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

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Abstract

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 into G0 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 lentiviral vectors that overcome this restriction by displaying early-acting cytokines on their surface. Display of thrombopoietin, stem cell factor or both cytokines on 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 colonies (LTC-ICs) and of long-term NOD/SCID repopulating cells (SRCs) in vivo.
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 upon NOD/SCID repopulating cell (SRC) assays, defined as a measure for long-term human repopulating cells, HSC in G0 represent the most primitive, uncommitted hematopoietic progenitors and thus, the preferred targets for gene transfer.

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 non-dividing cells such as neurons, fibroblasts and hepatocytes, while resting lymphocytes are refractory to LV-mediated gene transfer. We and others have shown that stimulation into the G1b phase of the cell cycle is sufficient to allow productive transduction of quiescent T-cells with lentiviral vectors, while these conditions are insufficient for transduction with murine leukemia virus (MLV)-based retroviral vectors. Whether HIV or the lentiviral vectors can integrate into the genome of a truly quiescent cell, in particular an early progenitor HSC in G0, remains controversial. Compared to their MLV counterparts, LVs do not need extended cytokine stimulation in order to transduce HSCs, avoiding strong cell proliferation, which might lead to loss of stem cell potential. While 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. 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. Moreover, TPO and SCF act in synergism for these characteristics. 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 hematopoietic stem cells, as defined by their capacity to repopulate NOD/SCID mice.
**Results**

**Functional display of SCF and TPO on HIV-1 derived vectors.** To improve gene transfer into HSCs, we displayed two early acting cytokines, TPO and SCF, on the surface of lentiviral particles. We fused either of two TPO truncated forms (163 and 171 amino-acid-long), previously shown to have a 3-fold higher biological activity than wild type TPO\textsuperscript{33,34}, to the N-terminus of the MLV envelope glycoprotein (gp). These env chimeras are called TPO163SU and TPO171SU, respectively. Additionally, we fused the TPO171 form to the influenza hemaglutinin (HA) glycoprotein (TPOHA). Both fusion partners, the MLV and HA gps incorporate efficiently on LVs\textsuperscript{6,7,35}. The second cytokine, SCF, was fused to the MLV env gp (SCFSUx)\textsuperscript{36}.

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 (Fig 1A). Survival was significantly reduced only at low doses of virus/cell. In contrast, we observed that TPO presentation on LV, by fusion to the HA gp, 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 (Fig 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 (Fig 1B). In order to evaluate a potential synergistic effect between SCF and TPO, we produced TPO/SCF-co-displaying vectors using the combination of VSV-G/TPOHA/SCFSUx gps. 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 (Fig 1A and B).

Figure 1. Functional display of TPO and SCF on HIV-derived vectors.
(A) BAF3-Mpl cells expressing the TPO receptor were incubated with lentiviral vectors presenting either of two
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).
Incubation were also performed with vectors co-displaying TPO and SCF. After a 72 hr incubation with
decreasing doses of vector (multiplicity of infection (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 non-concentrated TPO-displaying vectors. 

(B) BAF3-cKit cells expressing the SCF receptor were incubated with lentiviral vectors presenting SCF at their surface by fusion to the amphotropic MLV glycoprotein (G/SCFSUx) or co-presenting TPO and SCF at their surface. After 72 hr 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 to 5 ng/ml). G/SCFSUx vectors were concentrated (G/SCFSUx-conc) by ultracentrifugation and incubations were performed under the same conditions as for non-concentrated SCF-displaying vectors. 

(C) Immunoblots of lentiviral vector particles displaying TPOHA, 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 chimeric 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 hr 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.

Efficient incorporation of TPOHA and SCFSUx chimera on LV particles was demonstrated by immunoblot detection of highly purified viral pellets with antibodies against MLV gp and HA gp (Fig 1C). Since lentiviral vectors displaying these chimeric envelope glycoproteins alone showed reduced infectivity, the chimera were incorporated together with the vesicular stomatitis virus G protein (VSV-G) in all experiments described below. 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 gps were detected in the fraction containing the viral particles and that such association to the viral surface was not disrupted upon co-expression 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-c-kit cells, respectively (G/TPOHA-conc in Fig 1A; G/SCFSUx-conc in Fig 1B). Altogether, these results indicated that the biological 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 cord blood (CB) CD34+ cells against apoptosis and this to an extent equivalent to that observed following incubation with high levels of their recombinant cytokine counterparts (Fig 1D).

