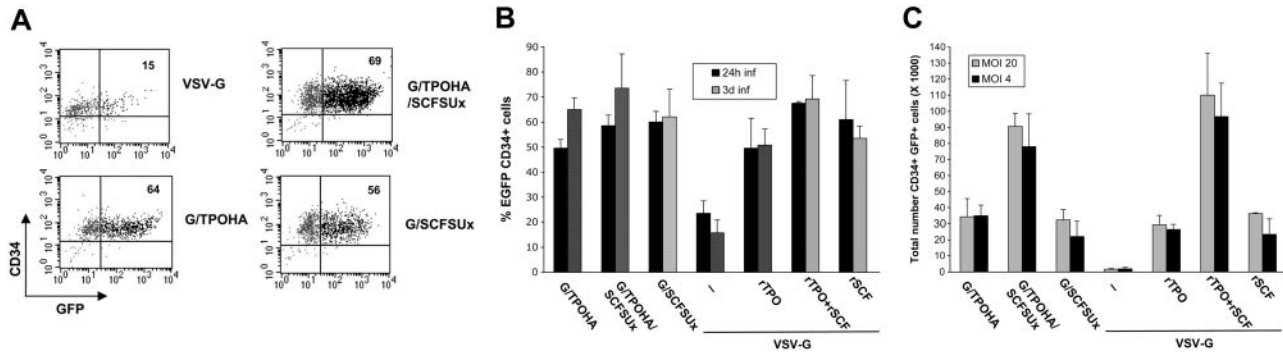
Figure 2. TPO-, SCF-, and TPO/SCF-displaying HIV-1–derived vectors promote high-level gene transfer into CB CD34+ cells. (A) CD34+ CB cells were incubated for 72 hours with VSV-G/TPO-displaying (G/TPOHA), VSV-G/SCF-displaying (G/SCFSUx), or VSV-G/TPO/SCF-displaying (G/TPOHA/SCFSUx) vectors at an MOI of 20. Cells were analyzed for the presence of the CD34+ surface marker and GFP expression by FACS analysis. The percentage of GFP+CD34+ cells is indicated into the upper right quadrant. The data presented are representative of 6 independent experiments. (B) CD34+ CB cells were incubated for 24 hours versus 3 days with TPO-, SCF-, or TPO/SCF-displaying vectors at an MOI of 20. Control incubations were performed with VSV-G–pseudotyped vectors in the absence or presence of cytokines (rTPO = 10 ng/mL; rSCF = 50 ng/mL). Cells transduced for 24 hours were washed and maintained for another 48 hours in serum-free medium in the absence of cytokines. Cells were analyzed for GFP expression 72 hours after the start of transduction by FACS analysis. Data are shown as means ± SD, n = 3. (C) CD34+ CB cells were incubated with TPO-, SCF-, and TPO/SCF-displaying LVs at MOIs of 20 and 4. Control incubations were performed with VSV-G–pseudotyped vector in the absence (−) or presence of recombinant cytokines (rTPO = 10 ng/mL; rSCF = 50 ng/mL). After a 72-hour incubation, the total number of transduced CD34+ CB cells was calculated (number of cells at start of infection [5×10^4] × cell expansion × % cell transduction × % cell survival; data are shown as means ± SD, n = 4).

TPO-, SCF-, or TPO/SCF-displaying vectors in the absence of serum or other stimuli promoted significantly high transduction levels in these cells as compared to VSV-G LVs (Figure 2A), which were less efficient as expected and reported previously.[19] Because CD34 is often used as a marker of early progenitors, we confirmed by counterstaining that the GFP+ cells retained CD34+ expression. Thus, TPO-, SCF-, or TPO/SCF-displaying vectors not only improved survival of CD34+ CB but also promoted high transduction levels of these cells, in a manner similar to VSV-G–pseudotyped LVs in the presence of recombinant cytokines (Figure 2B-C).

We sought to determine if a short exposure to TPO-, SCF-, or TPO/SCF-displaying LVs would be sufficient to render CD34+ CB cells more susceptible to gene transfer. CD34+ CB cells were exposed for 24 hours to the cytokine-displaying and VSV-G LVs, washed, and cultured for a further 48 hours in the complete absence of cytokines, vectors, and serum. Low transduction was detected following infection with LVs displaying VSV-G glycoprotein (G; 23.2% ± 4.9%), whereas transduction with cytokine-displaying vectors resulted in high CD34+ cell transduction efficiency (G/TPOHA, 49.5% ± 3.5%; G/SCFSUx, 60.0% ± 4.2%; G/TPOHA/SCFSUx, 58.5% ± 4.2%; Figure 2B). It should be recalled, however, that leaving the CD34+ cells for 3 days in the absence of cytokines compromised their survival as indicated in Figure 1D, whereas the cytokine-displaying vectors induced remarkable cell survival. Interestingly, no significant loss in transduction efficiency of CD34+ CB cells was detected for a 24-hour versus a 3-day incubation with TPO-, SCF-, or TPO/SCF-displaying vectors. Control incubations with VSV-G–displaying LVs in the presence of rTPO or rSCF resulted in equivalent transduction levels, as for the corresponding TPO-, SCF-, or TPO/SCF-displaying vectors (Figure 2B).

We then analyzed the absolute numbers of transduced CB CD34+ cells (calculated by: initial number of cells × transduction (%) × cell survival (%) × expansion; Figure 2C) after incubation with the LVs at MOIs of 20 and 4. Compared to unmodified LVs, TPO- or SCF-displaying vectors resulted in a higher number of transduced CD34+ CB cells (Figure 2C). Interestingly, LVs codisplaying SCF and TPO demonstrated a clear additive effect between the 2 cytokines, resulting in 3-fold more transduced CD34+ cells as compared to TPO- or SCF-displaying vectors. Even at very low vector input (MOI = 4), TPO/SCF-displaying vectors demon-strated this additive effect (Figure 2C). Additionally, TPO-, SCF-, or TPO/SCF-displaying vectors transduced equivalent number of CD34+ cells as unmodified LVs in the presence of their counterpart recombinant cytokines, rTPO or rSCF (Figure 2C). The significant increase in the number of transduced cells obtained with TPO/SCF codisplaying LVs compared to unmodified LVs resulted from a combination of higher transduction efficiency (Figure 2A-B), higher cell survival (Figure 1D), and slight expansion. Indeed, alternative transduction protocols using extensive stimulation by cytokines (TPO, SCF, Flk-3) led to over 10-fold expansion (mean = 10.10 ± 0.50; data not shown), whereas TPO-, SCF-, or TPO/SCF-displaying LVs resulted in low expansions ranging from 1.6- to 2.6-fold (G/TPOHA = 1.72 ± 0.26; G/TPOHA/SCF-SUx = 2.62 ± 0.49; G/SCFSUx = 1.65 ± 0.25) cell expansions, similar to incubations with low-dose rTPO or rSCF (data not shown).

