

Transduced CD34⁺ Cells from Adrenoleukodystrophy Patients with HIV-Derived Vector Mediate Long-Term Engraftment of NOD/SCID Mice

Sonia Benhamida,¹ Françoise Pflumio,² Anne Dubart-Kupperschmitt,² Jing-Chao Zhao-Emonet,¹ Marina Cavazzana-Calvo,³ Francis Rocchiccioli,¹ Serge Fichelson,² Patrick Aubourg,¹ Pierre Charneau,⁴ and Nathalie Cartier^{1,*}

¹INSERM U561, Hôpital Saint Vincent de Paul, 75014 Paris, France

²Département d'Hématologie, Institut Cochin, 75014 Paris, France

³Laboratoire de Thérapie Cellulaire et Génique, Hôpital Necker, Paris, France

⁴Unité d'Oncologie Virale, Institut Pasteur, 75724 Paris Cedex 15, France

*To whom correspondence and reprint requests should be addressed. Fax: (33) 1 4048 8352. E-mail: cartier@cochin.inserm.fr.

X-linked adrenoleukodystrophy (ALD), an inherited demyelinating disorder of the central nervous system, can be corrected by allogeneic bone marrow transplantation, likely due to the turnover of brain macrophages that are bone marrow derived. ALD is characterized by an accumulation of very long chain fatty acids (VLCFA) due to the deficiency of an ATP binding cassette transporter that imports these fatty acids in peroxisomes. Murine retroviral transduction results in metabolic correction of ALD CD34⁺ cells *in vitro* but reinfusion of these cells into ALD patients would not provide clinical benefit owing to the absence of selective advantage conferred by transgene expression. High-efficiency transduction of ALD CD34⁺ peripheral blood mobilized cells was achieved using an HIV-based vector driving ALD gene expression under the elongation factor 1 α promoter and a protocol without prestimulation of CD34⁺ cells with cytokines prior to transduction to preserve their stem cell properties. Efficient expression of the ALD gene was demonstrated in monocytes/macrophages derived from cultures of transduced ALD CD34⁺ cells and in long-term culture initiating cells. VLCFA metabolism was corrected in transduced CD34⁺, CFU-derived, and LTC-derived cells, indicating that the vector-encoded ALD protein was fully functional. Transplantation of transduced ALD CD34⁺ cells into NOD/SCID mice resulted in long-term expression of ALD protein in monocytes/macrophages derived from engrafted stem cells.

INTRODUCTION

X-linked adrenoleukodystrophy (ALD) is a genetic disorder (1/17,000 males) characterized by progressive demyelination within the central nervous system (CNS) [1,2]. The cerebral form of ALD affects boys between 5 and 12 years of age and leads to vegetative stage or death within 2–5 years. Adult ALD males develop initially spinal cord involvement between 20 and 30 years of age but 35% of them show later cerebral demyelination. Overall, 65% of ALD patients are at risk to develop fatal cerebral demyelination during childhood or adulthood. Cerebral demyelination is diffuse at a late stage of the disease but CNS myelin is normal until patients develop the disease. Brain magnetic resonance (MR) imaging, MR spectroscopy, and neuropsychological tests can detect demyelinating lesions years before clinical symptoms become evident [1,2]. There is thus a window for therapeutic intervention in ALD patients for whom cerebral demyelination can be diagnosed at an early stage.

The ALD protein is expressed in glial cells [3], including microglia, and belongs to the superfamily of ATP-binding cassette transporters [4]. ALD protein is an integral membrane protein localized in the peroxisomal membrane that plays a role in the transport of very long chain fatty acids that accumulate in the white matter of ALD patients [1,2].

Allogeneic bone marrow transplantation (BMT) is the only therapeutic approach proven to be effective in the cerebral form of ALD [5,6]. When performed at an early stage of cerebral ALD, BMT can stabilize or even reverse cerebral demyelination. The long-term benefits of BMT [6] are likely due to the turnover of brain macrophages (microglia) that are bone marrow derived [7,8].

However, the efficacy of allogeneic BMT is limited by the lack of donors and the mortality risk of the procedure. Targeting the ALD gene into hematopoietic stem cells (HSC) of ALD patients followed by reinfusion of modified

TABLE 1: Expression of ALD protein (ALDP) in human ALD CD34⁺ cells after lentiviral-mediated ALD gene transfer

ALD patient	% of cells expressing ALDP 72 h after transduction	% of individual CFU-GM colonies expressing ALDP	% of individual CFU-GM colonies derived from LTC expressing ALDP
1	56.5 ± 13.1 (n = 4)	38.5 ± 16.1 (n = 3)	41 ± 2 (n = 2)
2	37.5 ± 3.5 (n = 2)	39 ± 8.5 (n = 2)	50 (n = 1)
3	47.5 ± 3.5 (n = 2)	32.5 ± 3.5 (n = 2)	ND

Results are expressed as means ± SD of *n* performed experiments. ND, not done.

cells would circumvent most of the problems associated with allogeneic BMT. The source of HSC would be the CD34-positive fraction purified from bone marrow or from cytokine-mobilized peripheral blood cells.