**TPO/SCF-displaying lentiviral vectors 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 the green fluorescent protein (GFP). A single exposure to either 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 (Fig 2A), which were less efficient as expected and reported previously19. Since 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 (Fig 2B and 2C).
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 hr with VSV-G/TPO- (G/TPOHA), VSV-G/SCF- (G/SCFSUx) or VSV-G/TPO/SCF- (G/TPOHA/SCFSUx) displaying 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 hr vs. 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=10ng/ml; rSCF=50 ng/ml). Cells transduced for 24 hr were washed and maintained for another 48h in serum-free medium in the absence of cytokines. Cells were analyzed for GFP expression 72 hr 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 lentiviral vectors at MOIs of 20 and 4. Control incubations were performed with VSVG pseudotyped vector in the absence (-) or presence of recombinant cytokines (rTPO= 10 ng/ml; rSCF =50 ng/ml). After a 72 hr incubation, the total number of transduced CD34+ CB cells was calculated (= number of cells at start of infection (5x10^4) x cell expansion x % cell transduction x % cell survival; data are shown as means±SD, n=4).

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 hr to the cytokine displaying and VSV-G LVs, washed and cultured for a further 48 hr in the complete absence of cytokines, vectors and serum. Low transduction was detected following infection with LVs displaying VSV-G gp (G: 23.2± 4.9 %), while 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%; Fig 2B). It should be reminded, however, that leaving the CD34+ cells for 3 days in absence of cytokines compromised their survival as indicated in Fig 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 hr versus a 3 days incubation with TPO-, SCF- or TPO/SCF-displaying vectors. Control incubations with VSV-G displaying LVs in the presence of rTPO and/or rSCF resulted in equivalent transduction levels, as for the corresponding TPO-, SCF- or TPO/SCF-displaying vectors (Fig 2B).

We then analyzed the absolute numbers of transduced CB CD34+ cells (= initial number of cells x transduction (%) x cell survival (%) x expansion; Fig 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 (Fig 2C). Interestingly, LVs co-displaying SCF and TPO demonstrated a clear additive effect between the two 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 demonstrated this additive effect (Fig 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 and/or rSCF (Fig 2C). The significant increase in the number of transduced cells obtained with TPO/SCF co-displaying LVs compared to unmodified LVs resulted from a combination of higher transduction efficiency (Fig 2A and B), higher cell survival (Fig 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), while TPO-, SCF- or TPO/SCF-displaying LVs resulted in low expansions ranging from 1.5- to 2.6-fold (G/TPOHA = 1.72±0.26; G/TPOHA/SCFSUx = 2.62±0.49; G/SCFSUx=1.65±0.25) cell expansions, similar to incubations with low dose rTPO and/or rSCF (data not shown).

To verify whether the high transduction efficiencies obtained for CD34+ CB cells persisted in their derived colony forming cell (CFCs) 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-, TPO/SCF- LVs was reflected in equivalent percentages of transduced CFC colonies (Fig 3). Again, the additive effect of TPO and SCF was reproduced since a 4-fold higher number of GFP+ CFC derived colonies were detected for TPO/SCF-co-displaying 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 and/or rSCF.
Figure 3. SCF/TPO surface-engineered lentiviral vectors induce high levels of gene transfer in CB CD34+ short-term colony forming cell (CFCs)

CD34+ CB cells were incubated with TPO- (G/TPOHA), SCF- (G/SCFSUx) single or co- (G/TPOHA/SCFSUx) displaying HIV-derived vectors for 72 hr. The gene transfer efficiency in the bulk CD34+ cell population (% GFP+ cells) and their CFCs derived transduced colonies (% GFP+ colonies) are shown. Data are shown as means±SD, n=3.