To verify whether the high transduction efficiencies obtained for CD34+ CB cells persisted in their derived CFC colonies, CD34+ cells were incubated with TPO, SCF, TPO/SCF surface-engineered LVs and VSV-G LVs and subsequently plated in methylcellulose with cytokines that support outgrowth of human progenitors. Importantly, the percentage of initially transduced CD34+ CB cells with TPO, SCF, or TPO/SCF LVs was reflected in equivalent percentages of transduced CFCs (Figure 3). Again, the additive effect of TPO and SCF was reproduced because a 4-fold higher number of GFP+ CFC-derived colonies was detected for TPO/SCF-codisplaying vectors as compared to LVs displaying either cytokine (data not shown). Compared to these vectors, equivalent percentages of transduced CFCs were obtained for VSV-G–displaying LVs in the presence of rTPO or rSCF.

TPO/SCF-displaying LVs preferentially transduce and promote survival of CD34+ cells in G0

Most CD34+ cells from mobilized peripheral blood or BM with NOD/SCID repopulating potential, reside in the G0 phase of the cell cycle.[1] Because CD34+ cells from CB in either G0 or G1 contain SRCs,[38,39] we assessed the efficacy of gene transfer by the cytokine-displaying vectors in either population. CD34+ CB cells were stained with PY to discriminate between quiescent cells (G0), which have minimal RNA content, and those in early or late G1/S/G2+M (G1+), which are highly enriched in RNA
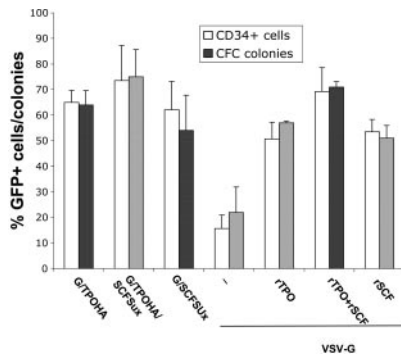
Figure 3. SCF/TPO surface-engineered LVs induce high levels of gene transfer in CB CD34$^+$ short-term CFCs. CD34$^+$ CB cells were incubated with TPO-displaying (G/TPOHA), SCF-displaying (G/SCFSUx), or codisplaying (G/TPOHA/SCFSUx) HIV-derived vectors for 72 hours. The gene transfer efficiency in the bulk CD34$^+$ cell population (% GFP$^+$ cells) and their CFC-derived transduced colonies (% GFP$^+$ colonies) are shown. Data are shown as means ± SD, n = 3.

(Figure 4A). We confirmed the purity of the sorted G_0 and $G_1/S/G_2+M$ populations by PY/7-AAD staining (Figure 4A). Both CD34$^+$ CB cell populations were incubated with TPO-, SCF-, or TPO/SCF-displaying vectors or VSV-G–pseudotyped vectors. In
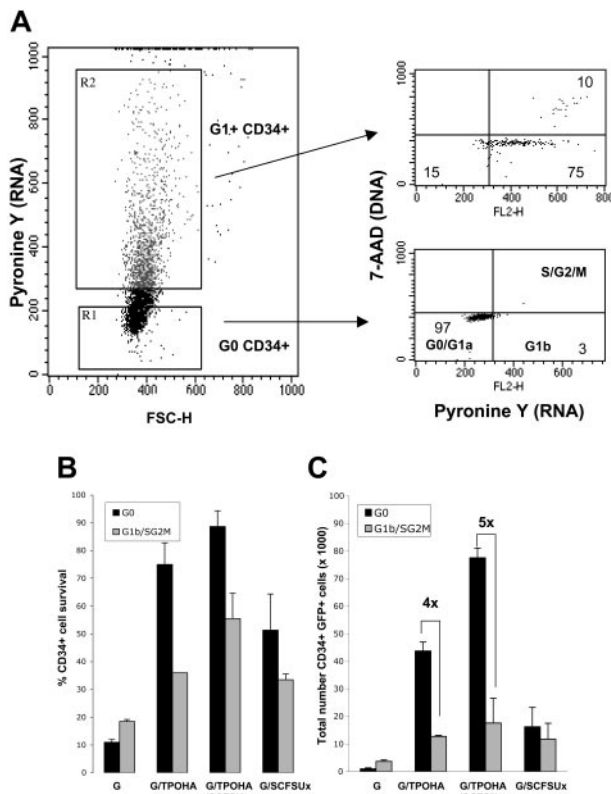
the absence of cytokines, we observed drastic cell death for the G_0-sorted cells after incubation with VSV-G LVs (G, 11% ± 1% cell survival), as expected, whereas TPO- and TPO/SCF-displaying vectors ensured a 7- to 8-fold higher protection of G_0 cells (G/TPOHA, 75.0% ± 7.8% cell survival; G/TPOHA/SCFSUx, 88.6% ± 5.6% cell survival; Figure 4B). SCF-displaying vectors induced survival of G_0 CD34$^+$ CB cells to a lower extent (G/SCFSUx, 51.3% ± 13.0% cell survival; Figure 4B). This emphasized that quiescent CD34$^+$ CB cells require minimal stimulation by "early-acting cytokines" for survival. The cytokine-displaying vectors even preferentially promoted survival of cells in G_0 over those that had entered into the cycle (Figure 4B), which probably depend on other additional cytokines for survival.

Most importantly, TPO-, SCF-, and TPO/SCF-displaying vectors transduced more efficiently the G_0-sorted cells than the cycling CD34$^+$ cells (Figure 4C). Indeed, a single exposure with TPO- or TPO/SCF-engineered vectors could, respectively, transduce 4- and 5-fold more CD34$^+$ cells of the resting cell population (G_0) than of the cycling population. Thus, the cytokine-displaying LVs allowed preferential transduction and survival of quiescent CD34 CB cells, a compartment highly enriched in HSCs.

