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Preclinical Safety and Efficacy of Human CD34+ Cells Transduced With Lentiviral Vector for the Treatment of Wiskott-Aldrich Syndrome

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Gene therapy with *ex vivo*-transduced hematopoietic stem/progenitor cells may represent a valid therapeutic option for monogenic immunohematological disorders such as Wiskott-Aldrich syndrome (WAS), a primary immunodeficiency associated with thrombocytopenia. We evaluated the preclinical safety and efficacy of human CD34+ cells transduced with lentiviral vectors (LV) encoding WAS protein (WASp). We first set up and validated a transduction protocol for CD34+ cells derived from bone marrow (BM) or mobilized peripheral blood (MPB) using a clinical grade, highly purified LV. Robust transduction of progenitor cells was obtained in normal donors and WAS patients' cells, without evidence of toxicity. To study biodistribution of human cells and exclude vector release *in vivo*, LV-transduced CD34+ cells were transplanted in immunodeficient mice, showing a normal engraftment and differentiation ability towards transduced lymphoid and myeloid cells in hematopoietic tissues. Vector mobilization to host cells and transmission to germline cells of the LV were excluded by different molecular assays. Analysis of vector integrations showed polyclonal integration patterns *in vitro* and in human engrafted cells *in vivo*. In summary, this work establishes the preclinical safety and efficacy of human CD34+ cells gene therapy for the treatment of WAS.

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INTRODUCTION

Transplantation of autologous hematopoietic stem cells (HSC) genetically modified *ex vivo* using viral vectors represents a potentially curative treatment for inherited disorders of the hematopoietic system, including primary immunodeficiencies.[1,2] Clinical trials for severe combined immunodeficiency (SCID) due to adenosine deaminase deficiency[3] or IL2RG-deficiency (X-linked severe combined immunodeficiency (SCID-X1))[4,5] have shown that gene therapy with retrovirally transduced HSC results in long-term clinical efficacy. However, gene modification by γ-retroviral vectors, likely in combination with disease-specific factors, resulted in insertional mutagenesis in the clinical trials for SCID-X1[6,7] and chronic granulomatous disease.[8] Self-inactivating lentiviral vectors (LV) may represent a substantial improvement in terms of safety profile while offering a more robust transduction and better transcriptional control of transgene expression in HSC.[9] Two recent studies for the treatment of X-linked adrenoleukodystrophy[10] and β-thalassemia[11] have provided information on the experimental clinical use of LV-transduced CD34+ cells. In the adrenoleukodystrophy trial, highly polyclonal multilineage engraftment of gene corrected cells at substantial levels (15%) and clear therapeutic benefits were observed. In the β-thalassemia study, transduced CD34+ cells failed to engraft in the first patient while the second patient showed oligoclonal HSC engraftment associated with a dominant clone reaching ~15% of the cells and leading to independency from red blood cell transfusion.[11]

Wiskott-Aldrich Syndrome (WAS) is an excellent candidate for a gene therapy approach in patients lacking a suitable donor or at high risk of complications.[12,13] WAS is a rare and severe X-linked immunodeficiency characterized by eczema, platelet defects, recurrent infections, autoimmunity, and increased susceptibility to develop tumors, in particular lymphomas.[14,15] The disorder is caused by mutations in the gene encoding for the WAS protein (WASp),[16] preferentially expressed in hematopoietic cell lineages and involved in actin cytoskeleton reorganization, leading to different cellular dysfunctions such as migration, signal transduction, and activation.[17,18]

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The recent report that WAS-gene transfer using a γ-retroviral vector resulted in improved platelet counts and immune functions further supports the rationale for a gene therapy strategy for this disease.[19,20] On the other hand, the occurrence of leukemia associated to an insertion near the LMO2 gene[21] in this trial reinforces the need for a potentially safer vector platform to deliver the WAS transgene.

We have developed an HSC gene therapy approach based on lentiviral-mediated gene transfer of WAS complementary DNA under the control of a 1.6 kb fragment of the endogenous WAS promoter. We previously showed that transplantation of gene corrected WAS-deficient murine HSC in non-lethally irradiated *Was*$^{-/-}$ mice resulted in long-term multilineage engraftment of WASp-expressing cells and functional correction of lymphocyte and dendritic cells with a lack of observable toxicity over a short- and long-term period.[22,23] Moreover, the vector was able to restore *in vitro* expression of WASp in CD34$^+$ cells and mature cells from WAS patients.[24,25]

The recently issued European regulation on Advanced Therapy Medicinal Products[26] define genetically modified HSC as the Medicinal Product, and guidelines detailing nonclinical aspects of medicinal products containing genetically modified cells are in preparation by regulatory authorities. Crucial parameters to be assessed include the dose of vector required for therapeutic efficacy, the *in vitro* toxicity of the vector, the profile of vector integration, the dose of transduced cells available at the end of the manufacturing process, the *in vivo* biodistribution of transduced cells, and the potential for vector release and germline transmission. Although *in vitro* tests for human HSC are well defined, surrogate *in vivo* assays may rely on transplantation of transduced CD34$^+$ cells in humanized immunodeficient mouse models.[27,28] Several investigators have employed these assays to study the properties of transduced cells as part of their preclinical studies,[29–31] but not all aspects related to pharmacological and toxicological properties of transduced CD34$^+$ cells have been previously addressed.

The aim of our study was to develop a clinically applicable transduction protocol for human CD34$^+$ cells using a purified good manufacturing practice (GMP)-grade lentiviral vector for the treatment of WAS and to assess the safety and efficacy in relevant *in vitro* and *in vivo* preclinical assays. Our results show robust transduction of CD34$^+$ cells from bone marrow (BM) and mobilized peripheral blood (MPB) of healthy donors (HD) and WAS patients, in the absence of detectable toxicity, allowing to validate manufacturing process. Transduced CD34$^+$ cells infused in immunodeficient mice were able to repopulate and differentiate into multiple lineages with a polyclonal profile of vector integrations, in the absence of vector shedding and germline transmission. Results from this and previous studies have provided the rationale and preclinical data to launch a clinical trial for HSC gene therapy of WAS.

RESULTS

Effect of prestimulation time and multiplicity of infection of LV on transduction efficiency, growth, and progenitor cell content

We aimed at identifying the culture conditions and vector exposure for clinically applicable efficient LV gene transfer into CD34$^+$ cells without toxicity using serum-free medium, early-acting cytokines, and retronectin. We first investigated the effect of different vector doses on human HD CD34$^+$ cells from BM or MPB prestimulated for 24 hours. Cells were analyzed by PCR for vector copy number (VCN), 12–15 days after transduction when only integrated copies are detected by the assay (**Supplementary Figure S1**). As shown in **Figure 1a**, we found a dose effect for both vector and number of exposures, reaching the highest transduction efficiency (0.84 ± 0.63) at two rounds of transduction with a multiplicity of infection of 100 (corresponding to a 1 × 10^8 TU/ml), with a cumulative dose of 2 × 10^8 TU/ml.