TPO/SCF-displaying lentiviral vectors preferentially transduce and promote survival of CD34+ cells in G0. Most CD34+ cells from mobilized peripheral blood or bone marrow with NOD/SCID repopulating potential, reside in the G0 phase of the cell cycle. As CD34+ cells from cord blood in either G0 or G1 contain SRCs, we assessed the efficacy of gene transfer by the cytokine-displaying vectors in either population. CD34+ CB cells were stained with pyronin-Y (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 (Fig 4A). We confirmed the purity of the sorted G0 and G1/S/G2+M populations by PY/7AAD
staining (Fig 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 G0-sorted cells after incubation with VSV-G LVs (G: 11±1% cell survival), as expected, while TPO- and TPO/SCF-displaying vectors ensured a 7 to 8-fold higher protection of G0 cells (G/TPOHA: 75.0±7.8% cell survival; G/TPOHA/SCFSUx: 88.6±5.6% cell survival; Fig 4B). SCF-displaying vectors induced survival of G0 CD34+ CB cells to a lower extent (G/SCFSUx: 51.3±13.0 % cell survival; Fig 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 G0 over those that had entered into cycle (Fig 4B), which probably depend on other additional cytokines for survival.

Most importantly, TPO-, SCF- and TPO/SCF-displaying vectors transduced more efficiently the G0-sorted cells than the cycling CD34+ cells (Fig 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 (G0) 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.
Figure 4. TPO/SCF-displaying lentiviral vectors promote transduction and survival of immature quiescent G0 CD34+ cells.

(A) FACS analysis of CD34+ CB cells stained with Pyronin Y as described in the experimental procedure. Quiescent cells residing in the G0 phase of the cell cycle (G0 CD34+) have minimal RNA content, as indicated by low PY staining, whereas cells residing into G1b, S, G2+M phase (G1+) have a high RNA content. Sort windows to collect G0 and G1+ 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 two cell categories. The G0 population was checked for purity by 7AAD (DNA)/PY (RNA) staining. The G0/G1a, G1b and SG2M phases are indicated. In (B) and (C) the G0 and G1+ CD34+ populations were transduced for 72 hr with TPO-, SCF-, SCF/TPO-displaying vectors or with VSV-G-pseudotyped vectors. The percentage of CD34+ cell survival is indicated in (B), and the numbers of GFP+ CD34+ marked cells (= number of cells at start of infection (5x10^4) x cell expansion x % cell transduction x % cell survival) for the G0 and G1+ populations are shown in (C). Data are shown as means±SD, n=3.
TPO-, SCF- and TPO/SCF-displaying LVs ensure superior transduction levels in immature CD34+ cells and their progeny as compared to LVs in presence of recombinant cytokines. As a measure of transduction of primitive progenitor/stem cells, long-term-culture initiating cells (LTC-ICs) 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 (Fig 5).

Figure 5. SCF/TPO-surface-engineered lentiviral vectors selectively promote gene transfer in CB CD34+ long-term culture-initiating cell derived colonies (LTC-IC).
CD34+ CB cells were incubated with TPO- (G/TPOHA), SCF- (G/SCFSUx) single or co- (G/TPOHA/SCFSUx) displaying HIV-derived vectors for 72 hr. The gene transfer efficiency in the CD34+ long-term culture-initiating cell derived colonies (LTC-IC) (% GFP+ colonies) are shown. Data are shown as means±SD, n=3.

Indeed, TPO-displaying 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 (Fig 5). This demonstrated that TPO-, SCF-, or SCF/TPO-displaying vectors transduced early progenitors, while unmodified vectors in the presence or absence of recombinant cytokines (rTPO and/or SCF) 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 and/or SCF for transduction efficiency of their CD34+ derived progeny 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, irrespective 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 and/or SCF (Fig 6A and B). TPO/SCF-co-displaying 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 speudotyped 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 (Fig 6A and 6B). 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).
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 hr at 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 hr 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 three independent experiments are shown. The absolute number of transduced differentiated cells is calculated e.g. for B-cells as % CD19 in total population x % transduction x expansion of the cells after culture. The number of transduced granulo-monocytes is in a lymphoid/myeloid culture on MS5 in the presence of FCS, IL-2, IL-15 and SCF is shown for two independent experiments.