TPO-, SCF-, and TPO/SCF-displaying LVs ensure superior transduction levels in immature CD34$^+$ cells and their progeny as compared to LVs in the presence of recombinant cytokines

As a measure of transduction of primitive progenitor/stem cells, LT-CICs were derived from the transduced CD34$^+$ CB cells. Remarkably, TPO-, SCF-, or TPO/SCF-displaying vectors resulted in a higher level of transduced LTC-IC–derived colonies as compared to VSV-G pseudotypes, even when the latter vectors were used in the presence of the recombinant cytokines (Figure 5).

Indeed, TPO- and TPO/SCF-displaying LVs resulted in a 2.5-fold higher transduction of LTC-IC colonies as compared to unmodified vectors in the presence of rTPO or rTPO and rSCF, respectively. SCF-engineered vectors even allowed 3-fold higher transduction of LTC-ICs as compared to unmodified vectors in the presence rSCF (Figure 5). This demonstrated that TPO-, SCF-, or SCF/TPO-displaying vectors transduced early progenitors, whereas unmodified vectors in the presence or absence of recombinant cytokines (rTPO or SCF or both) resulted in a lower transduction or quicker loss of these cells.

Importantly, we compared TPO-, SCF-, or TPO/SCF-displaying and unmodified VSV-G LVs in the absence or presence of rTPO or SCF for transduction efficiency of their CD34$^+$-derived progeny

Figure 4. TPO/SCF-displaying LVs promote transduction and survival of immature quiescent G_0 CD34$^+$ cells. (A) FACS analysis of CD34$^+$ CB cells stained with PY as described in "Materials and methods." Quiescent cells residing in the G_0 phase of the cell cycle (G_0 CD34$^+$) have minimal RNA content, as indicated by low PY staining, whereas cells residing into G_{1b}, S, G_2+M phase (G_1+) have a high RNA content. Sort windows to collect G_0 and G_1+ cells are shown in the dot blot (A) as R1 and R2, respectively. R1 and R2 were kept well separated to avoid contamination of the 2 cell categories. The G_0 population was checked for purity by 7-AAD (DNA)/PY (RNA) staining. The G_0/G_{1a}, G_{1b}, and SG_2M phases are indicated. In panels B and C, the G_0 and G_1+ CD34$^+$ populations were transduced for 72 hours with TPO-, SCF-, or SCF/TPO-displaying vectors or with VSV-G–pseudotyped vectors. The percentage of CD34$^+$ cell survival is indicated in panel B, and the numbers of GFP$^+$ CD34$^+$ marked cells (number of cells at start of infection [5 × 10^4] × cell expansion × % cell transduction × % cell survival) for the G_0 and G_1+ populations are shown in panel C. Data are shown as means ± SD, n = 3.

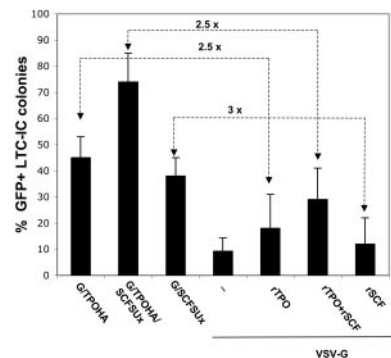**Figure 5. SCF/TPO surface-engineered LVs selectively promote gene transfer in CB CD34$^+$ LTC-IC–derived colonies.** CD34$^+$ CB cells were incubated with TPO-displaying (G/TPOHA), SCF-displaying (G/SCFSUx), or codisplaying (G/TPOHA/SCFSUx) HIV-derived vectors for 72 hours. The gene transfer efficiency in the CD34$^+$ LTC-IC–derived colonies (% GFP$^+$ colonies) are shown. Data are shown as means ± SD, n = 3.

Figure 6. Multilineage in vitro differentiation of transduced CB CD34⁺ cells. (A) In vitro differentiation of CB CD34⁺ cells transduced with TPO-, SCF-, or TPO/SCF-displaying HIV-derived vectors for 72 hours at an MOI of 20. Control incubations with VSV-G–displaying HIV vectors in the absence (−) or presence of recombinant cytokines (rTPO = 10 ng/mL; rSCF = 50 ng/mL; Flk-3 = 100 ng/mL) for 72 hours were performed. The obtained cell lineages are the results of a 2-week in vitro lymphoid culture in Iscove medium on MS5 cells. In this culture, the total number of transduced B cells (CD19⁺ cells) and residual progenitors (CD34⁺ cells) for 3 independent experiments are shown. The absolute number of transduced differentiated cells is calculated, for example, for B cells as percent CD19 in total population × percent transduction × expansion of the cells after culture. The number of transduced granulo-monocytes in a lymphoid/myeloid culture on MS5 in the presence of FCS, IL-2, IL-15, and SCF is shown for 2 independent experiments. (B) In vitro differentiation of CB CD34⁺ cells transduced for 72 hours with TPO-displaying LVs or VSV-G–displaying vectors in the presence of rTPO (10 ng/mL). Differentiation was performed in 2 steps: (1) lymphoid/myeloid culture in presence of FCS, SCF, IL-15, and IL-2 on MS5 cells for 15 days, followed by (2) culture in the presence of Epo and IL-3 for 5 days. After step 1 of culture, differentiation into natural killer cells (CD56⁺) and B cells (CD19⁺) is shown. After step 2 of culture, maturation into erythrocytes (GPA⁺) and residual progenitors (CD34⁺) are indicated. For each cell lineage the GFP⁺ cells are indicated in the upper right quadrant (data presented are representative of 3 different experiments).
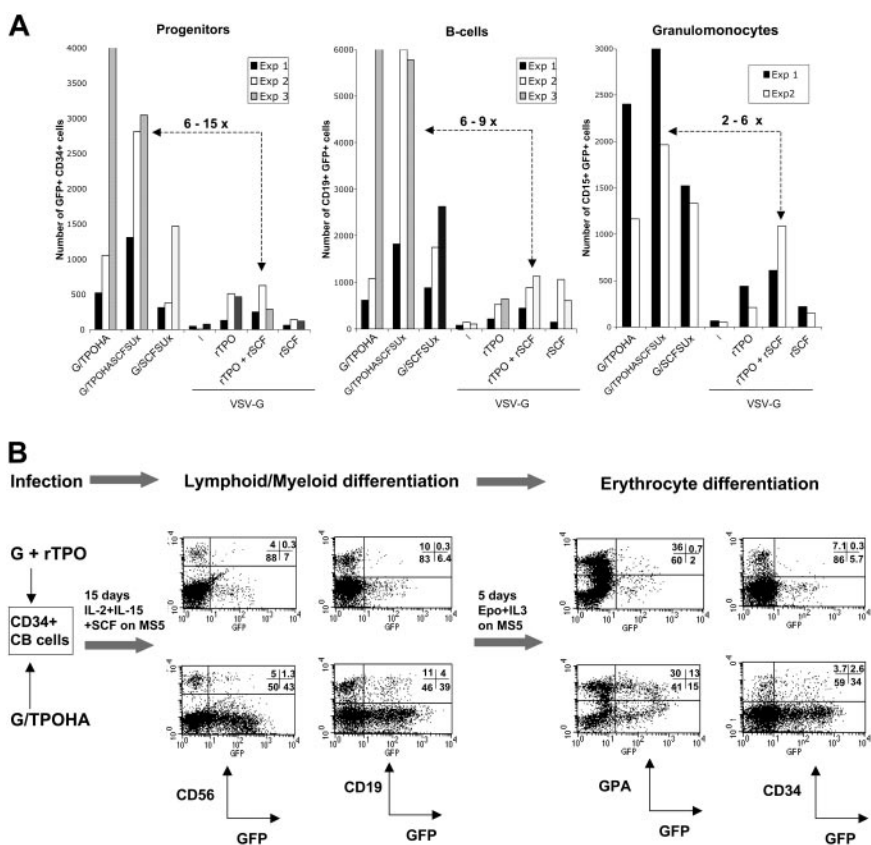
into various hematopoietic lineages to evaluate if the earliest progenitors, HSCs, were transduced in the total CD34⁺ population. Both TPO-, SCF-, and TPO/SCF-displaying LVs and unmodified LVs used with recombinant cytokines gave rise to equivalent expansion and multilineage differentiation after the in vitro cultures. However, after in vitro differentiation, regardless of the lineage marker considered, the level of transduced cells was consistently higher for the TPO-, SCF-, TPO/SCF-displaying LVs as compared to unmodified LVs in the presence of recombinant TPO or SCF (Figure 6A-B).