We have previously demonstrated *in vitro* the correction of 15–20% of ALD CD34⁺ cells using a Moloney-derived retrovirus vector with stable expression of the ALD gene in CFU-derived colonies after 5 weeks of long-term culture (LTC) [9]. We have demonstrated that macrophages, the therapeutic cells of our approach, are corrected and that recombinant ALD protein is functional [9]. However, normal cells have no selective advantage over ALD cells and transduction of HSC with murine retrovirus vectors requires that one use a high cytokine concentration to cycle the CD34⁺ cells. As a result, HSC enter into differentiation, leading to loss of ALD gene expression in the long term [10].

In contrast, human immunodeficiency virus (HIV)-based vectors are able to transduce nondividing cells [11], including HSC [12–15], especially vectors that include the central polypurine tract (cPPT) and the central termination sequence (CTS) [16–18], leading to an increase in gene transduction efficiency in human CD34⁺ cells [17,18], making these vectors promising for future gene therapy of HSC.

We show that an HIV-based vector containing the cPPT-CTS tract is able to transduce ALD-mobilized peripheral blood CD34⁺ cells capable of long-term human hematopoiesis in NOD/SCID mice. High-efficiency transduction occurred using a protocol minimizing the use of cytokines to preserve the properties of HSC, resulting in ALD transgene expression in NOD/SCID mice-engrafted macrophages. These results obtained by the use of the improved lentiviral vector described here are an important step toward successful ALD gene therapy.

RESULTS

Lentiviral Vector-Mediated ALD Gene Transfer into ALD CD34⁺ Cells

We used a modified HIV-based TRIP-vector (TRIP-ΔU3-EF1α-ALD) that includes a deletion of the enhancer/promoter of the 3' LTR and the elongation factor 1α (EF1α) promoter. This vector was previously shown to efficiently transduce human cord blood HSC with enhanced green

fluorescent protein (EGFP) transgene expression in CD34⁺-derived T, B, NK, and myeloid hematopoietic cells [19].

Taking advantage of the ability of the lentiviral vector to transduce nondividing cells and to avoid terminal differentiation of CD34⁺ cells before and during transduction, we used a protocol characterized by the absence of prestimulation with cytokines prior to incubation with the vector and a short (36 h) vector incubation time with low-cytokine serum-free medium.

Cytokine-mobilized peripheral CD34⁺ cells from three ALD patients whose ALD gene mutation led to a complete absence of ALD protein were transduced with the TRIP-ΔU3-EF1α-ALD vector. After transduction, cells were incubated for 72 h in LTC medium without cytokines. Transduction efficacy was then analyzed by the expression of ALD protein using immunocytochemistry. ALD protein was expressed by 37.5 to 56.5% (mean 47.2%) of ALD cells (Table 1).

To examine the transduction efficiency in colony-forming cells (CFCs), we plated transduced ALD CD34⁺ cells on methylcellulose immediately after transduction and cultured for 14–16 days. The number of individual CFC that expressed ALD protein ranged from 32.5 to 39% (Table 1) (mean 36.6%). No difference either in plating efficiency or in CFU-GM/BFU ratio was observed in transduced vs nontransduced cells (data not shown).

In Vitro Analysis of Transduced ALD Hematopoietic Cells

In the perspective of clinical application, the main goal of ALD gene transfer is to target immature hematopoietic stem cells with proliferating and differentiating potentials in monocytes/macrophages. To demonstrate that the TRIP-ΔU3-EF1α-ALD lentiviral vector was able to transduce the ALD gene into such cells, we used different approaches (Fig. 1).