(B) In vitro differentiation of CB CD34+ cells transduced for 72 hr with TPO-displaying lentiviral vectors or VSV-G displaying vectors in the presence of rTPO (10 ng/ml). Differentiation was performed in two 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).

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 (Fig 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 (Fig 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 hematopoietic stem cells. Since 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 \textit{in vivo}. Thus, to address this question, we transplanted CD34+ CB cells transduced with the different LVs into sub-lethally irradiated NOD/SCID mice. The efficiency of gene transfer in SCID repopulating cells (SRCs) was determined 6-7 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 dosis (24 hr at
MOI=4) was performed (Fig 7A and Table 1). For the cytokine-displaying vector we additionally performed a 72 hr transduction at higher vector dose (MOI= 20).

Table 1. Engraftment and transduction efficiency of SRCs

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Protocol: 2×10^5 cord blood CD34+ cells were transduced with the indicated vector pseudotypes (G=VSV-G) at an MOI of 4 for 24 hr or at an MOI of 20 for 72 hr. (a) incubation with cytokine displaying lentivectors without addition of recombinant cytokines; (b) incubation with lentivectors and addition of indicated cytokines (10 ng TPO/ml; 50 ng SCF/ml; 100 ng Flk-3/ml). After 72 hr or 24 hr of incubation with vector, CD34+ cells were washed and injected by tail vein injection into sublethally irradiated NOD/SCID mice. The animals were sacrificed 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 lineages positive markers as indicated and for each lineage the percentage EGFP+ cells were analysed by FACs. Independent experiments were performed with different CD34+ CB samples and different preparations of each vector.

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 hr) resulted in a more variable and lower repopulation efficiency than the short one (24 hr). 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 rTPO and rSCF, the latter resulting in a more variable engraftment efficiency (Fig 7A and Table 1). Furthermore, a better human cell engraftment was found using cells transduced with the TPO-displaying vectors as compared to unmodified vectors in presence of rTPO, while 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 (Fig 7A and 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+; Fig 7A and Table 1).
Figure 7. Preferential transduction of NOD/SCID repopulating cells by TPO- and SCF- or TPO/SCF-displaying lentiviral vectors.
NOD/SCID mice that received 2x10^5 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 post-transplantation.
(A) FACs analysis of multilineage engraftment by CB CD34+ cells transduced for 24 hr 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 hr 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 the counterpart cytokines. After repopulation into NOD/SCID mice, transduction levels of total SRCs (hCD45+) and the subpopulation of human progenitor cells (hCD34+) were compared.

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 hr transduction in Table 1 and Fig 7A and 7B). 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 (Fig 7B and 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 (Fig 7B and 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 and/or SCF on the LV surface as compared to LVs in the presence of rTPO and/or rSCF.
Discussion

Lentiviral vectors are invaluable tools for several gene therapy approaches involving non-dividing cells\textsuperscript{2-4}. However, a major limitation of these conventional LVs hampers their application for hematopoietic stem cell gene therapy, as they do not allow efficient gene transfer into a subpopulation of non-dividing cells, the quiescent (G0) 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 IL6 or IL3) and in combination with spinoculation or multiple administration of vector to achieve high gene transfer rates in HSCs\textsuperscript{11,12,19-21,39}. However, an undesirable effect of extended cytokine stimulation is a decrease of the multipotentiality of HSCs\textsuperscript{18,40}. Moreover, a too high vector dose poses the risk for multicopy integration, as under these conditions, insertional mutagenesis cannot be neglected\textsuperscript{41,42}.