TPO/SCF-codisplaying vectors allowed transduction of up to 15-fold higher number of progenitors, up to 9-fold higher number of B cells, and up to 6-fold higher number of monocytes as compared to VSV-G–pseudotyped vectors in the presence of rTPO and rSCF. Similarly increased transduction levels in progenitors and differentiated cells were found for TPO- and SCF-displaying vectors as compared to unmodified vectors used with either recombinant cytokine (Figure 6A-B). Cell transduction assays performed in the presence of a stronger cytokine cocktail (rTPO + rSCF + rFlk-3) resulted, at best, in transduction levels equivalent to those obtained with TPO-, SCF-, or TPO/SCF- displaying vectors (data not shown).

In addition, we found that TPO-displaying vectors resulted in highly superior transduction levels than LVs in the presence of rTPO after lymphoid/myeloid culture (Figure 6B; G/TPOHA, 20% GFP⁺CD56⁺ cells and 26.6% GFP⁺CD19⁺ cells versus G+rTPO, 6.9% GFP⁺CD56⁺ cells and 3% GFP⁺CD19⁺ cells). After further maturation of these cultures in erythrocytes, a highly increased number of transduced CD34⁺ early progenitors were still present in the cultures for the cytokine-displaying vectors as compared to transduction with LVs in presence of recombinant cytokines (Figure 6B and data not shown; G/TPOHA, 41% GFP⁺CD34⁺

versus G+rTPO, 4% GFP⁺CD34⁺). In summary, much higher transduction levels were found in all CD34⁺-derived lineages for the cytokine-displaying vectors as compared to LVs in the presence of recombinant cytokines. Additionally, these new vectors showed a superior transduction of LTC-ICs, indicating that very early progenitors were transduced.

TPO-, SCF-, and TPO/SCF-engineered LVs selectively promote survival and transduction of NOD/SCID repopulating human HSCs

Because TPO-, SCF-, and TPO/SCF-displaying LVs promoted high transduction levels of CD34⁺ cells accompanied by very limited expansion, this might imply that differentiation of CD34⁺ HSCs is limited during the gene transfer procedure and that the long-term repopulating capacity of these transduced cells is better preserved in vivo. Thus, to address this question, we transplanted CD34⁺ CB cells transduced with the different LVs into sublethally irradiated NOD/SCID mice. The efficiency of gene transfer in SRCs was determined 6 to 7 weeks after transplantation. For the cytokine-displaying vectors and the unmodified vector in the presence or absence of recombinant cytokines a short transduction protocol at low vector doses (24 hours at MOI of 4) was performed (Figure 7A; Table 1). For the cytokine-displaying vector we additionally performed a 72-hour transduction at higher vector doses (MOI of 20).