First, we cultured transduced ALD CD34⁺ cells (patient 3) under conditions that promote lymphomyeloid differentiation [18,19]. We obtained 1.5% NK and 85% CD15⁺ myeloid cells, of which 68% were macrophages (CD15⁺CD14⁺) and 32% granulocytes (CD15⁺CD14⁻). Monocytes/macrophages were further identified with an anti-CD68 antibody, and double immunostaining with anti-ALD protein antibody showed that 15% of mono-

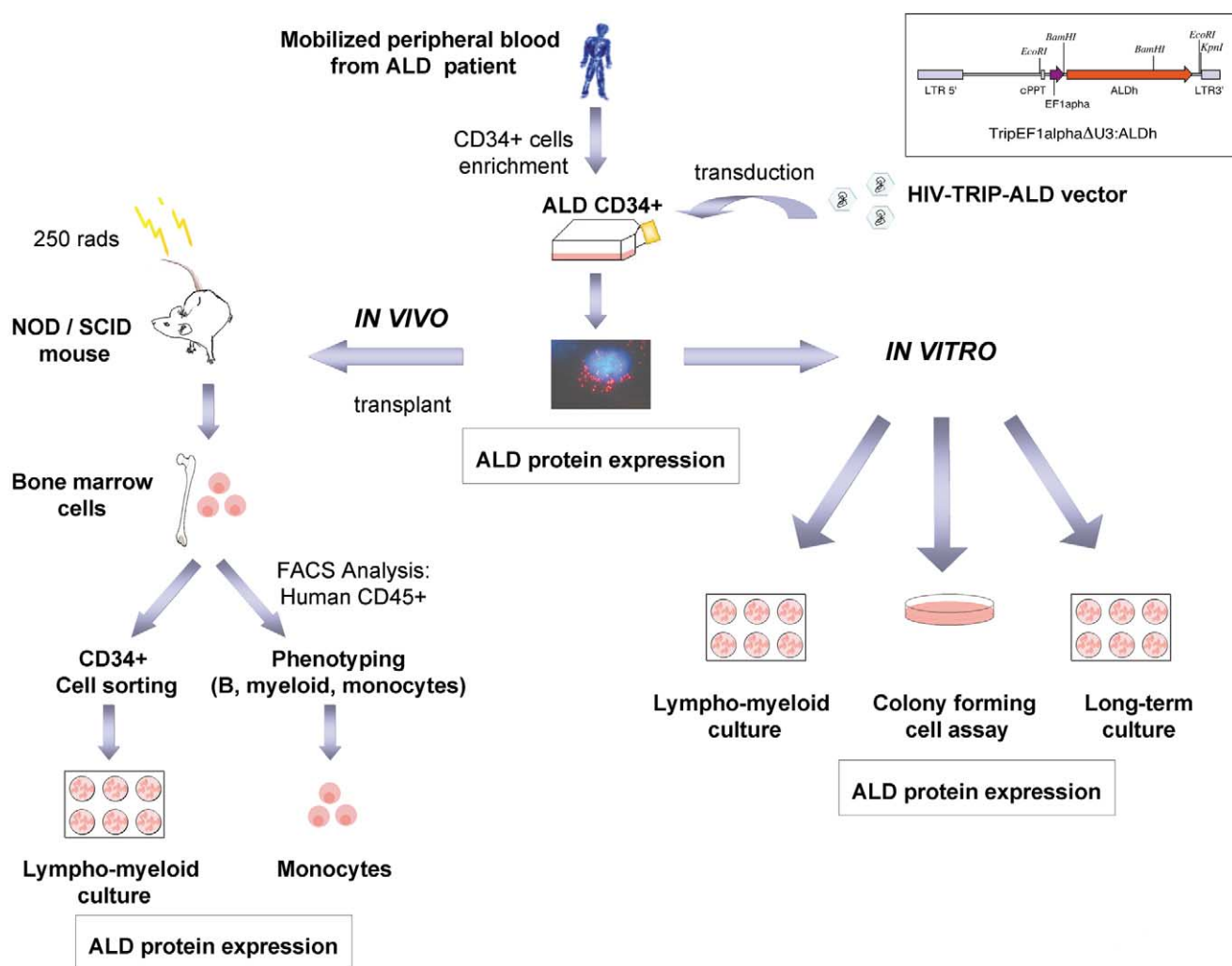


FIG. 1. Diagram of experimental strategy to evaluate transduction of ALD CD34⁺ cells.

cytes/macrophages expressed ALD protein. An average 0.5 copy of the provirus per cell was found to be integrated in these cells as evaluated by semiquantitative PCR analysis (data not shown).

Second, we maintained transduced CD34⁺ cells from two ALD patients (Nos. 1 and 2) in LTC for 5 weeks. ALD protein was detected in 35% of 3000 peroxisome-containing cells that were examined at the end of the two LTC experiments with no variation of this percentage between LTC experiments.