We then studied the effect of different prestimulation times (24 or 48 hours) on CD34$^+$ cells followed by a single hit of transduction in comparison to two hits of transduction. After a single hit, VCN was similar after 24 or 48 hour prestimulation. Two hits exposure to LV resulted in higher VCN versus one hit for both MPB and BM, with BM being transduced at an increased efficiency with respect to MPB (1.1 versus 0.5, paired *t*-test; *P* value = 0.003) (**Figure 1b,c**).

Expression of CD34 cell surface marker, clonogenic potential, and yield of recovered CD34$^+$ cells after culture did not show significant differences compared to untransduced (UT) cells in all conditions (**Supplementary Tables S1** and **S2**). As expected, there was a tendency toward a decrease in the percentage of CD34$^+$ cells after 48 hours prestimulation in the two hits protocols due to the longer period of culture (**Supplementary Table S2**).

Finally, we showed that transduced CD34$^+$ cells display a normal content in primitive progenitor cells (long-term culture-initiating cell (LTC-IC)) as compared to UT (**Figure 1d**). In conclusion, a 60-hour culture and two hits exposure under clinical grade conditions results in efficient transduction of CD34$^+$ cells without detectable impact on growth and differentiation capacity of transduced hematopoietic stem and progenitor cells.

Efficient transduction of WAS patients' CD34$^+$ cells using a clinical grade LV

Next, we transferred the optimized protocol to WASp-deficient CD34$^+$ cells obtained from patients' BM or MPB and investigated the efficiency of gene transfer in bulk cultures. Similarly to HD, also WAS CD34$^+$ cells exposed to two hits of infections had a significantly increased VCN as compared to one hit (1.44 ± 0.48 versus 0.64 ± 0.31) (**Figure 2a**). To verify transduction efficiency at the single progenitor cell level, we measured the percentage of LV positive colony-forming cell (CFC) colonies by quantitative PCR (qPCR). As shown in **Figure 2b**, the second hit of LV increased the percentage of transduction from 33.5 ± 13.7 to 53.8 ± 15.8 in HD and from 49.0 ± 20.3 to 79.9 ± 14.1 in WAS patients. Intriguingly, WAS patients' CD34$^+$ cells showed a higher VCN and gene transfer into progenitor cells as compared to both HD BM- and MPB-derived cells in all conditions. Importantly, there were no marked differences between transduced and UT cells in terms of growth, CD34 expression, total number of CFC colonies, and proportion of progenitors (erythroid, myeloid or mixed colonies) (**Table 1** and data not shown). In WAS BM CD34$^+$ cells, we observed a reduced recovery of cells after thawing and a decreased growth capacity and number of colony-forming cells versus HD CD34$^+$ cells, without difference between UT and transduced cells (data not shown).

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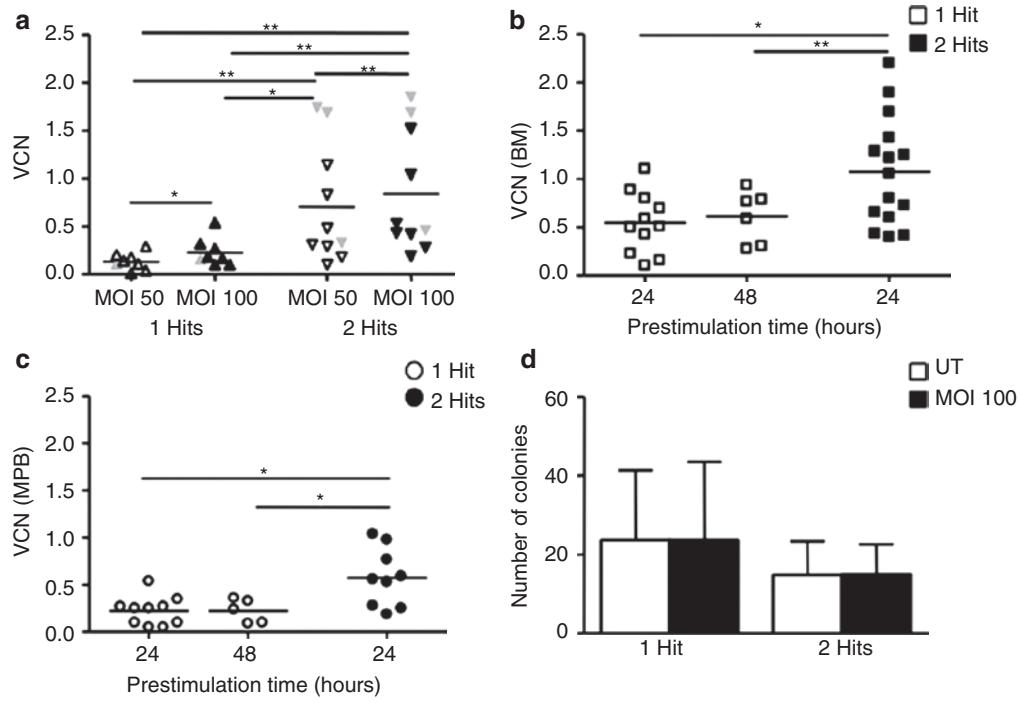


Figure 1 Optimization of transduction protocol using pre-GMP vectors. (**a**) VCN on CD34⁺ cells transduced with 1 or 2 hits of infection with two different MOI ($n = 10$ for 1 hit and $n = 12$ for 2 hits). Statistical analysis for significance was performed by Wilcoxon-matched paired test. In gray are indicated VCN of transduced CD34⁺ cells from WAS patients' BM or MPB. Lines indicate mean values. (**b**) CD34⁺ cells derived from healthy donors BM or (**c**) MPB were transduced with 1 or 2 hits after a prestimulation of 24 hours (BM, $n = 11$; MPB, $n = 10$) and 48 hours (BM, $n = 6$; MPB, $n = 5$) for 1 hit, and 24 hours (BM, $n = 15$; MPB, $n = 9$) for 2 hits. VCN/cell was calculated on cultured cells. (**d**) Total number of colonies derived from LTC-IC are reported for 1 ($n = 5$) and 2 hits ($n = 5$) of transduction at MOI 100, after 24 hours of prestimulation in the presence of cytokines. Results are reported as mean ± SD. *P value < 0.05, **P value < 0.01. BM, bone marrow; LTC-IC, long-term culture-initiating cell; MOI, multiplicity of infection; MPB, mobilized peripheral blood; UT, untransduced; VCN, vector copy number; WAS, Wiskott-Aldrich syndrome.

