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## Evaluation of new lentiviral vector pseudotypes for gene transfer into hematopoietic cells

Anaïs Girard Gagnepain

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# THÈSE

En vue de l'obtention du grade de

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par Mme Anaïs Girard, épouse Gagnepain

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## EVALUATION DE NOUVEAUX PSEUDOTYPES DE VECTEURS LENTIVIRAUX POUR LE TRANSFERT DE GENES DANS LES CELLULES HEMATOPOIETIQUES

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工欲善其事，必先利其器

« De bons outils pour un bon travail »,

*Extrait des Analectes de Confucius*



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Au plus important,  
Stephen et Manu,



## RESUME

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Le transfert de gènes dans les cellules souches hématopoïétiques par des vecteurs lentiviraux s'inscrit dans les protocoles actuels de traitement par thérapie génique de plusieurs maladies monogéniques (B-thalassémie, Adréno-leucodystrophie, SCID...). De même, le transfert de gènes dans les lymphocytes T et B ouvre des perspectives tant au niveau de la thérapie génique que pour l'immunothérapie.

Nous avons mis au point des vecteurs lentiviraux pseudotypés par des glycoprotéines chimérique (BaEV/TR) et mutante (BaEVRLess) du rétrovirus endogène de babouin.

Nous avons montré que ces nouveaux vecteurs peuvent transduire de manière plus efficace les cellules souches hématopoïétiques stimulées et quiescentes que les vecteurs pseudotypés par la glycoprotéine du virus de la stomatite vésiculaire (VSV-G). Il en est de même pour les vecteurs développés récemment et pseudotypés par les Glycoprotéines H et F du virus de la rougeole.

Nous avons aussi comparé la capacité de ces derniers vecteurs à ceux pseudotypés par les glycoprotéines BaEV/TR et BaEVRLess dans le transfert de gènes dans les lymphocytes B et T ainsi que dans l'ensemble des cellules de la lignée T. Nous sommes désormais en mesure de proposer des vecteurs adaptés au transfert de gènes à chaque étape de la différenciation des cellules CD34+ en thymocytes ainsi qu'en lymphocytes T matures. Ceci pourrait permettre de proposer de nouveaux protocoles cliniques en thérapie génique avec une co-transplantation de cellules souches génétiquement modifiées et de cellules T différenciées à partir de ces cellules. Ceci permettrait notamment de réduire les phases d'aplasie actuellement nécessaires pour la greffe de cellules souches.

## ABSTRACT

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Lentiviral vectors and their ability to transfer gene into hematopoietic stem cells are currently evaluated for the cure of several single-gene diseases (eg : B-thalassemia, Adrenoleucodystrophy, SCID). Likewise, gene transfer into B and T lymphocytes is of major interest in gene therapy and immunotherapy.

We engineered new lentiviral vectors pseudotyped by some chimeric (BaEV/TR) and mutant (BaEVRLess) glycoproteins from the baboon endogenous retrovirus. We demonstrated that these new vectors can transduce more efficiently resting and mild stimulated hematopoietic stem cells than obtained with lentivectors pseudotyped by the glycoprotein G from the vesicular stomatitis virus (VSV-G). It is the same with the recently developed lentiviral vectors pseudotyped by the H and F glycoprotein from measles virus (H/F-LVs).

We also compared the ability of the H/F-LVs with the BaEV/TR and BaEVRLess lentiviral vector pseudotype to transfer genes into B and T lymphocytes and into the whole T lineage. From now on, we are able to propose adapted vectors for gene transfer at each stage of differentiation from CD34+ cells to thymocytes and mature T cells. This could allow us to propose some new clinical protocols in gene therapy with a co-transplantation of genetically modified stem cells and their differentiated T progenitors in order to reduce the aplasia stage induced by current transplantation protocols.