As indicated in Table 1, display of cytokines on LVs allowed efficient engraftment of transduced human CD34⁺ cells in NOD/SCID mice. The long-term transduction (72 hours) resulted in a more variable and lower repopulation efficiency than the short one (24 hours). Interestingly, NOD/SCID repopulation with human CD34⁺ cells transduced by TPO/SCF-displaying vectors was similar to that of cells transduced with VSV-G LVs in presence of
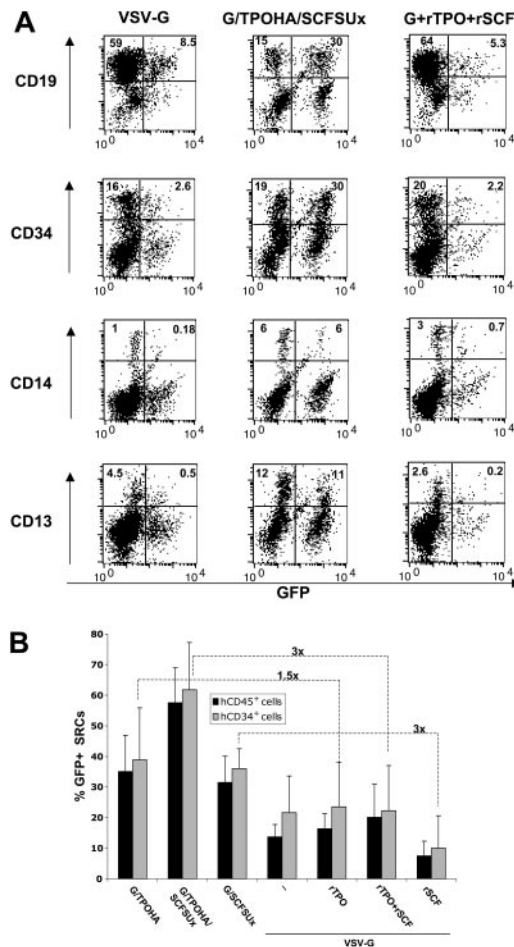
Figure 7. Preferential transduction of NOD/SCID repopulating cells by TPO- and SCF- or TPO/SCF-displaying LVs. NOD/SCID mice that received 2×10^5 CD34+ CB cells transduced with TPO- (G/TPOHA), SCF- (G/SCFSUx), TPO/SCF- (G/TPOHA/SCFSUx), or VSV-G–pseudotyped vectors were analyzed for human engraftment in femur BM (hCD45+) at 7 weeks after transplantation. (A) FACS analysis of multilineage engraftment by CB CD34+ cells transduced for 24 hours at an MOI of 4 with TPO/SCF-displaying lentiviral (G/TPOHA/SCFSUx) or unmodified vectors in absence (VSV-G) or presence of rTPO (10 ng/mL) and rSCF (50 ng/mL). The upper right quadrant shows the GFP+ cells within the CD19+, CD14+, CD34+, CD13+ cells in the human graft (hCD45+ cells). The FACS profiles are representative of each transduction condition. (B) CB hCD34+ cells were transduced for 24 hours at an MOI of 4 with TPO- (G/TPOHA), SCF- (G/SCFSUx), TPO/SCF- (G/TPOHA/SCFSUx), or VSV-G–pseudotyped vectors in the absence (−) or presence of counterpart cytokines. After repopulation into NOD/SCID mice, transduction levels of total SRCs (hCD45+) and the subpopulation of human progenitor cells (hCD34+) were compared.

rTPO and rSCF, the latter resulting in a more variable engraftment efficiency (Figure 7A; Table 1). Furthermore, a better human cell engraftment was found using cells transduced with the TPO-displaying vectors as compared to unmodified vectors in the presence of rTPO, whereas for the SCF-displaying vectors, the opposite was true.

Detailed flow cytometry analysis revealed that TPO-, SCF-, and TPO/SCF-displaying vectors promoted multilineage engraftment (Figure 7A; Table 1). Gene-marked cells were able to repopulate all hematopoietic lineages in the same manner as unmarked cells, indicating that the differentiation capacity of the transduced cells was not impaired. For TPO-, SCF-, and TPO/SCF-displaying LVs, a high level of GFP expression was detected in B cells (CD45+CD19+), monocytes (CD45+CD14+), immature progenitors (CD45+CD34+), and progenitors/monocytes/granulocytes (CD45+CD13+; Figure 7A; Table 1).

Most importantly, we observed a striking preferential transduction of SRCs with the "early-acting cytokine"-displaying vectors as compared to VSV-G–displaying LVs, even when the latter vectors were used to transduce the CD34+ cells in the presence of recombinant cytokines (see 24-hour transduction in Table 1 and Figure 7A-B). Indeed, TPO-, SCF-, and TPO/SCF-displaying vectors could transduce 1.5- to 3-fold more human repopulating cells (hCD45+) and human progenitors (hCD34+) as compared to unmodified LVs in the presence of cytokines (Figure 7B; Table 1). For the cytokine-displaying vectors, high levels of GFP+ CD34+ immature cells were detected in the BM of all recipients, in addition to differentiated cells, indicating that immature CD34+ cells had been transduced (Figure 7B; Table 1). Overall these data clearly demonstrated that efficient lentiviral gene transfer in primitive hematopoietic progenitors (SRCs), in addition to more differentiated cells, is highly improved by display of TPO or SCF or both on the LV surface as compared to LVs in the presence of rTPO or rSCF or both.

Discussion

LVs are invaluable tools for several gene therapy approaches involving nondividing cells.[2-4] However, a major limitation of these conventional LVs hampers their application for HSC gene therapy because they do not allow efficient gene transfer into a subpopulation of nondividing cells, the quiescent (G_0) HSCs. To overcome this limitation, many studies using LVs required very high vector input (MOIs of 60 up to 3000) in the presence of strong cytokine cocktails (TPO, SCF, Flk-3, and IL-6 or IL-3) and in combination with spinoculation or multiple administration of vector to achieve high gene transfer rates in HSCs.[11,12,19-21,40] However, an undesirable effect of extended cytokine stimulation is a decrease of the multipotentality of HSCs.[18,41] Moreover, a too high vector dose poses the risk for multicopy integration, because under these conditions, insertional mutagenesis cannot be neglected.[42,43]

To overcome these problems, we designed a novel generation of LVs that are surface-engineered for display of "early-acting cytokines" to target and promote gene transfer in the resting (G_0) HSCs with the specific aim to preserve their stem cell potential. Previous results from our laboratory have shown that the display of lymphokines (IL-7) on the surface of LV particles promoted efficient transduction of quiescent human T cells without changing their phenotype.[6] Here, LVs engineered to display a single cytokine, TPO or SCF, allowed, via a single round of transduction, efficient gene transfer in CD34+ CB cells as compared to unmodified LVs. Importantly, TPO/SCF-codisplaying LVs demonstrated a clear additive effect of both cytokines on transduction efficiency, which resulted in 3-fold more transduced CD34+ cells as compared to TPO- or SCF-displaying vectors. This effect was evident even at low vector doses. These high transduction levels obtained with the cytokine-displaying vectors were confirmed in the progeny of the CD34+ cells, that is, short-term CFCs, in vitro derived hematopoietic lineages, and LTC-ICs. Most importantly, the new LVs performed better than unmodified vectors in the presence of recombinant cytokines. Indeed, in vitro differentiation of transduced CD34+ CB cells by TPO-, SCF-, or TPO/SCF-displaying vectors revealed transduction levels that were highly superior as compared to those obtained with unmodified vectors in the presence of rTPO or rSCF or both in all hematopoietic lineages (Figure 5A-B). In addition, in long-term in vitro cultures, high transduction levels of residual early progenitors were exclusively