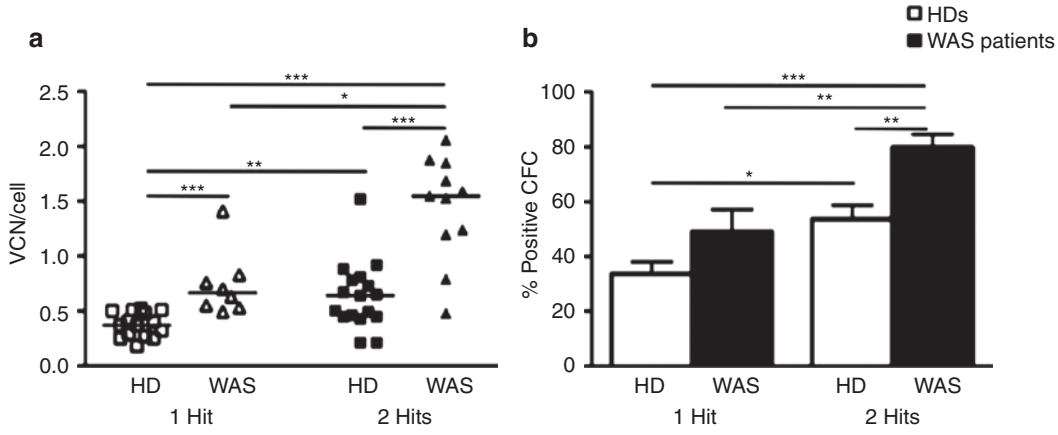


Figure 2 Efficient transduction of CD34⁺ cells by clinical LV vectors. BM or MPB from healthy donors (squares/white columns) or WAS patients (triangles/black columns) were transduced with 1 or 2 hits using GMP-grade lots of LV vector encoding WAS. (**a**) VCN/cell were calculated by qPCR on cultured CD34⁺ cells and (**b**) single transduced colonies. n for 1 hit trasduction protocol = 14 (6 MPB and 8 BM) and 8 (1 MPB and 7 BM) for HDs and WAS patients respectively, and n for 2 hits protocol = 17 (7 MPB and 10 BM) for HDs and 11 (2 MPB and 9 BM) for WAS patients. Lines indicate median values. *P value < 0.05, **P value < 0.01, ***P value < 0.001. BM, bone marrow; CFC, colony-forming cell; GMP, good manufacturing practice; HD, healthy donors; LV, lentiviral vectors; MPB, mobilized peripheral blood; qPCR, quantitative PCR; VCN, vector copy number; WAS, Wiskott-Aldrich syndrome.

Large scale GMP production of LV-transduced CD34⁺ cells

To further validate the selected protocol for use in WAS patients, three experiments were performed under large scale GMP conditions on BM CD34⁺ cells. CD34⁺ cells (12.5×10^6 cells) from HD were cultured in retronectin-coated bags in serum-free medium supplemented with cytokines and exposed twice to GMP grade purified vector at multiplicity of infection of 100, for a total culture time of 60 hours. As reported in **Table 2**, CD34 cell surface expression (72.8 ± 4.2%) and yield at the end of culture (1.22 ± 0.20)

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were in the range of small scale experiments, with no evidence of toxicity on bulk cultures or on clonogenic progenitors. *In vitro* cultured CD34$^+$ cells remained always cytokine-dependent (data not shown). Analysis of integrated vector by qPCR in transduced cells performed on single CFC colonies, showed robust and reproducible levels of transduction (53–67%). The mean VCN/cell in liquid bulk cultures maintained for 15 days after gene transfer was 0.70 ± 0.10. All quality tests on transduced CD34$^+$ cells were in agreement with the specification of the medicinal drug product, including cell viability, transgene expression, sterility, mycoplasma, endotoxin, replication competent LV, *in vitro* viral contaminants, and process contaminants (E1A DNA, large T antigen protein and plasmid DNA, free LV in cell supernatant) (**Table 2** and data not shown).

LV gene transfer restores WASp expression in differentiated cells from transduced CD34$^+$ cells

To determine if the clinical grade w1.6W LV vector allowed restoration of WASp expression, we performed transduction on different human cell lines and evaluated the presence of the protein by western blot or flow cytometric analysis. As shown in **Figure 3**, WASp expression was restored to normal levels after transduction of WASp-deficient untransformed T-cell lines (**Figure 3a**) and Epstein–Barr virus-transformed B cell line (**Figure 3b**).

We then analyzed WASp expression in transduced CD34$^+$ cells cultured in presence of cytokines promoting the differentiation to the myeloid lineage and specifically to megakaryocytes (MKs). Two hits transduction with w1.6W LV restored WASp expression in BM or MPB CD34$^+$ cells differentiated from three patients, reaching levels comparable to those of HD (**Figure 3c**) at a VCN of 0.7–1.9. Differentiated MKs from UT and transduced WASp-deficient CD34$^+$ cells were then evaluated for colocalization of WASp with the MKs marker CD41 by confocal microscopy. Remarkably, two hits transduction with the LV WAS allowed expression of WASp in MKs (**Supplementary Figure S2**).

Table 1 Characterization of CD34$^+$ cells transduced by clinical LV vectors

Source	Culture hours	Transduction	*n*	%CD34 ± SD	Yield ± SD	CFC ± SD
MPB	36	UT	7	94.5 ± 3.5	0.71 ± 0.23	126.3 ± 130.0
		1 hit	9	89.6 ± 22.2	0.69 ± 0.33	100.2 ± 71.1
	60	UT	6	86.9 ± 10.2	1.20 ± 0.75	82.9 ± 49.4
		2 hits	7	88.4 ± 5.5	1.23 ± 0.75	138.6 ± 115.4
BM	36	UT	6	88.6 ± 10.3	0.97 ± 0.21	89.0 ± 76.0
		1 hit	9	93.1 ± 4.8	1.13 ± 0.21	137.4 ± 98.5
	60	UT	6	74.0 ± 17.1	0.84 ± 0.47	154.3 ± 137.8
		2 hits	9	83.4 ± 11.2	0.98 ± 0.47	152.7 ± 117.7
WAS	36	UT	6	91.1 ± 3.5	0.65 ± 0.19	37.4 ± 30.5
		1 hit	7	93.1 ± 3.7	0.54 ± 0.17	35.7 ± 30.2
	60	UT	9	72.9 ± 15.9	0.47 ± 0.22	31.2 ± 44.4
		2 hits	9	75.5 ± 17.3	0.46 ± 0.25	35.8 ± 35.5

Abbreviations: BM, bone marrow; CFC, colony-forming cell; LV, lentiviral vectors; MPB, mobilized peripheral blood; UT, untransduced; WAS, Wiskott-Aldrich syndrome.
CD34 expression, yield (ratio of final and initial CD34$^+$ cell number) and total number of CFC colonies were evaluated on untransduced and transduced CD34$^+$ cells derived from healthy donors and patients' BM and MPB.