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## LIST OF ABBREVIATIONS

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✓ 7-AAD :	7-Aminoactinomycin D
✓ APC :	Antigen presenting cell
✓ BM :	Bone marrow
✓ BCR :	B cell Receptor
✓ CD :	Cluster of differentiation
✓ CFU :	Colony forming unit
✓ CLP :	Common lymphoid progenitor
✓ CMP :	Common myeloid progenitor
✓ CMV :	Cytomegalovirus
✓ CTL :	Cytotoxic T lymphocytes
✓ CTP :	T-lineage committed progenitors
✓ DC :	Dendritic cell
✓ DL :	Delta-like ligand
✓ DN :	Double negative cell
✓ DNA :	Deoxyribonucleic acid
✓ DP :	Double positive cell
✓ EBV :	Epstein Barr Virus
✓ EGF :	Epidermal growth factor
✓ ELP :	Early lymphoid progenitors
✓ ES cell :	Embryonic stem cell
✓ ETP :	Early thymic progenitor
✓ F :	Measles Virus fusion protein
✓ FA :	Fanconi anemia
✓ FACS :	Fluorescence activated cell sorting
✓ Flt3 :	Fms-related tyrosine kinase 3
✓ G-CSF :	Granulocyte-colony stimulating factor
✓ GFP :	Green Fluorescent Protein
✓ GH :	Growth hormone
✓ Gp :	Glycoprotein
✓ GvHD :	Graft-versus-host disease
✓ GvL :	Graft-versus-leucemia
✓ GvT :	Graft versus tumor
✓ H :	Measles Virus Hemagglutinin
✓ HIV :	Human Immunodeficiency Virus
✓ HLA :	Human leukocyte antigen (HLA)
✓ HSC :	Hematopoietic stem cell
✓ IL :	Interleukin
✓ IPS cell:	Induced pluripotent stem cell
✓ LAM PCR:	Linear amplification mediated PCR
✓ LCMV :	Lymphocytic choriomeningitis virus
✓ LCR :	Locus Control Region
✓ LDL :	Low-Density Lipoprotein
✓ LTC-IC :	Long term culture-initiating cell
✓ LV :	Lentiviral Vector
✓ MLP :	Multilymphoid progenitor
✓ MLV :	Murine Leukemia Virus
✓ MV :	Measles Virus
✓ PBMC :	Peripheral blood mononuclear cell
✓ PERT :	Product enhanced reverse transcriptase
✓ PCR :	Polymerase chain reaction
✓ RNA :	Ribonucleic acid
✓ SCF :	Stem cell factor
✓ SCID :	Severe combined immunodeficiency
✓ SRC :	<i>scid</i> repopulating cells
✓ TCR :	T-cell Receptor
✓ TPO :	Thrombopoietin
✓ UCB :	Umbilical cord blood
✓ VSV-G :	Vesicular stomatitis virus glycoprotein





## PREAMBLE

---

Hematopoietic cells are major targets for gene therapy and immunotherapy. Nevertheless, few efficient tools have been described for gene delivery into these cells. New generations of lentiviral vectors are powerful tools for the targeting of both stimulated and resting cells. This is of interest in the field of hematology where the hematopoietic cell activation status plays a major role in the hematopoiesis fate.

During the past twenty years, several lentiviral vector pseudotypes have been designed in order to develop an optimal tool. Until now, the lentiviral vector pseudotyped with the vesicular stomatitis virus glycoprotein (VSV-G-LV) is the best described LV pseudotype in particular for the transduction of hematopoietic stem cells and it is currently used in the field of gene therapy. However, numerous limitations of this vector prevent its extensive use in hematopoietic cell gene delivery. In particular, it is not possible to stably produce this vector that could decrease the important costs induced by treatment protocols in gene therapy. Moreover, it does not recognize resting hematopoietic stem cells, T and B lymphocytes and it does not efficiently transduce these cells upon mild stimulation. Furthermore, the preservation of hematopoiesis functionality is based on the respect of the hematopoietic cell activation status. Although VSV-G-LVs had been useful allowing the development of new therapies, it became necessary to develop new tools in order to improve efficiency and safety of tomorrow's treatments.

Therefore, the objective of this thesis was to engineer, evaluate and optimize some new lentiviral vectors for gene transfer into human hematopoietic cells. We propose to extend the evaluation of the lentiviral vectors pseudotyped by modified measles virus glycoprotein which were previously developed in our group. We also engineered a novel promising lentiviral vector pseudotyped by the baboon endogenous retrovirus glycoprotein that hold great promises for the development of gene therapy and immunotherapy of hematopoietic cells.