Table 1. Engraftment and transduction efficiency of SRCs

Pseudotype, incubation time, MOI, and mouse no.	Total hCD45$^+$ cells, %	Transduced hCD45$^+$ cells, %	Transduced hCD19$^+$ cells, %	Transduced hCD34$^+$ cells, %	Transduced hCD14$^+$ cells, %
VSV-G*					
24 h, 4					
1A	4	11	25	40	27
2A	37	11.5	12	12	25
3A	4.4	10	14	12	10
4A	2	19	20	27	40
5A	17.5	17	13	17.5	18
G/TPOHA*					
24 h, 4					
1A	18	25	13	23	23
2A	12	21	12	24	30
3A	3	36	26	33	50
4A	7	48	41	56	33
5A	5	45	43	58	51
72 h, 20					
1B	21	59	58	48	63
2B	1	45	45	41	37
3B	2.5	9	7	25	44
G/TPOHA/SCFSUx*					
24 h, 4					
1A	20	42	40	58	53
2A	17	57	40	46	42
3A	11	62	66	60	50
4A	4	69	76	83	90
72 h, 20					
1B	13	67	70	48	36
2B	1	4	9	44	8
G/SCFSUx*					
24 h, 4					
1A	1.2	38	30	36	12
2A	10	20	12	27	3
3A	1	38	21	39	36
4A	3.5	30	28	42	20
72 h, 20					
1B	1	20	42	61	37
2B	1	37	51	57	41
3B	0.5	31	68	59	68
G+rTPO†					
24 h, 4					
1A	7	22	25	36	22
2A	3	13	7	7.5	10
3A	2	14	13	27	0
4A	1	0	0	0	0
G+rTPO+rSCF†					
24 h, 4					
1A	2	21	32	25	25
2A	30	35	37	42	41
3A	5	10	2	11	17
4A	21	15	8	11	20
G+rSCF†					
24 h, 4					
1A	3	10	8	10	0
2A	24	13	11	25	11
3A	9.5	4	5.5	3	7.2
4A	14	3	4	2.5	0

Protocol: 2^{E}5 cord blood CD34$^+$ cells were transduced with the indicated vector pseudotypes (G indicates VSV-G) at an MOI of 4 for 24 hours or at an MOI of 20 for 72 hours. After 72 hours or 24 hours of incubation with vector, CD34$^+$ cells were washed and injected by tail vain injection into sublethally irradiated NOD/SCID mice. The animals were humanely killed 7 weeks later and the BM was harvested and assessed for levels of human engraftment and GFP-expressing human cells. Multilineage engraftment was demonstrated by lineage-positive markers as indicated and for each lineage the percentage EGFP$^+$ cells was analyzed by FACS. Independent experiments were performed with different CD34$^+$ CB samples and different preparations of each vector.

*Incubation with cytokine displaying lentivectors without addition of recombinant cytokines.

†Incubation with lentivectors and addition of indicated cytokines (TPO, 10 ng/mL; SCF, 50 ng/mL; Flk-3, 100 ng/mL).

detected for TPO-, SCF- and TPO/SCF-displaying vectors (Figure 5B). Moreover, these findings were confirmed by the fact that our cytokine-displaying vectors resulted in up to 3-fold higher transduction of LTC-IC–derived colonies as compared to unmodified LVs in the presence of recombinant cytokines (Figure 4). Theoretically, assuming a maximum of 100 to 200 glycoprotein trimers per viral particle and a ratio of physical-to-infectious particles of 100:1000, under our conditions of transduction with the TPO- or SCF-displaying vectors (MOI of 4), we estimated that 6-to 100-fold fewer TPO molecules and 50- to 200-fold fewer SCF molecules were present on the surface of LVs as compared to transduction performed with VSV-G vectors in the presence of 10 ng/mL rTPO or 50 ng/mL SCF. This could indicate that the superior performance of TPO-, SCF-, and TPO/SCF-displaying LVs might be due to increased specific activity of the cytokines when presented on the viral surface as multivalent trimers or to increased targeting of HSCs. Of importance is that these novel gene transfer tools allowed efficient gene transfer into cells with long-term in vivo NOD/SCID mice repopulation capacity (SRCs). Thus, high-level lentiviral gene transfer in HSCs, indispensable for clinical applications, is guaranteed through minimal stimulation by TPO- and TPO/SCF-displaying vectors that replaces the use of a complex cocktail of cytokines. The latter is a major technical advantage because our new LV system does not depend on clinical-grade–produced cytokines at all.

Although others have suggested that transduction of CD34$^+$ CB cells is possible without cytokine stimulation,[11-15] we clearly demonstrated here that the new "early-acting cytokine"-displaying vectors relieved a partial resistance of nonstimulated HSCs to lentiviral transduction, consistent with other reports.[16,17,19] Indeed, among the more primitive progenitor subsets, SRCs, transduction with unmodified LVs in absence of cytokines was poor, only achieving 11% to 19%. This compared to the high gene transfer levels achieved with the TPO-, SCF-, and TPO/SCF-displaying vector reaching up to 50% to 70% in SRCs. In addition, we showed here that TPO-, SCF-, and SCF/TPO-displaying vectors led to very limited expansion (1.7-fold, 1.6-fold, and 2.6-fold, respectively) of CD34$^+$ CB cells during transduction, where complex cytokine cocktails result in a 10-fold expansion. This limited expansion might explain the increased multilineage engraftment ability of the transduced cells. The levels of GFP-expressing cells were similar in both lymphoid and myeloid lineages indicating that primitive SRCs were transduced with sustained long-term transgene expression in vivo. Most importantly, we observed a striking preferential transduction of SRCs with the "early-acting cytokine"-displaying

vectors as compared to VSV-G–displaying LVs in the absence or even in the presence of cytokine stimulation (Table 1; Figure 7A-B). In addition, we achieved high transduction efficiencies even by using low MOI (4) and short transduction (24 hours) conditions for our new vectors. Our data are in accordance with those obtained by Zielske and Gerson,[16] who demonstrated that rSCF alone enhanced LV transduction of CD34$^+$ cells. Recently, MLV-derived vectors engineered to display hSCF were described by us[37] and others.[44,45] Although their functional interaction with the c-kit receptor expressed on cell lines could be demonstrated,[37,44,45] such vectors only allowed gene delivery to hCD34$^+$ primary cells that were prestimulated with a cocktail of recombinant cytokines, in contrast to the vector described in this report.