LTC-derived cells were then plated on methylcellulose and CFU-GM colonies were individually and randomly picked and scored for ALD expression. From a total of 90 CFU-GM colonies derived from three LTCs (30 colonies for each experiment), 44% expressed ALD protein (Table 1), indicating that LTC initiating cells (LTC-ICs) had been transduced. To determine the transduction efficacy in

LTC-ICs, we performed LTC of transduced ALD CD34⁺ cells in limiting dilution. After 5 weeks, cells from each well were plated on methylcellulose and the number of CFU-GM colonies was scored after 15 days. The percentage of wells, originally seeded with one cell, that gave rise to CFU-GM colonies is representative of the LTC-IC frequency. The results indicate that 20% of transduced ALD CD34⁺ cells were LTC-ICs, similar to the percentage observed with nontransduced peripheral blood normal CD34⁺ cells cultured under the same conditions (not shown).

We scored ALD protein expression in CFU-GM colonies derived from 10 cells per well, which represents two LTC-ICs/well according to the LTC-IC frequency. Colonies from each methylcellulose plate were pooled and analyzed. ALD protein-expressing cells were found in every plate, indicating that at least 50% of LTC-ICs had been transduced.

TABLE 2: Correction of very long chain fatty acid metabolism in ALD CD34⁺ cells after transduction with a lentiviral vector and in derived CFU-GM colonies and LTC cells

	Observed C _{26:0} /C _{22:0}			% of transduced ALD cells expressing ALDP	% of biochemical correction
	Control cells	Nontransduced	ALD cells Transduced		
CD34 ⁺ cells	0.041 ± 0.018 (n = 4)	0.192 ± 0.0421 (n = 2)	0.118 ± 0.016 (n = 2)	40	49
CFU-GM-derived cells	0.042 ± 0.016 (n = 2)	0.121 (n = 1)	0.072 (n = 1)	45	62
Cells derived from 5-week LTC	0.021 ± 0.012 (n = 2)	0.113 (n = 1)	0.088 (n = 1)	16.5	27

The percentage of biochemical correction is calculated by linear regression from C_{26:0}/C_{22:0} values observed in nontransduced and transduced cells [9].

These results indicate that the LTC-IC compartment is well maintained during the transduction protocol and that these immature progenitors have been efficiently transduced.

Functional Correction of ALD Biochemical Defect in Hematopoietic Cells *in Vitro*

ALD is biochemically characterized by the accumulation of VLCFAs that involves mainly hexacosanoic (C_{26:0}) acid, whereas the concentration of docosanoic (C_{22:0}) acid remains normal. The C_{26:0}/C_{22:0} ratio thus reflects the ability of cells to metabolize very long chain fatty acids (VLCFA) in the presence of functional ALD protein [1,2].

Table 2 shows that the C_{26:0}/C_{22:0} ratio decreased proportionally to the percentage of cells that expressed ALD protein in ALD CD34⁺ transduced cells, 72 h after transduction, in CFU-GM-derived cells and in transduced ALD CD34⁺ cells cultured for 5 weeks (LTC). The correction of C_{26:0}/C_{22:0} ratio was greater than expected with respect to the number of cells expressing ALD protein, suggesting that overexpression of ALD protein leads to increased VLCFA degradation, as previously shown using murine retroviral-mediated transfer of the ALD gene into ALD CD34⁺ cells [9]. These results indicate that lentiviral vector-encoded ALD protein is functional in peroxisomes of transduced hematopoietic ALD cells.

Engraftment of Transduced ALD CD34⁺ Cells in NOD/SCID Mice

Long-term *in vivo* transplantability of human hematopoietic cells in NOD/SCID mice is considered a hallmark of cell immaturity [20]. We therefore injected 10⁶ to 1.5 × 10⁶ ALD CD34⁺ cells immediately after transduction into 18 NOD/SCID mice (Fig. 1).

Eighteen weeks after transplantation, we sacrificed the mice and analyzed human hematopoietic engraftment by fluorescence-activated cell sorting (FACS) of bone marrow cells with anti-human CD45 antibody. Five of the 18 NOD/SCID mice were engrafted with transduced ALD human CD34⁺ cells in proportions ranging from 3 to 75%

(Table 3 and Fig. 2A). The variability of human engraftment reflects oligoclonal rather than polyclonal engraftment of NOD/SCID repopulating cells and the fact that reconstitution of NOD/SCID mice with peripheral mobilized blood CD34⁺ cells is less efficient than with cord blood CD34⁺ cells. Recombinant ALD protein was expressed in 30 to 85% of bone marrow cells from recipient mice (Table 3 and Fig. 2B).

Bone marrow cells of NOD/SCID mouse 3 engrafted with 75% CD45⁺ human cells were phenotyped with specific human CD11b, CD14, CD15, and CD19 antibodies (Fig. 2). As expected, the majority (58%) of human CD45⁺ cells were B lymphocytes (CD19⁺) (Fig. 2C); 10% were myeloid cells (CD15⁺) (Fig. 2C) and 1.75% monocytes (CD14⁺, CD11⁺) expressing ALD protein (Figs. 2D and 2E). Interestingly, bone marrow from mouse 3 contained 1% human CD34⁺/CD38⁻ cells (Fig. 3A), indicating that early human hematopoietic progenitors engrafted the mouse bone marrow.