# INTRODUCTION

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## PART 1 - LENTIVIRAL VECTORS

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### 1) LENTIVIRAL VECTOR FEATURES

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#### 1.1) BASICS IN LENTIVIROLOGY

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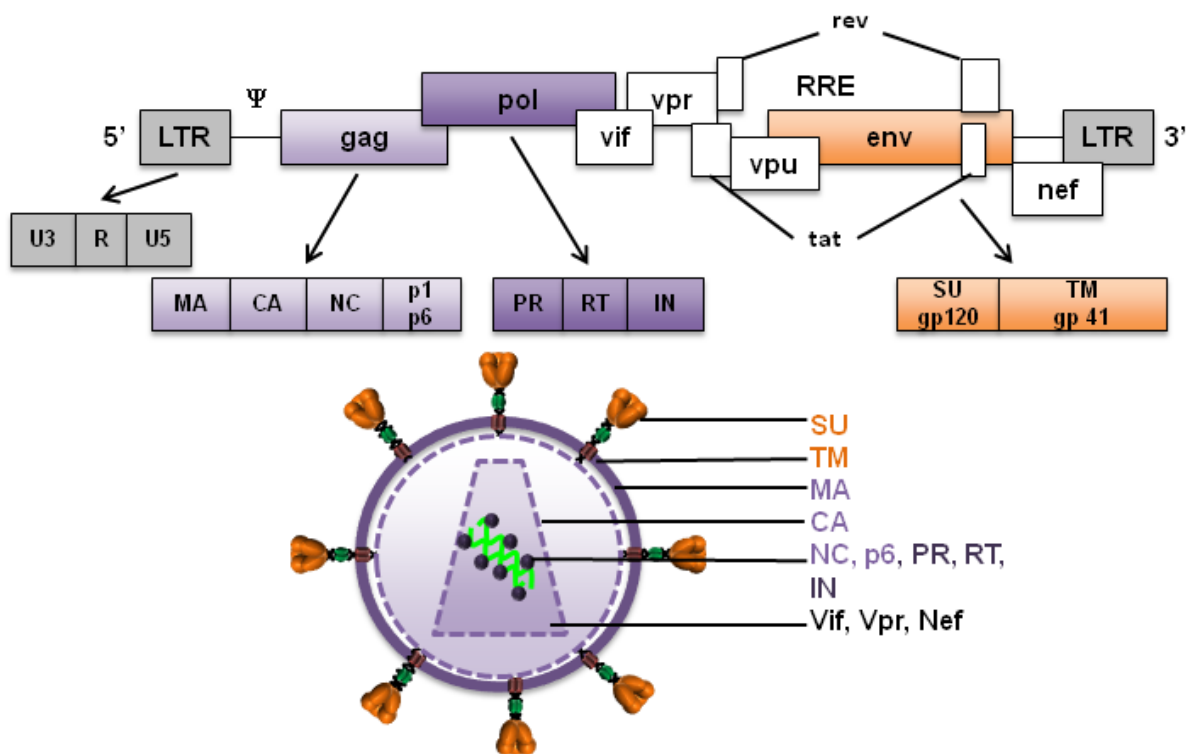
Lentiviruses are complex retroviruses studied since the early 1980s, when the AIDS pandemic emerged due to the current well known Human Immunodeficiency Virus Type 1 (HIV-1). Lentivirus is a genus of the family Retroviridae that causes slow (lentus= slow in Latin) progressive human and animal diseases. They are enveloped RNA viruses. Five serogroups were identified: Bovine, Equine, Feline, Ovine/Caprine lentiviruses and the primate lentiviruses, HIV and SIV. Most of the lentiviral vectors are derived from the primate lentivirus group, which includes the Human immunodeficiency virus (HIV) types 1 to 3, the Simian immunodeficiency virus and the Simian AIDS retrovirus SRV-1.

In order to understand the manipulation of lentiviral vectors (LV), it is important to have a good comprehension of the viral components (Coiras et al. 2009).

##### 1.1.1) LENTIVIRAL GENOMIC STRUCTURE AND PROTEINS

---

HIV-1 genome is composed of two identical single stranded positive RNA molecules of approximately 9kb and encodes at least nine viral proteins (Figure 1). It is important to distinguish the *cis*- and *trans*-acting components of the HIV genome. The *trans*-acting components are composed of nine open reading frames. The three major open reading frames encode the gag, pol and env polypeptides and are also characteristic for MLV-based vectors. The six others encode lentivirus specific genes such as Tat and Rev, the viral regulatory proteins, and the four accessory proteins: Vpr, Vpu, Nef and Vif. The *cis*-acting elements consist of the LTRs, the Rev responsive elements (RRE), the splicing sites and the packaging signal ( $\Psi$ ).