It is known that in their native state, most HSCs are quiescent. Srour and colleagues reported that CD34$^+$ SRCs from BM and mobilized blood reside predominantly in the G$_0$ phase of the cell cycle.[1,46] Cellular factors that contribute to reverse transcription, nuclear translocation, or integration may be inactive or absent in these quiescent cells, which, therefore, inefficiently support LV-mediated gene transfer.[9,47] We demonstrate here that TPO-, SCF-, and TPO/SCF-displaying LVs remarkably rescued transduction and survival of the G$_0$ CD34$^+$ cells, a population highly enriched in HSCs. Indeed, the new TPO- or TPO/SCF-engineered vectors promoted gene transfer in a 4- to 5-fold higher number of resting CD34$^+$ CB cells than cycling cells. Moreover, the novel vectors preferentially promoted transduction and survival of the most immature G$_0$ cells over cycling cells.

In conclusion, the novel "early-acting cytokines"-displaying LVs described here outperform conventional VSV-G–pseudotyped LVs in the presence of recombinant "early-acting cytokine" stimulation in that they target high transduction to the most immature hematopoietic cells characterized as LTC-ICs and SRCs. They provide simplified, reproducible gene transfer protocols that ensure efficient gene transfer in SRC HSCs. As such, these novel reagents bring us one step closer to gene therapy protocols whereby gene transfer could be achieved directly by in vivo inoculation.

Acknowledgments

References

1. Gothot A, van der Loo JC, Clapp DW, Srour EF. Cell cycle-related changes in repopulating capacity of human mobilized peripheral blood CD34(+) cells in non-obese diabetic/severe combined immune-deficient mice. Blood. 1998;92:2641-2649.

2. Blomer U, Naldini L, Kafri T, Trono D, Verma IM, Gage FH. Highly efficient and sustained gene transfer in adult neurons with a lentivirus vector. J Virol. 1997;71:6641-6649.

3. Naldini L, Blomer U, Gallay P, et al. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science. 1996;272: 263-267.

4. Reiser J, Harmison G, Kluepfel-Stahl S, Brady RO, Karlsson S, Schubert M. Transduction of nondividing cells using pseudotyped defective high-titer HIV type 1 particles. Proc Natl Acad Sci U S A. 1996;93:15266-15271.

5. Kafri T, Blomer U, Peterson DA, Gage FH, Verma

IM. Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. Nat Genet. 1997;17:314-317.

6. Verhoeyen E, Dardalhon V, Ducrey-Rundquist O, Trono D, Taylor N, Cosset FL. IL-7 surface-engineered lentiviral vectors promote survival and efficient gene transfer in resting primary T lymphocytes. Blood. 2003;101:2167-2174.

7. Maurice M, Verhoeyen E, Salmon P, Trono D, Russell SJ, Cosset FL. Efficient gene transfer into human primary blood lymphocytes by surface-engineered lentiviral vectors that display a T cell-activating polypeptide. Blood. 2002;99:2342-2350.

8. Ducrey-Rundquist O, Guyader M, Trono D. Modalities of interleukin-7-induced human immunodeficiency virus permissiveness in quiescent T lymphocytes. J Virol. 2002;76:9103-9111.

9. Korin YD, Zack JA. Progression to the G1b phase

of the cell cycle is required for completion of human immunodeficiency virus type 1 reverse transcription in T cells. J Virol. 1998;72:3161-3168.

10. Dardalhon V, Jaleco S, Kinet S, et al. IL-7 differentially regulates cell cycle progression and HIV-1-based vector infection in neonatal and adult CD4$^+$ T cells. Proc Natl Acad Sci U S A. 2001;98: 9277-9282.

11. Case SS, Price MA, Jordan CT, et al. Stable transduction of quiescent CD34(+)CD38(−) human hematopoietic cells by HIV-1-based lentiviral vectors. Proc Natl Acad Sci U S A. 1999;96:2988-2993.

12. Miyoshi H, Smith KA, Mosier DE, Verma IM, Torbett BE. Transduction of human CD34$^+$ cells that mediate long-term engraftment of NOD/SCID mice by HIV vectors. Science. 1999;283:682-686.

13. Guenechea G, Gan OI, Inamitsu T, et al. Transduction of human CD34$^+$ CD38$^-$ bone marrow

and cord blood-derived SCID-repopulating cells with third-generation lentiviral vectors. Mol Ther. 2000;1:566-573.

14. Uchida N, Sutton RE, Friera AM, et al. HIV, but not murine leukemia virus, vectors mediate high efficiency gene transfer into freshly isolated G0/G1 human hematopoietic stem cells. Proc Natl Acad Sci U S A. 1998;95:11939-11944.

15. Follenzi A, Ailles LE, Bakovic S, Geuna M, Naldini L. Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences. Nat Genet. 2000;25:217-222.

16. Zielske SP, Gerson SL. Cytokines, including stem cell factor alone, enhance lentiviral transduction in nondividing human LTCIC and NOD/SCID re-populating cells. Mol Ther. 2003;7:325-333.

17. Sutton RE, Reitsma MJ, Uchida N, Brown PO. Transduction of human progenitor hematopoietic stem cells by human immunodeficiency virus type 1-based vectors is cell cycle dependent. J Virol. 1999;73:3649-3660.

18. Kittler EL, Peters SO, Crittenden RB, et al. Cyto-kine-facilitated transduction leads to low-level engraftment in nonablated hosts. Blood. 1997;90:865-872.

19. Ailles L, Schmidt M, Santoni de Sio FR, et al. Mo-lecular evidence of lentiviral vector-mediated gene transfer into human self-renewing, multi-potent, long-term NOD/SCID repopulating hema-topoietic cells. Mol Ther. 2002;6:615-626.

20. Robert D, Mahon FX, Richard E, Etienne G, de Verneuil H, Moreau-Gaudry F. A SIN lentiviral vector containing PIGA cDNA allows long-term phenotypic correction of CD34⁺-derived cells from patients with paroxysmal nocturnal hemo-globinuria. Mol Ther. 2003;7:304-316.

21. Amsellem S, Ravet E, Fichelson S, Pflumio F, Dubart-Kupperschmitt A. Maximal lentivirus-mediated gene transfer and sustained transgene expression in human hematopoietic primitive cells and their progeny. Mol Ther. 2002;6:673-677.