Normal biodistribution of LV-transduced CD34$^+$ cells in Rag2$^{-/-}$/γc$^{-/-}$ mice

In order to investigate whether transduction with the w1.6W LV clinical vector affects the ability of human HSC to engraft and differentiate *in vivo*, we injected transduced HD umbilical cord blood (UCB)-derived CD34$^+$ cells into sublethally irradiated Rag2$^{-/-}$/γc$^{-/-}$ neonate mice (VCN of CD34$^+$ cells differentiated *in vitro* 1.8 ± 1.5). Control animals were transplanted with the same amount of UT and unmanipulated (UM) UCB CD34$^+$ cells. After 8 weeks, the biodistribution of human-derived cells was analyzed in different organs by flow cytometry (a representative example of the analysis is shown in **Supplementary Figure S3**). As shown in **Figure 4a**, the engraftment of human CD45$^+$ cells was comparable to mice injected with UCB-derived UM and UT cells in all organs tested (BM, thymus, spleen, and liver). As expected, we found a majority of CD19$^+$ cells, and a small proportion of CD34$^+$ progenitor cells and CD13$^+$ myeloid cells in the BM and spleen of transplanted mice (**Figure 4b,c**). In the thymus, we observed a normal pattern of T-cell differentiation with presence of all subsets (CD4/CD8 double positive cells, single positive) (**Figure 4d**). No significant differences were observed between different treatment groups either in terms of level of engraftment or differentiation pattern in tested organs. To confirm the engraftment capacity of BM-derived cells, we injected transduced BM CD34$^+$ obtained from large scale transduction validation experiments (VCN: 0.7 ± 0.1), for which UM control cells were not available in sufficient number. As expected, BM-derived cells had lower levels of engraftment with respect to UCB (**Figure 4e**) with a similar pattern of differentiation (**Figure 4f**). As shown in

Table 2 Large scale validation transduction of CD34$^+$ cells derived from healthy donor BM

Run	% CD34	Yield	VCN/genome	% Of transduction	E1A DNA[a]	LargeT Ag (ng/ml)	Plasmid DNA (VSV-G)[a]
I	74.00	1.31	0.80	67%	<LLOQ	<LLOQ	<LLOQ (9,736)
II	68.10	0.99	0.60	56%	<LLOQ	<LLOQ	<LLOQ (6,234)
III	76.20	1.35	0.70	53%	<LLOQ	<LLOQ	<LLOQ (8,844)

Abbreviations: BM, bone marrow; CFC, colony-forming cell; LLOQ, lower limit of quantification; LV, lentiviral vectors; VCN, vector copy number; VSV-G, vesicular stomatitis virus G.
CD34 expression, recovery, integrated copy numbers per cell, and % of transduction on single CFC colonies, were evaluated on CD34$^+$ cells derived from healthy donor BM transduced with a clinical grade LV in three different large scale experiments. In plasmid DNA column, in brackets are reported values calculated immediately after transduction.
[a]Copies/75,000 cells.

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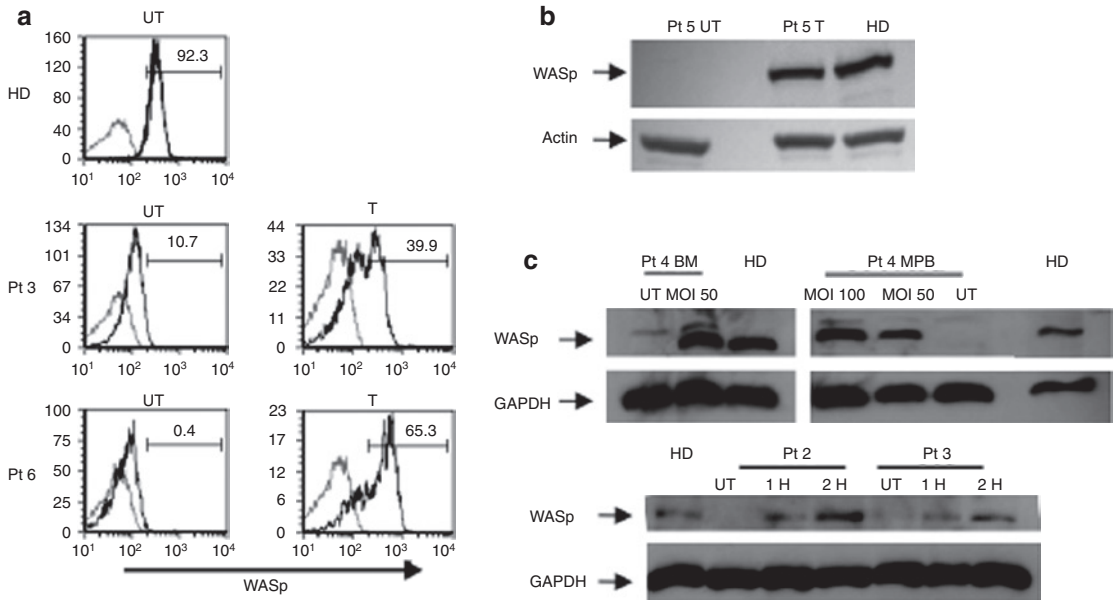


Figure 3 Restoration of WASp expression in transduced cells. (**a**) The expression of WASp was evaluated after transduction of untransformed T cells (FACS analyses), (**b**) EBV-transformed B cell lines (western blot), and (**c**) CD34$^+$ cells differentiated into myeloid cells (western blot) from normal donors and WAS patient(s). In **a**, in gray are indicated control cells, black lines indicated stained cells. Numbers represent the percentage of WASp positive cells. BM, bone marrow; EBV, Epstein–Barr virus; FACS, fluorescence-activated cell sorting; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HD, healthy donors; Pt, patient; UT, untransduced; WASp, WAS protein.

Figure 4g, LV-transduced cells were present in all hematopoietic organs, including BM (VCN 1.86 ± 1.31 for UCB and 1.07 ± 0.56 for BM), thymus (VCN 1.11 ± 0.92 for UCB and 1.08 ± 0.56 for BM), spleen (VCN 1.95 ± 1.78 for UCB and 0.48 ± 0.18 for BM), and liver (VCN 1.09 ± 0.87 for UCB and 0.43 ± 0.11 for BM).

Analyses of vector shedding, mobilization, and germline transmission of LV-transduced CD34$^+$ cells

To assess the safety of the drug product, we first performed experiments to investigate whether infectious vector particles were able to remain associated with gene corrected cells and potentially transduce bystander cells, as shown previously in a mouse model.[32] To exclude the occurrence of *in vivo* generation of replication competent lentivirus (RCL), vector mobilization or shedding to nontarget cells including germline, we followed different approaches exploiting the model of immunodeficient mice transplanted with transduced CD34$^+$ cells.