To overcome these problems, we designed a novel generation of lentiviral vectors that are surface-engineered for display of “early-acting-cytokines” in order to target and promote gene transfer in the resting (G0) HSCs with the specific aim to preserve their stem cell potential. Previous results of our laboratory have shown that the display of lymphokines (IL-7) on the surface of lentiviral vector particles promoted efficient transduction of quiescent human T cells without changing their phenotype\textsuperscript{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 co-displaying 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+
Most importantly, the new LVs performed better than unmodified vectors in the presence of recombinant cytokines. Indeed, \textit{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 and/or rSCF in all hematopoietic lineages (Fig 5A and B). In addition, in long term \textit{in vitro} cultures, high transduction levels of residual early progenitors were exclusively detected for TPO-, SCF- and TPO/SCF-displaying vectors (Fig 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 (Fig 4). Theoretically, assuming a maximum of 100-200 gp trimers per viral particle and a ratio of physical to infectious particles of 100-1,000, under our conditions of transduction with the TPO- and/or SCF-displaying vectors (MOI of 4), we estimated that 6-100 fold less TPO molecules and 50-200 fold less SCF molecules were present on the surface of LVs as compared to transduction performed with VSV-G vectors in the presence of 10ng/ml rTPO or 50 ng/ml SCF. So this could indicate that the superior performance of TPO-, SCF- and TPO/SCF-displaying lentiviral vector 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 high importance is that these novel gene transfer tools allowed efficient gene transfer into cells with long-term \textit{in vivo} NOD/SCID mice repopulation capacity (SRCs). Thus, high-level lentiviral gene transfer in HSC, indispensable for clinical applications is guaranteed through minimal stimulation by TPO- and TPO/SCF-displaying vectors which replace the use of a complex cocktail of cytokines. The latter is a major technical advantage since 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\textsuperscript{11-15}, we clearly demonstrated here that the new early-acting-cytokine-displaying vectors relieved a partial resistance of non-stimulated HSCs to lentiviral transduction, consistent with other reports\textsuperscript{16,17,19}. Indeed, among the more primitive progenitor subsets, SRCs, transduction with unmodified LVs in absence of cytokines was poor, only achieving 11-19%. This compared to the high gene transfer levels achieved with the TPO-, SCF- and TPO/SCF-displaying vector reaching up to 50-70% in SRCs. In addition, we showed here that TPO-, SCF- and SCF/TPO-displaying vectors led to very limited expansion (1.5-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 \textit{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 and Fig 7A and 7B). In addition, we achieved high transduction efficiencies even by using low-MOI and short transduction conditions for our new vectors (24 hr, MOI of 4). Our data are in accordance with those obtained by Zielke and Gerson\textsuperscript{16}, who demonstrated that rSCF alone, enhanced LV transduction of CD34+ cells. Recently, MLV-derived vectors engineered to display hSCF were described by us\textsuperscript{36} and others\textsuperscript{43,44}. While their functional interaction with the c-kit receptor expressed on cell lines could be demonstrated\textsuperscript{36,43,44}, 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 cells from bone marrow and mobilized blood reside predominantly in the G0 phase of the cell cycle. 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. We demonstrate here that TPO-, SCF- and TPO/SCF-displaying LVs remarkably rescued transduction and survival of the G0 CD34+ cells, a population highly enriched in HSCs. Indeed, the new TPO or TPO/SCF-engineered vectors promoted gene transfer in a 4-5 fold higher number of resting CD34+ CB cells than cycling cells. Moreover, the novel vectors preferentially promoted transduction and survival of the most immature G0 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 hematopoietic stem cells. 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.
Methods

Envelope construction. The TPO C-terminal truncation mutants (163aa and 171aa) were obtained by PCR with primers encoding for the SfiI and NotI sites at the 5' and 3' ends, respectively. The SfiI/NotI PCR fragment was fused to the 4070A (amphotrophic) MLV env gene at position + 1 of the surface SU subunit using the SfiI/NotI backbone fragment of CMVOKT3SU. The resulting chimeric glycoproteins were called TPO163SU and TPO171SU. The SfiI/NotI fragment from TPO171SU was fused to the hemaglutinin (HA) env gene using the SfiI/NotI backbone fragment of CMVEGFPHA. The SUx mutation, 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 gp were expressed in the phCMV-G expression vector backbone.