Bone marrow-engrafted human CD34⁺ cells from mouse 3 were sorted by flow cytometry and cultured under conditions that promote lymphomyeloid differentiation [18,19]. Seventy-five percent NK and 18% myeloid (CD15⁺) cells were obtained, of which 72% were macrophages (CD15⁺, CD14⁺) and 28% granulocytes (CD15⁺, CD14⁻). Ten per-

TABLE 3: Analysis of ALD protein (ALDP)-positive cells in bone marrow from transplanted NOD/SCID mice

NOD/SCID mouse	% of human CD45 ⁺ cells in bone marrow	% of human CD45 ⁺ cells expressing ALDP in bone marrow
3	75	30
8	25	80
9	10	85
13	7	57
17	3	73

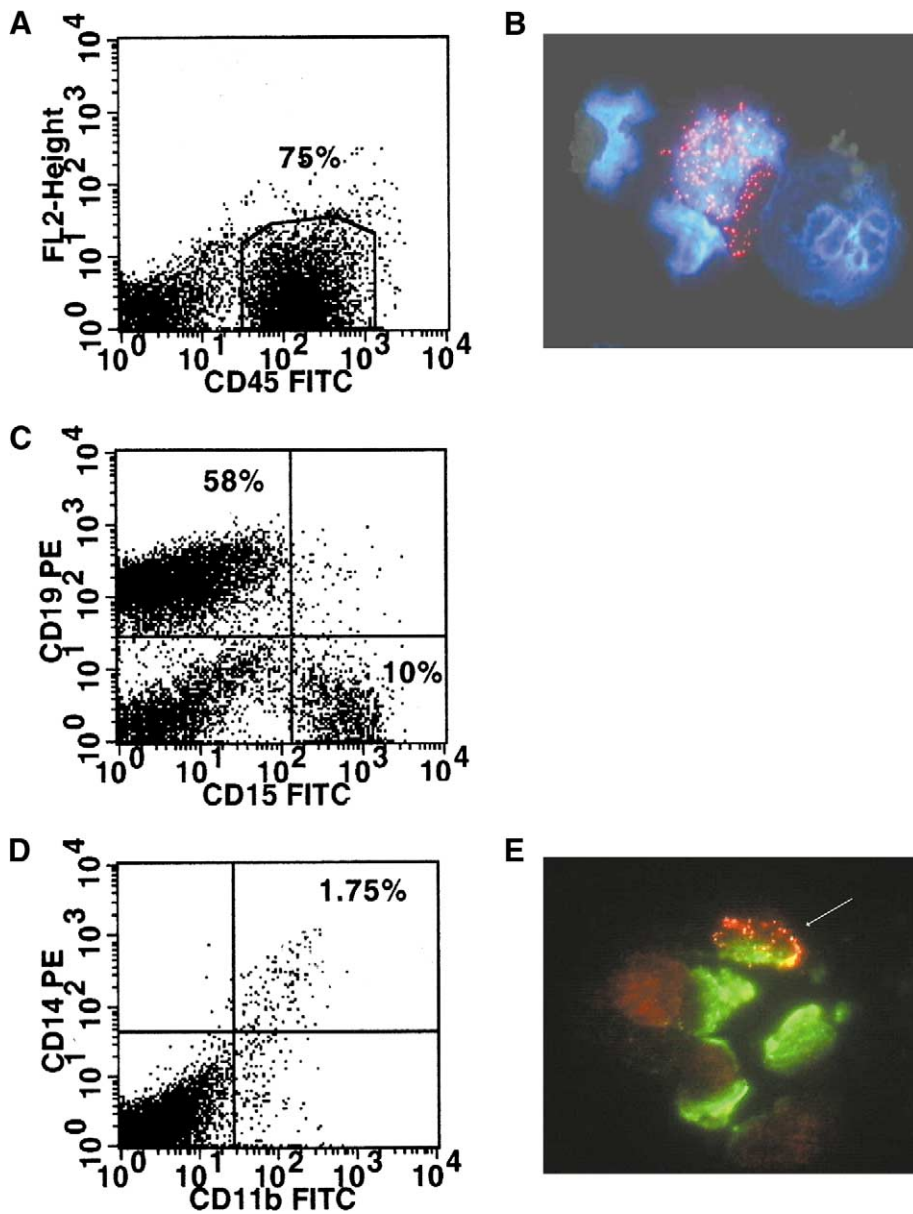
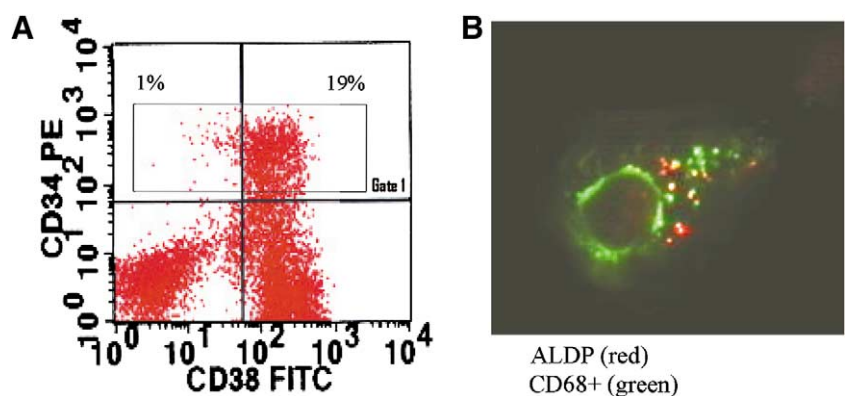