To investigate vector shedding and germline transmission, we designed specific experiments collecting gonads and brains of male mice (n = 7) injected with human BM- or UCB-derived transduced CD34$^+$ cells and perfused with saline solution, before harvest, to reduce organ contamination with circulating hematopoietic cells. As negative control, brains were used. No vector signal was detected by qPCR (sensitivity threshold: <16 copies in 10^5 cells) in these organs, indicating the absence of detectable vector shedding to nonhematopoietic tissues, including the germline (data not shown).

To exclude the transfer of packaging function or generation of RCL, we then measured gag p24 HIV by enzyme-linked immunosorbent assay (ELISA) in the plasma of 17 mice transplanted with LV-WAS transduced CD34$^+$ cells. All animals tested were negative except for a mouse that displayed a low-level of protein (0.4 ng/ml). HIV-1 *gag* sequence was detected by PCR on the BM of positive mice, but there was no evidence of *gag* integrated in the infused CD34$^+$ cells and the RCL culture assays were negative both on infused CD34$^+$ cells and on BM from the transplanted mouse. More detailed analyses using specific PCR primers indicated the presence of gag-pol packaging plasmid sequences in the original configuration, but not recombined with viral long terminal repeat or transfer vector sequences in the mouse BM (data not shown).

To assay the potential mobilization or shedding of vector cells to bystander cells, we designed a novel PCR assay (B2-SINE) that distinguishes between LV integrated in murine cells versus human cells. This method has a high sensitivity (up to 0.25% transduction, 35 transduced genomes into 14,000 UT ones) and specificity (no signal is detected in 100% human transduced cells) (**Supplementary Figure S4**). No evidence of vector sequences integrated in the murine genome from human to murine cells was reported in murine tissues (**Supplementary Figure S5a**), including BM from the mouse that was found positive for p24 (**Supplementary Figure S5b**).

LV insertion profile from *in vitro* cultured CD34$^+$ cells and human cells engrafted *in vivo* are polyclonal

In order to study the integration profile of w1.6W LV vector and the clonal repertoire of transduced cells, we collected insertion sites *in vitro* and *in vivo* through the combination of ligation-mediated/linear amplification-mediated PCR (LM/LAM-PCR) and pyrosequencing. We analyzed the distribution of the vector in CD34$^+$ cells from a pool of four HD BM and transduced human UCB *in vitro* and *in vivo* upon engraftment in Rag2$^{-/-}$/γc$^{-/-}$ mice. The insertion profile *in vitro* was typical of the LV vectors with significant preference for transcriptional units (from 74.5 to 77.0%

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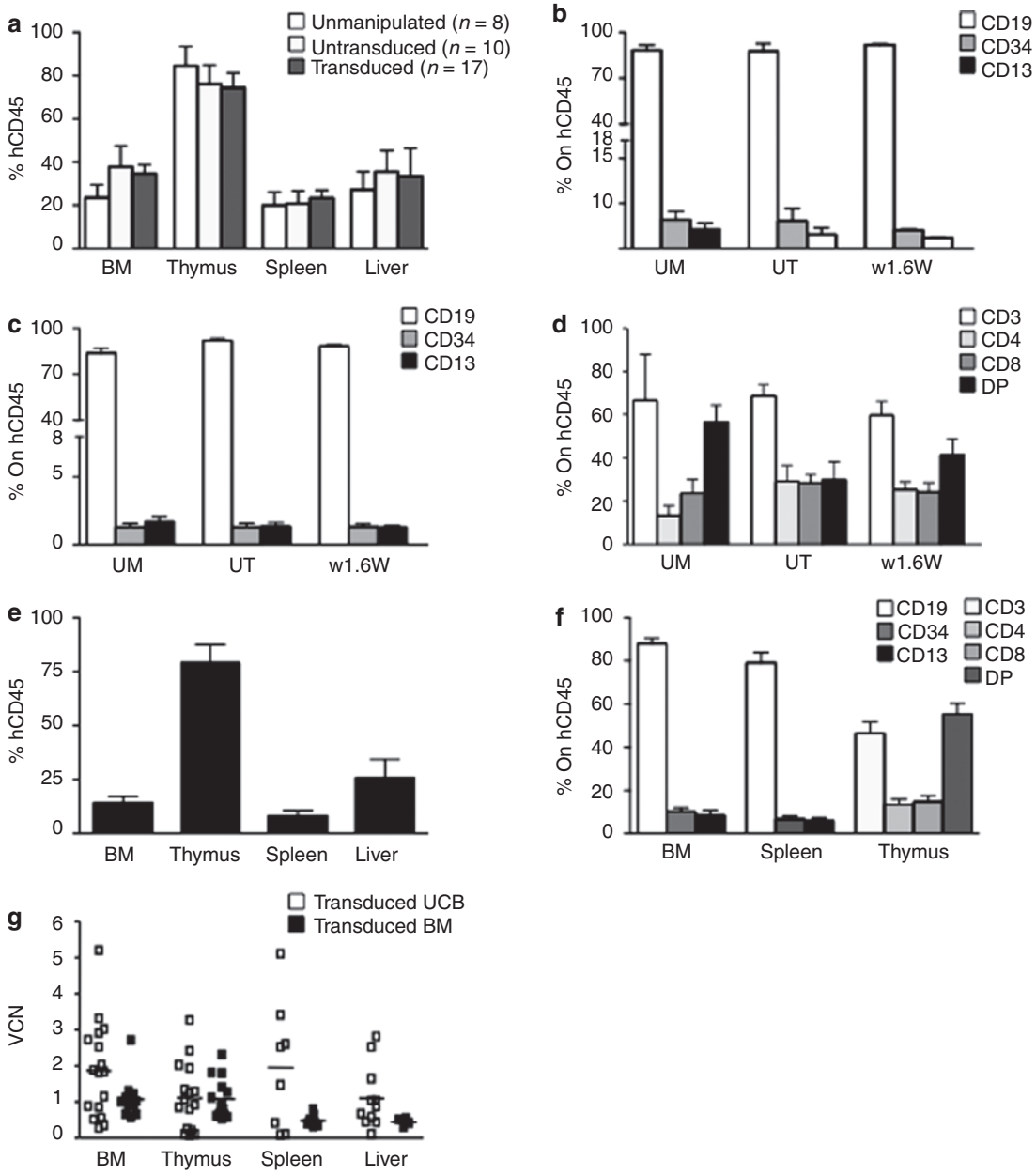


Figure 4 Engraftment of transduced human HSC in Rag2$^{-/-}$/γc$^{-/-}$ mouse model. Rag2$^{-/-}$/γc$^{-/-}$ mice were injected after sublethal irradiation with human UCB CD34^{+} cells either UM, UT or LV transduced. (**a**) Engraftment of human cells was calculated in terms of CD45^{+} cell in different organs. The percentage of most representative subpopulations in (**b**) BM, (**c**) spleen, and (**d**) thymus were evaluated by FACS on human CD45^{+} cells. (**e**) The percentage of engrafted BM-derived transduced CD34^{+} cells and (**f**) subpopulation distribution were evaluated on specified organs. (**g**) qPCR analysis was performed on cells from different organs or BM. BM, bone marrow; FACS, fluorescence-activated cell sorting; HSC, hematopoietic stem cells; LV, lentiviral vectors; qPCR, quantitative PCR; UCB, umbilical cord blood; UM, unmanipulated; UT, untransduced; VCN, vector copy number.

of integrations) as compared to *in silico* generated random insertions (n = 100,000, 40.7%) ($P < 0.01$, *z*-test for two proportions) (**Supplementary Table S3**).