Production of retroviral vectors. Self-inactivating HIV-1-derived vectors were generated as previously described by transient transfection of 293T cells. For co-display of VSV-G, SCFSUx and TPOHA 1.5 ug of each envelope plasmid was co-transfected with the Gag-Pol packaging construct and the 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. The plasmid encoding neuraminidase was as a control co-transfected in all other vector preparations (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 IL-3 for growth. BAF-Mpl cells and BAF3-cKit cells were washed twice in PBS to remove IL-3 and make them exclusively dependent on TPO or SCF, respectively. They were plated at 5x10^4 cells/24 well in RPMI/10% FSC and incubated with decreasing doses of fresh lentiviral vector supernatant or concentrated over a sucrose cushion (MOI = 20, 10, 4, 2 or 1) for 72 hours. Cells were stained with propidium iodide (PI) (1µg/ml) in PBS and the amount of living cells was assessed by FACS analysis.

CD34+ CB cells were seeded at 5x10^4 cells/24 well in serum-free medium (Cellgro, Cellgenix, Germany) and incubated for 72 hr with LVs at MOIs 20 or 4. After 72 hr of incubation the cells were washed, 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 rTPO (10 ng/ml) and/or 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 anti-coagulant. Low-density cells were separated over Ficoll-Hypaque. CD34+ isolation was performed by means of positive selection using magnetic cell separation (Myltenyi MACs) columns according to the manufacturer’s instructions. Purity of the selected CD34+ fraction was assessed by FACS analysis with a PE-conjugated anti-CD34 antibody (BD Pharmingen) 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. Multiplicities of infection (MOIs) were determined on proliferating HeLa cells and are indicated in all transduction experiments.
5x10^4 CD34+ CB cells were transduced with fresh lentiviral vector supernatant at an MOIs of 20 or 4 in serum-free medium (CellGro, CellGenix, Germany) in 48-well plates. 24 hr transductions were washed and resuspended in serum-free Cellgro-medium for a further 48 hr before transduction efficiency was determined by flow cytometry.

**Cell-cycle fractionation by Pyronin Y staining.** To distinguish between cells in G0 or G1/S/G2+M, RNA staining with Pyronin Y (PY) was performed. Briefly, CD34+ cells were re-suspended at concentration of 2x10^6 cells/ml in a buffer containing Hanks Balanced Salt Solution, 20 mmol/L Hepes, 1g/L glucose, 10% FCS. PY was added at a concentration of 1µg/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). The living CD34+ cells were gated and in this gate cells in G0 were identified by their minimal RNA content, whereas cells in G1/S/G2+M phase were defined as those with high or maximal PY staining, thus allowing isolation of viable CD34+ cells in G0 or G1/S/G2+M. The two sorting gates were well separated. After each sorting experiment, the two populations were checked for purity by a Pyronine Y (RNA-)/7AAD (DNA-) staining (Fig 4A)^16,17,19

**Clonogenic cell assays.** Assays for CFCs and LTCIC 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 rhu-IL2 (5 ng/ml), -IL15 (10 ng/ml) and -SCF (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 Epo (2U/ml) to assess erythroid maturation.

**NOD/SCID repopulating assays.** After a 72 hr (MOI of 20) or 24 hr (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. 6-8 weeks post-transplantation, the bone marrow (BM) from femurs was harvested and assessed for levels of GFP-expressing human cells. Cells were prepared for three color flow cytometry to detect the percentage of EGFP+ lineage positive cells as described below.

**Immunophenotyping by flow cytometry.** EGFP expression in the CD34+ CB cell population was analyzed 72 hr post transduction by flow cytometry after immunolabeling with an anti-CD34-PE (BD Pharmingen) 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), hCD56- and hCD34-PE-Cy5 (Immunotech). Three-color flow cytometry was used to detect GFP+ human cells of various lineages in NOD/SCID bone marrow using anti-hCD45-Cychrome and anti-hCD19-, anti-hCD14-, anti-hCD13 and anti-hCD34-PE antibodies. In all cases, corresponding PE conjugated mouse IgG controls were used.
References


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

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