FIG. 2. Phenotype and expression of ALD protein in bone marrow cells from NOD/SCID mouse transplanted with human ALD CD34⁺ cells transduced with HIV-derived TRIP- Δ U3-EF1 α -ALD vector. Dot-plot analyses show the percentages of human CD45⁺ hematopoietic cells (A), CD19⁺ B lymphocytes (C, left upper quadrant), CD15⁺ myeloid cells (C, right lower quadrant), and CD14⁺/CD11b⁺ monocytes (D, right upper quadrant) recovered from the bone marrow of one NOD/SCID mouse (No. 3, Table 3), 18 weeks after transplantation with transduced ALD CD34⁺ cells. (B) Human ALD protein expression was studied by immunocytochemistry on at least 500 bone marrow cells. A punctuated staining corresponding to the peroxisomal expression of ALD protein (Cy3, in red) is observed in transduced cells (overlay with nucleic acid stained with DAPI in blue). (E) Monocytes/macrophages recovered from NOD/SCID mouse bone marrow and expressing recombinant ALD protein were identified by double immunostaining with antibodies against human ALD protein (Cy3, in red, arrow) and CD68 (FITC, in green).

FIG. 3. Transduced ALD CD34⁺ cells capable of macrophage differentiation long term engraft the bone marrow of NOD/SCID mice. (A) This dot-plot analysis shows that bone marrow of a NOD/SCID mouse transplanted with transduced ALD CD34⁺ cells contains 20% CD34⁺ cells, among which 1% do not express the CD38 surface antigen marker (left upper quadrant), 18 weeks after transplantation. CD34⁺ cells were sorted by flow cytometry (Gate 1). (B) Transduced CD68⁺ (FITC, in green) monocytes/macrophages expressing human ALD protein (Cy3, in red) were identified in sorted CD34⁺ cells cultured under conditions that promote lymphomyeloid differentiation.



cent CD68-positive monocytes/macrophages present in this culture expressed ALD protein (Fig. 3B).

Altogether, these results show that ALD HSC capable of long-term NOD/SCID bone marrow engraftment have been indeed transduced. Moreover, these cells have the capacity to differentiate both *in vivo* and in culture into monocytic/macrophagic cells that express recombinant ALD protein.

DISCUSSION

Therapeutic trials targeting hematopoietic cells with murine retroviral vectors have been initiated for several genetic disorders, including combined immunodeficiency diseases, Gaucher disease, and chronic granulomatous disease [reviewed in 21]. One major pitfall of these trials resides in the very low percentage of corrected cells (usually <1%) in peripheral blood several months after reinjection of transduced hematopoietic stem cells. Because murine retrovirus vectors are not able to transduce nondividing cells, the transduction of HSC with such vector requires the use of high cytokine concentration to trigger mitosis. As a result, transduced HSC enter into differentiation, leading to loss of transgene expression in the long term. One exception is the case for SCID-X in which gene therapy was able to provide full correction of disease phenotype and, hence, clinical benefit, owing to the selective advantage conferred by transgene expression [22].