This general bias was maintained *in vivo*, 8 weeks after transduced cell infusion in Rag2$^{-/-}$/γc$^{-/-}$ mice. As for the frequency of integration sites in proximity of genes involved in "Cancer" disease category from IPA software (Ingenuity Systems, http://www.ingenuity.com) we did not found any enrichment *in vivo* as compared to random frequency (6.3% in both HD and WAS patients versus 16.1% in random *in silico* insertions dataset; hit "Cancer" genes are listed in **Supplementary Table S4**). The number of unique insertions retrieved was in agreement with the overall profile of the LAM-PCR products run on Spreadex gels (Elchrom Scientific, Zurich, Swizerland), showing a polyclonal repertoire both *in vitro* in human CD34^{+} cells from HD and WAS patients (**Figure 5a**) and *in vivo* upon infusion in Rag2$^{-/-}$/γc$^{-/-}$ mice (**Figure 5b**). In addition, by comparing the insertions *in vivo* in BM and thymus from individual treated mice or in BM, thymus, and spleen from a pool of three mice, we observed the presence of shared identical integrants among the different hematopoietic compartments analyzed (**Figure 5c**).

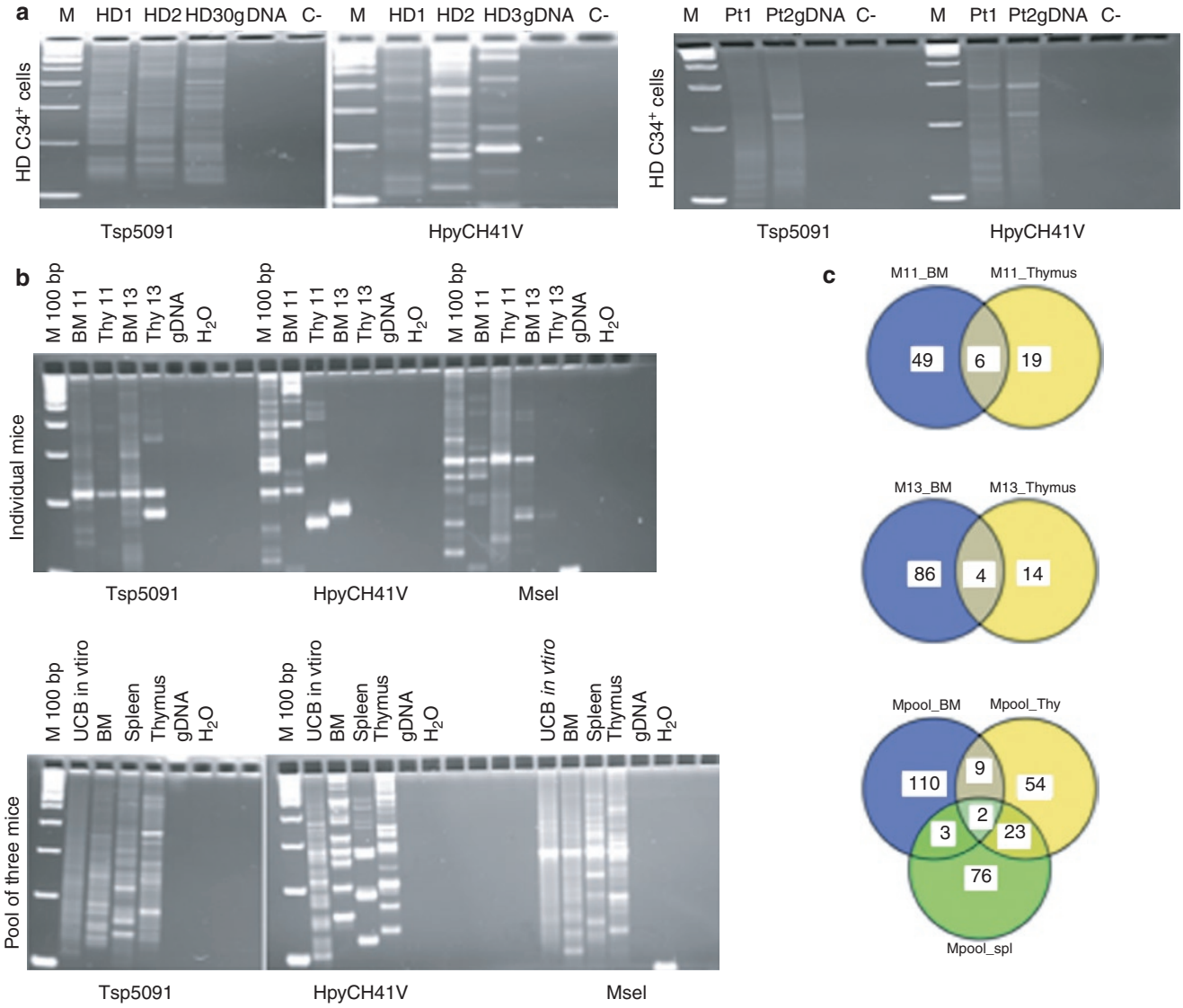
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DISCUSSION

Transplant of *ex vivo* gene corrected HSC could represent a valid therapeutic option for patients affected by WAS. Our results indicate that the clinical grade WAS-encoding LV under the control of its own promoter can transduce at high efficiency human CD34$^+$ progenitor cells from HD and WAS patients' in a dose-dependent manner, and drive protein expression in differentiated cells. The two hit protocol allowed us to reach 1–2 copies of vector per cells, shown to be required to achieve functional correction of the WAS defect,[23,24] and was thus selected for validation and clinical development.

No detectable toxic effect on the growth and *in vitro* or *in vivo* differentiation of CD34$^+$ cells were observed. We cannot rule out the two hits protocol (60 hours culture), may reduce the *in vivo* engraftment properties of hematopoietic stem and progenitor cells with respect to one hit protocol. It should be noted that the selected protocol is shorter than the one previously adopted in γ-retroviral vector-based trials. Long-term studies in humans will allow to determine the engraftment capacity of transduced CD34$^+$ cells at the moment of the infusion.