22. Mantel C, Hendrie P, Broxmeyer HE. Steel factor regulates cell cycle asymmetry. Stem Cells. 2001;19:483-491.

23. Kirito K, Fox N, Kaushansky K. Thrombopoietin stimulates Hoxb4 expression: an explanation for the favorable effects of TPO on hematopoietic stem cells. Blood. 2003;102:3172-3178.

24. Kaushansky K. Thrombopoietin: accumulating evidence for an important biological effect on the hematopoietic stem cell. Ann N Y Acad Sci. 2003;996:39-43.

25. Borge OJ, Ramsfjell V, Cui L, Jacobsen SE. Abil-ity of early acting cytokines to directly promote

survival and suppress apoptosis of human primi-tive CD34⁺CD38⁻ bone marrow cells with multi-lineage potential at the single-cell level: key role of thrombopoietin. Blood. 1997;90:2282-2292.

26. Fox N, Priestley G, Papayannopoulou T, Kaush-ansky K. Thrombopoietin expands hematopoietic stem cells after transplantation. J Clin Invest. 2002;110:389-394.

27. Ema H, Takano H, Sudo K, Nakauchi H. In vitro self-renewal division of hematopoietic stem cells. J Exp Med. 2000;192:1281-1288.

28. Yagi M, Ritchie KA, Sitnicka E, Storey C, Roth GJ, Bartelmez S. Sustained ex vivo expansion of hematopoietic stem cells mediated by thrombo-poietin. Proc Natl Acad Sci U S A. 1999;96:8126-8131.

29. Kimura S, Roberts AW, Metcalf D, Alexander WS. Hematopoietic stem cell deficiencies in mice lack-ing c-Mpl, the receptor for thrombopoietin. Proc Natl Acad Sci U S A. 1998;95:1195-1200.

30. Solar GP, Kerr WG, Zeigler FC, et al. Role of c-mpl in early hematopoiesis. Blood. 1998;92:4-10.

31. Ramsfjell V, Borge OJ, Veiby OP, et al. Thrombo-poietin, but not erythropoietin, directly stimulates multilineage growth of primitive murine bone mar-row progenitor cells in synergy with early acting cytokines: distinct interactions with the ligands for c-kit and FLT3. Blood. 1996;88:4481-4492.

32. Luens KM, Travis MA, Chen BP, Hill BL, Scollay R, Murray LJ. Thrombopoietin, kit ligand, and flk2/flt3 ligand together induce increased num-bers of primitive hematopoietic progenitors from human CD34⁺Thy-1⁺Lin⁻ cells with preserved ability to engraft SCID-hu bone. Blood. 1998;91:1206-1215.

33. Maurice M, Mazur S, Bullough FJ, et al. Efficient gene delivery to quiescent interleukin-2 (IL-2)-dependent cells by murine leukemia virus-derived vectors harboring IL-2 chimeric envelope glycop-roteins. Blood. 1999;94:401-410.

34. Sandrin V, Boson B, Salmon P, et al. Lentiviral vectors pseudotyped with a modified RD114 en-velope glycoprotein show increased stability in sera and augmented transduction of primary lym-phocytes and CD34⁺ cells derived from human and nonhuman primates. Blood. 2002;100:823-832.

35. Muto T, Feese MD, Shimada Y, et al. Functional analysis of the C-terminal region of recombinant human thrombopoietin. C-terminal region of thrombopoietin is a "shuttle" peptide to help se-cretion. J Biol Chem. 2000;275:12090-12094.

36. Kato T, Matsumoto A, Ogami K, Tahara T, Morita H, Miyazaki H. Native thrombopoietin: structure and function. Stem Cells. 1998;16:322-328.

37. Fielding AK, Maurice M, Morling FJ, Cosset F-L, Russell SJ. Inverse targeting of retroviral vectors: selective gene transfer in a mixed population of hematopoietic and nonhematopoietic cells. Blood. 1998;91:1802-1809.

38. Wilpshaar J, Falkenburg JH, Tong X, et al. Similar repopulating capacity of mitotically active and resting umbilical cord blood CD34(+) cells in NOD/SCID mice. Blood. 2000;96:2100-2107.

39. Wilpshaar J, Bhatia M, Kanhai HH, et al. Engraft-ment potential of human fetal hematopoietic cells in NOD/SCID mice is not restricted to mitotically quiescent cells. Blood. 2002;100:120-127.

40. Sirven A, Ravet E, Charneau P, et al. Enhanced transgene expression in cord blood CD34(+)-derived hematopoietic cells, including developing T cells and NOD/SCID mouse repopulating cells, following transduction with modified trip lentiviral vectors. Mol Ther. 2001;3:438-448.

41. Glimm H, Oh IH, Eaves CJ. Human hematopoi-etic stem cells stimulated to proliferate in vitro lose engraftment potential during their S/G(2)/M transit and do not reenter G(0). Blood. 2000;96:4185-4193.

42. Woods NB, Muessig A, Schmidt M, et al. Lentivi-ral vector transduction of NOD/SCID repopulating cells results in multiple vector integrations per transduced cell: risk of insertional mutagenesis. Blood. 2003;101:1284-1289.

43. Hacein-Bey-Abina S, Von Kalle C, Schmidt M, et al. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Sci-ence. 2003;302:415-419.

44. Chandrashekran A, Gordon MY, Casimir C. Tar-geted retroviral transduction of c-kit+ hematopoi-etic cells using novel ligand display technology. Blood. 2004;104:2697-2703.

45. Chandrashekran A, Gordon MY, Darling D, Farza-neh F, Casimir C. Growth factor displayed on the surface of retroviral particles without manipulation of envelope proteins is biologically active and can enhance transduction. J Gene Med. 2004;6:1189-1196.

46. Jetmore A, Plett PA, Tong X, et al. Homing effi-ciency, cell cycle kinetics, and survival of quies-cent and cycling human CD34(+) cells trans-planted into conditioned NOD/SCID recipients. Blood. 2002;99:1585-1593.

47. Bukrinskaya AG, Ghorpade A, Heinzinger NK, Smithgall TE, Lewis RE, Stevenson M. Phosphor-ylation-dependent human immunodeficiency vi-rus type 1 infection and nuclear targeting of viral DNA. Proc Natl Acad Sci U S A. 1996;93:367-371.