Adrenoleukodystrophy is a genetic disorder of CNS myelin that can be corrected by allogeneic BMT when this procedure is performed at an early stage of the disease [5,6]. Although the biological mechanisms allowing CNS correction are not fully understood, the effect of BMT is likely mediated through the replacement of endogenous mutated microglia by normal ALD protein-expressing microglial cells that derive from donor hematopoietic stem cells [10]. However, normal or transduced ALD hematopoietic stem cells have no selective advantage over ALD cells and therapeutic trials with murine retroviral vectors would not provide clinical benefit. HIV-based vectors have the ability to cross through the nuclear pores of nondividing cells and therefore may alleviate the requirement for target HSC cycling. The longevity and persistence of the vectors in human hematopoietic stem cells grown in extended long-term culture [14] and engrafted for several months in NOD/SCID mice [13,19,23–25] indirectly demonstrate integration into the stem cell genome.

Using a protocol in which CD34⁺ cells were not activated with cytokines prior to transduction and in which very low concentrations of cytokines (10 ng/ml stem cell factor (SCF), Flt3 ligand (FL), interleukin (IL)-3, and pegylated megakaryocyte growth and differentiation factor (hereafter named TPO)) were added during a short period of transduction (36 h) to preserve the stem cell properties of CD34⁺ cells, we demonstrate that an HIV-based TRIP- Δ U3-EF1 α -ALD vector allows transduction of ALD CD34⁺

peripheral blood mobilized cells, with efficient transduction of primitive cells. The EF1 α promoter driving the expression of ALD cDNA was chosen to allow high transgene expression in all hematopoietic cell types [19].

Indeed, among these ALD CD34⁺ cells, 36.5% of the granulomacrophagic and erythrocytic clonogenic progenitor-derived colonies expressed recombinant ALD protein. A limiting dilution assay allowed us to demonstrate that at least 50% of the LTC-ICs were transduced. The transduced ALD CD34⁺ cells differentiated normally *in vitro* and recombinant ALD protein was detected in at least 15% of the monocytes and macrophages. Furthermore, VLCFA metabolism was corrected in ALD CD34⁺ cells and in derived CFU-GM colonies and LTC cells, indicating that the vector-encoded ALD protein was functional in these cells. The biochemical correction was demonstrated in cells lacking ALD protein. Recombinant ALD protein was markedly overexpressed and likely sufficient to correct ALD cells with present although nonfunctional ALD protein. Some authors [26] have raised concerns about the ability to correct ALD cells with missense mutations and present although nonfunctional ALD protein, due to competition for insertion or function within the peroxisomal membrane of normal and mutant forms of ALD protein. However, we have previously shown that the expression of recombinant ALD protein in fibroblasts through an oncoretroviral vector was sufficient to correct to the same extent ALD cells with or without endogenous ALD protein [27]. In any case, this point will need to be checked for every patient with or without missense mutation for future gene therapy trial.

We used the NOD/SCID model to evaluate long-term ALD gene expression driven by the TRIP- Δ U3-EF1 α -ALD vector. We observed that ALD protein was expressed in 30 to 85% of bone marrow cells from engrafted NOD/SCID mice, 18 weeks after transplantation. Cells harvested from one NOD/SCID mouse bone marrow contained early human hematopoietic progenitor cells (i.e., CD34⁺/CD38⁻ cells) and NOD/SCID bone marrow-engrafted CD34⁺ cells derived from transduced ALD CD34⁺ cells were able to differentiate into monocytes/macrophages expressing vector-encoded ALD protein.

The high transduction efficiency of HIV-based TRIP- Δ U3-EF1 α -ALD vector and its capacity to induce *in vitro* and *in vivo* strong and long-lasting ALD protein expression in macrophages derived from transduced ALD CD34⁺ cells raises hopes for gene therapy in ALD. Efficiency of this vector to correct the ALD mouse model will be a further step toward this goal, encouraged by recent successes in murine models of sickle cell and β -thalassemic diseases [28,29].

MATERIAL AND METHODS

Lentiviral vector. TRIP- Δ U3-EF1 α -ALD lentiviral vector (see map of the vector in Fig. 1) was constructed by replacing the EGFP cassette (*Bam*HI/

KpnI) from the previously described TRIP- Δ U3-EF1 α -EGFP lentiviral vector [19] with a *Bam*HI-*Eco*RI fragment containing the coding sequence of the human ALD cDNA [4,9]. This self-inactivating vector in which the U3 region of the 3' LTR is deleted to improve the safety of the vector system includes the cPPT and the CTS [16] that increase gene transduction efficiency in human CD34⁺ hematopoietic cells [18]. The expression of the ALD gene was driven by the EF1 α promoter [19].

Preparation of high-titer virus vector. Lentivirus vectors were generated by transient calcium phosphate cotransfection of 293T cells, an encapsidation plasmid lacking all accessory HIV-1 proteins (p8.91), and a vesicular stomatitis virus envelope expression plasmid (pHCMV-G), as previously described [16,19]. Vector particles were normalized according to both p24 (HIV-1 capsid protein) content of supernatants [16] and measurement of infectious titers on murine 3T3 cells [27]. Viral titers varied from 5×10^8 to 10^9 IU/ml after concentration (400-fold) by ultracentrifugation.