WAS patient-derived cells displayed an overall reduced capacity to grow in culture when exposed to cytokines as compared to HD cells, likely as a result of a defect in cell signaling or proliferation. Indeed, a key role for WASp in signaling of the cytokine receptor for stem cell factor (c-Kit) has been identified in murine hematopoietic cells.[33] The higher efficiency of gene transfer into WAS CD34$^+$ cells as compared to HD may be related to a higher susceptibility to infection from LV of WASp-deficient cells, possibly


a
M HD1 HD2 HD30gDNA C-
M HD1 HD2 HD3gDNA C-
M Pt1 Pt2gDNA C-
M Pt1 Pt2gDNA C-
HD C34+ cells
Tsp5091
HpyCH41V
Tsp5091
HpyCH41V
b
M 100 bp
BM 11
Thy 11
BM 13
Thy 13
gDNA
H2O
Individual mice
Tsp5091
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related to alterations in entry and post-entry processing of LV due to the absence of the protein. However, we cannot exclude that *in vitro* corrected WAS cells might have a selective advantage over UT cells, in agreement with the observed *in vitro* selective advantage for BM cells from WASp-heterozygous mice.[33]

Since the experimental medicinal product is represented by the autologous transduced CD34$^+$ cells and there is limited clinical experience with the *ex vivo* use of LV, we designed assays to assess biodistribution and the potential risk of vector shedding, mobilization, and germline transmission using the relevant human target cells in *in vitro* and *in vivo* models.[27] Our results show that WAS LV transduced healthy HSC migrate to the lymphohematopoietic organs and undergo *in vivo* short-term differentiation similarly to UT and unmanipulated HSC. Despite their limitations, our data combined to previous observations[28,30,31] indicate that immunodeficient mice could represent a suitable model for assessing biodistribution and safety of human CD34$^+$ cells from different sources. The sensitivity and observation time may be further improved by the use of novel immunodeficient strains which allow higher engraftment of human cells.[34]

Even though viral vector particles might be associated to the drug product at the end of the manufacturing process, our data show that LV distributes along with human hematopoietic cells, remaining integrated in the human genome and is not mobilized or shed to murine cells, as assessed by different approaches. In addition to the classical techniques for the analysis of vector biodistribution, we developed a new technique adapting and improving the B2 SINE-PCR from Follenzi *et al.*[35] Differently from conventional methods, this approach allows to distinguish between episomal and integrated form in unwanted target. In addition, integrated vector can be detected in several tissues including germ lines where silencing of transgene could mask the vector presence. The detection of HIV *gag* sequences in the BM of one mouse together with the evidence of HIV p24 gag antigen in the plasma raised the remote possibility that a RCL may have been generated, although such an event should have occurred during cell transduction as the virus batch was tested for RCL and shown to be negative. However, the safety features of the third generation packaging system combined with a SIN transfer vector used in this study make it extremely unlikely that a productive viral recombinant is generated during vector production or cell transduction. Indeed, no RCL was detected in the mouse tissue samples by the RCL assay. Thus, the most likely interpretation of the findings is the occurrence of a stable transfection of the packaging plasmid in a rare cell engrafted in the mouse. This is possible given that the plasmids used for the transient transfection of the vector-producer cells represent a major contaminant of the final vector product, even if the downstream purification process removes almost 99.9% of the input. This event was not deemed to be associated with pathology because it did not represent RCL nor it would lead to vector mobilization because the Gag-containing particles released from the cells would lack an envelope to make them infectious as well as a viral RNA, as the transduced SIN vector would be resistant to packaging and mobilization. If such Gag-expressing cells were to be infused in a patient their output would be diluted in the larger body fluids to well below detection and, most likely, the cells would be cleared by viral antigen-directed immunity (at variance with its survival in the immunocompromised SCID mouse).

The analysis of vector integrations *in vitro* and *in vivo* showed a strong bias of WAS vector for coding regions without major preference for regions upstream of transcription start sites, a feature typical of lentiviral vectors[10,36] (**Supplementary Table S3**), and proto-oncogenes (**Supplementary Table S4**). The insertional profile was polyclonal in human CD34$^+$ cells both *in vitro* and after engraftment and selection *in vivo* in Rag2$^{-/-}$/γc$^{-/-}$ mice (**Figure 5b**), in agreement with the results of preclinical studies of WAS-LV integration in murine cells.[37]

A gene therapy approach based on a γ-retroviral vector encoding WASp under the control of a strong viral promoter (myeloproliferative sarcoma virus (MPSV)) showed the therapeutic potential[20] of this approach but was associated with the occurrence of leukemia in a patient.[21] In this regard, the implementation of a LV platform in combination with a moderately active internal promoter should confer a substantially improved safety profile[9,38] over the MLV approach.

In summary, our data demonstrate that the GMP grade WAS-encoding LV can efficiently transduce CD34$^+$ cells under validated clinical conditions, leading to restoration of protein expression with no toxicity. This study provides important information about the quality and preclinical safety and efficacy data on transduced HSC for WAS patients complementing previous *in vitro* and *in vivo* studies performed in the murine WAS KO model which showed efficient engraftment of vector-transduced cells and immune reconstitution without the occurrence of LV-induced tumors.[22,23] Phase I/II gene therapy clinical protocols based on infusion of LV-transduced autologous CD34$^+$ cells combined with a reduced intensity conditioning received approval from regulatory authorities and have recently started by our and other groups.[39,40]

MATERIALS AND METHODS

Patients' and healthy donor cells. HDs granulocyte colony-stimulating factor MPB- or BM- derived CD34$^+$ cells were purchased from Lonza (Basel, Switzerland). HD UCB and WAS patients' BM cells were obtained after written informed consent according to standard ethical procedure and with approval of the San Raffaele Institute Bioethical Committee (protocol TIGET-01 and -02). Biological material from six WAS patients (score 3–5), diagnosed on the basis of clinical symptoms and genetic analysis[41,42] (**Supplementary Table S5**), was obtained at the time of diagnostic or therapeutic procedures. WASp expression was not detectable by western blot in patients' cells except for a weak expression observed in patient 3. CD34$^+$ cells were purified from mononuclear cells by positive selection with immunomagnetic beads according to the manufacturer's procedure (Miltenyi Biotec, Bergisch-Gladbach, Germany).

LV. The vector used in this study (w1.6W) is a 3rd generation, self-inactivating vesicular stomatitis virus (VSV.G) pseudotype lentiviral vector constructed on a pCCL backbone, previously described[23] (**Supplementary Figure S6a**). The vector lots were produced by MolMed S.p.a. (Milan, Italy), using a large scale validated process. The vectors were produced following GMP guidelines, except for initial studies of gene transfer protocol optimization, which were research grade. Vector characterization is reported in **Supplementary Table S6**.

Cell culture and transduction. CD34$^+$ cells were seeded on non-tissue culture treated Retronectin (TaKaRa Bio, Shiga, Japan) coated plates (30 μg/ml) or, for GMP manufacturing validation, in VueLife bags (American Fluoroseal, Gaithersburg, MD) at 1 × 10^6 cells/ml in serum-free CellGro

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SCGM Medium (Cell Genix Technologies, Friburg, Germany) in the presence of cells culture grade stem cell factor 300 ng/ml (Amgen, Thousand Oaks, CA), FLT3-L 300 ng/ml, Thrombopoietin 100 ng/ml, and IL-3 60 ng/ml (all from Peprotech, Rocky Hill, NY). Following different prestimulation times, cells were transduced with w1.6W LV at different multiplicity of infection for one or two hits of transduction. For the two hits protocol, a wash period of 10–12 hours was performed between the vector exposures. At the end, cells were analyzed by flow cytometry for the expression of CD34 and CD45 cell surface markers, plated for colony-forming unit cells (CFU-C) and LTC-IC assays or cultured (described in **Supplementary Materials and Methods**) to perform proviral integration evaluation by qPCR, WASp expression measurement, and integration profile analysis. This culture method promotes the differentiation of $CD34^+$ cells toward myeloid lineage.

Epstein–Barr virus-transformed B cell lines and untransformed T-cell lines were established and transduced as previously described.[25]

Western blot analysis. Western blot analyses was performed as previously described,[24] using anti-hWASP (H250; Santa Cruz Biotechnologies, Santa Cruz, CA), anti-hGAPDH (Chemicon, Temecula, CA), and anti-hActin (Sigma-Aldrich, St Louis, MO) antibodies, followed by secondary HRP-coupled antibodies (DAKO A/S, Glostrup, Denmark).

Mice. $Rag2^{-/-}/\gamma c^{-/-}$ mice on BALB/c background were obtained from the Central Institute for Experimental Animals (Nogawa, Japan). All animals were bred and maintained in a specific pathogen-free animal facility. Procedures were performed according to protocols approved by the Committee for Animal Care and Use of San Raffaele Scientific Institute (Committee Protocol no. 318). Three to four days after birth, mice were irradiated with two doses of 3 and 2.5 Gy, given at 3 hours interval. Three hours after irradiation, mice were injected with 0.3×10^6 UCB cells or 0.7×10^6 BM cells in 25–30 µl phosphate-buffered saline into the temporal vein.

After 8 weeks, mice were euthanized and organs were collected and analyzed for the presence of human $CD45^+$ cells and the expression of different lineage-specific surface markers. For collection of gonads and brain, mice were perfused injecting saline solution for 10 minutes through blood vessels of mice to eliminate hematopoietic contamination from organs, before sacrifice. Cells were analyzed for proviral integration evaluation by qPCR and integration profile by LAM-PCR analyses.

Real-time PCR analysis. In order to evaluate the number of LV copies integrated per genome, a qPCR was performed using specific primer and probes for human telomerase, LV, and murine β-actin. A reference standard was obtained from serially diluted transduced human T-cell line carrying one copy of integrated LV. Results of integrated vector copies were normalized for the number of evaluated genomes. As negative control, samples of UT cells were used. All the reactions were performed according to the manufacturer's instructions and analyzed with an ABI PRISM 7900 sequence detection system (Applied Biosystems, Foster City, CA). Primers and probes sequences were reported in **Supplementary Table S7**

Flow cytometric analysis. Surface staining of transduced HSC was performed with anti-CD34-PE and CD45-PerCP monoclonal antibodies. Cells collected from the organs of $Rag2^{-/-}/\gamma c^{-/-}$ mice were stained for the expression of surface markers: CD45-APC, CD19-PC7, CD34-PC5, CD3-FITC, CD4-PerCP, CD8-PE, CD13-PE, and CD15-FITC (all from BD Biosciences, San Jose, CA). Detection of WASp expression was performed after permeabilization (Cytofix/Cytoperm kit; BD Biosciences) by a purified anti-hWASp monoclonal antibody (clone 5A5; BD Biosciences) followed by an Alexa 488 conjugated goat anti-mouse antibody (Invitrogen, Carlsbad, CA). Cells were acquired using a FACSCantoII (BD Biosciences, San Jose, CA) and analyzed with FCS Express Software (De Novo Software, Los Angeles, CA).

Evaluation of p24 by ELISA assay and RCL detection. HIV p24 protein concentration was measured in mice plasma collected 7 weeks after UCB-transduced $CD34^+$ cell injection or culture supernatants by Alliance HIV-1 p24 ELISA kit (Perkin Elmer, Waltham, MA) according to manufacturer's instructions

The RCL assay was performed according to published method.[43,44] Briefly, permissive C8166-45 cell line were cocultured with transduced $CD34^+$ cells and cultured for 28 days. At the end of the culture, HIV p24 protein presence was determined by ELISA assay on culture supernatant. Moreover, C8166-45 genomic DNA was extracted to verify the absence of VSV-G DNA by qPCR.

B2-SINE PCR for detecting murine-specific integrations. In order to detect the presence of integrated LV in murine genome after systemic injection of human $CD34^+$ transduced cells, we performed a modified and improved three-step B2 SINE-PCR technique[35] (**Supplementary Figure S6b**). Sequences of primers and PCR conditions are reported in **Supplementary Materials and Methods**.

Integration profile analyses. Integrations were retrieved by LAM-PCR, as previously described.[9,45] Briefly, after a linear PCR amplification of genome-vector junctions, followed by a magnetic capture and second strand reconstitution, a digestion with *Tsp*509I, *Hpy*CH4IV or MseI was performed. Digestion products were ligated with a restriction site-complementary linker cassette and underwent to two sequential rounds of exponential PCR steps. The LAM-PCR products were tagged with megaprimers and underwent 454 pyrosequencing. Resulting genomic sequences were automatically aligned to the human genome (assembly hg18 of the UCSC genome browser, http://genome.ucsc.edu/).

Statistical analysis. Statistical analysis was performed using GraphPad Prism software (GraphPad Software, La Jolla, CA), analyzing data sets for significance by Mann–Whitney two-tailed test, unless otherwise stated.

SUPPLEMENTARY MATERIAL

Figure S1. VCN evaluation of different time-point in transduced $CD34^+$ cells.
Figure S2. Restoration of WASp expression in transduced cells.
Figure S3. Engraftment of human HSC in $Rag2^{-/-}/\gamma c^{-/-}$ mouse model.
Figure S4. SINE-PCR resolution and sensitivity.
Figure S5. No vector sequence integrated in murine bystander cells.
Figure S6. Schematic representation of LVV and SINE-PCR method.
Table S1. Recovery and CFU numbers from healthy control's BM and MPB $CD34^+$ cells untransduced and transduced with different vector concentration.
Table S2. Recovery and CFU numbers of healthy control's BM and MPB-derived $CD34^+$ cells untransduced and transduced after different prestimulation time.
Table S3. Insertion profile in *in vitro*- and *in vivo*- transduced cells.
Table S4. Cancer genes.
Table S5. Summary of patients score and mutations.
Table S6. Characterization of GMP and pre-GMP vector.
Table S7. Sequence of primers for qPCR.
Materials and Methods.

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