Isolation of ALD CD34⁺ cells. CD34⁺ cells were isolated from granulocyte colony-stimulating factor-mobilized peripheral blood from ALD patients according to approved institutional guidelines. CD34⁺ cells were purified by immunomagnetic selection (Miltenyi Biotec, Paris, France) as previously described [18,19]. FACS analysis performed on a FACStar (Becton-Dickinson) showed over 90% purity of the CD34⁺ population. CD34⁺ cells were then stored in liquid nitrogen before use.

Transduction protocol. ALD CD34⁺ cells were not prestimulated with cytokines prior to incubation with lentiviral vector and therefore were incubated directly with lentiviral vector at 10^6 cells/ml in serum-free medium (Stem Cell Technologies, Vancouver, Canada) in the presence of four recombinant human cytokines at low concentrations: 10 ng/ml SCF (Amgen, Neuilly sur Seine, France), 10 ng/ml FL (Immunex, Seattle, WA), 10 ng/ml IL-3 (Novartis-France, Rueil-Malmaison, France), and 10 ng/ml TPO (Kirin Brewery, Tokyo, Japan). Lentiviral vector particles were added twice at 0 and 12 h at a multiplicity of infection of 5. After an overall 36-h transduction, CD34⁺ cells were washed. For *in vitro* studies, cells were further cultured for 72 h in H5100 long-term culture medium (Stem Cell Technologies) in contact with MS5 stromal cells, to allow maximal transgene expression and avoid pseudo-transduction. For NOD/SCID transplantation experiments, washed cells were immediately injected into irradiated animals. Transduction efficiency was evaluated by counting the number of CD34⁺ cells expressing recombinant ALD protein using immunocytofluorescence [9,29].

Hematopoietic cell cultures. CFCs and LTC-ICs were assayed as described [18,19]. Bulk and 1/10/50 per well LTC cells were independently studied. After 5 weeks, LTC cells were seeded in methylcellulose and colonies were assessed 15 days later for ALD protein expression.

Lymphoid and myeloid differentiation was assessed on MS5 stromal cells in the presence of SCF, FL, TPO, IL-15, and IL-2 as described [18,19].

Cells were phenotyped by FACS after 3–4 weeks of culture, using the following human-specific mouse monoclonal antibodies (mAbs): CD15-PE and CD14-PE (PharMingen, Pont de Claix, France) for granulocytes and macrophages/monocytes; CD56-PE-Cy5 (Immunotech, Villepinte-Roissy CDG, France) for NK cells, CD19-PE for B lymphocytes, CD11b-PE, and CD34-PE-Cy5 (Immunotech). Nonspecific staining was detected using irrelevant mouse IgG and IgM mAbs.

Detection of ALD protein in transduced ALD cells by immunocytochemistry. Expression of ALD protein in hematopoietic cells was scored using immunocytochemistry with a polyclonal anti-human ALD protein antibody that does not cross react with mouse ALD protein [9] and further incubation with biotinylated anti-rabbit IgG antibody and then with Cy3-conjugated streptavidin (Chemicon). Macrophages/monocytes were identified using a monoclonal anti-CD68 KP1 (Dako, Carpinteria, CA) antibody followed by incubation with horse anti-mouse IgG-FITC (H+L) antibody (Vector Laboratories). Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).

Transplantation of transduced ALD CD34⁺ cells into NOD/SCID mice. Immediately after transduction, 1.5×10^6 ALD CD34⁺ cells were intravenously injected into sublethally irradiated NOD-LtSz-*scid/scid* (NOD/SCID)

mice (3 Gy, at 0.43 Gy/mn; in an X-ray Phillips RT250 irradiator) [19]. Eighteen weeks later, bone marrow cells were harvested from recipient mice and the presence of human cells was assessed in individual mice by FACS using mouse anti-human CD45-FITC, CD38-FITC, CD19-PE, CD11b-FITC (Immunotech), CD14-PE, CD15-FITC, and CD34-PE-Cy5 mAbs. Human CD34⁺ cells were purified by cell sorting (Fig. 3, Gate 1) from the bone marrow of transplanted NOD/SCID mice and cultured under lymphomyeloid conditions [18,19].

Analysis of very long chain fatty acid concentrations in hematopoietic cells. VLCFA were extracted and measured by gas/liquid chromatography/mass spectrometry isotope dilution method as described [9]. Results are expressed as $C_{26:0}/C_{22:0}$ ratios.

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