**Figure 1. Schematic representation of the HIV-1 viral genome and virion structure.** The HIV-1 viral genome encodes structural (*gag*, *pol*, *env*), regulatory (*tat*, *rev*) and accessory (*vpr*, *vif*, *vpu*, *nef*) genes flanked by two long terminal repeats (LTRs). Inner and outer viral membranes are coated with matrix (MA) and envelope (Env) proteins, respectively. The two viral ssRNAs, the reverse transcriptase (RT), the integrase (IN) and protease (PR) and the accessory proteins are surrounded by capsid (CA).

#### 1.1.1.1) GAG, POL AND ENV ARE THE THREE MAJOR STRUCTURAL PROTEINS OF HIV-1

The gag precursor protein (p55) is involved in budding of the viral particle from the infected cell membrane by recruiting two copies of viral genomic RNA and other viral components. During the viral maturation, p55 is cleaved by the viral protease into MA (p17), CA (p24), NC (p9) and P6 (Figure 1). The MA protein stabilizes the viral particle and facilitates the active nuclear import of the viral genome, allowing HIV-1 to infect non-dividing cells. The CA protein interacts with p55, which favors viral replication. The NC protein recognizes the packaging signal of HIV and also facilitates reverse transcription. The p6 polypeptide allows the incorporation of Vpr into viral particles by interacting with p55 and Vpr and additionally it is required for the efficient release of viruses from the cell.

The gag-pol fusion protein (p160) is produced by a ribosomal frame shifting event. During viral maturation, the pol polypeptide is digested in protease (PR, p10),

Reverse transcriptase (RT, p50), RNase H (p15) and integrase (IN, p31) (Figure 1). The protease is required during viral maturation for Gag and Gag-pol precursors' cleavage. The Reverse transcriptase allows the polymerization of a double strand DNA copy of the HIV-1 genome with the help of the RNase H that removes the original RNA template from the first retro-transcribed DNA strand. The integrase allows the integration of the proviral DNA into the host genomic DNA via its exonuclease, endonuclease and ligase activities.

The Env polypeptide (gp 160) is cleaved after glycosylation in gp41 that contains the transmembrane domain (TMd) and in gp120 the surface (SU) domain, which is linked to gp41 by non-covalent interactions (Frankel et al. 1998).

#### 1.1.1.2) TAT AND REV, THE VIRAL REGULATORY PROTEINS OF HIV-1

Tat is a transcriptional transactivator essential for viral replication. It is an RNA binding protein that recognizes the transactivation response element (TRE) at the 5' end of HIV-1 RNA and strongly activates transcription from the HIV LTR.

Rev is also a RNA binding protein that recognizes the Rev responsive element (RRE) allowing the transition from the early to the late phase in HIV infection. Of note, this binding facilitates the export of unspliced or one fold spliced viral RNA to the cytoplasm using snRNAs and the ribosomal 5s RNA.

#### 1.1.1.3) VPR, VPU, NEF AND VIF, THE VIRAL ACCESSORY PROTEINS OF HIV1

Vpr is a member of the pre-integration complex (PIC) and plays a role in the infection of non-dividing cells by HIV-1 by facilitating the nuclear localization of this PIC. Vpu facilitates the particle assembly by triggering the degradation of intracellular Env-CD4 complexes. It also enhances virus release from the infected cell surface. Vif is necessary for the viral replication in peripheral blood lymphocytes and macrophages by inactivating the host antiviral factor APOBEC3G (Vicenzi et al. 2013). Nef has three major activities: it decreases the cell surface expression of CD4 and thus facilitates viral budding; it perturbs T cell activation and stimulates the HIV-1 virus infectivity (Harris et al. 2012).



#### 1.1.1.4) THE CIS-ACTING ELEMENTS

The LTRs contain three elements U3, R and U5. The U3 region, at the 5' end of each LTR has binding sites for multiple transcription factors, integration sites and includes most of the *cis*-acting DNA elements. The central region, R, contains a specific sequence (100bp) where transcription starts, whereas polyadenylation occurs just at the end of this region. The U5 region contains the Tat binding site. HIV genome transcription is allowed by a single promoter in the 5' LTR. The 3' LTR mediates the polyadenylation that stabilizes the transcripts. Duplication of LTR elements occurs during reverse transcription just before integration when the copied 3' LTR U3 sub-region is transferred to the 5' LTR.

The packaging element ( $\Psi$ ) is an important feature and consists of two short sequences flanked at each side of the major splice donor site that gives a structured RNA element and allows the recognition and packaging of genomic RNA into the capsid (CA) just before assembly.

The role of the RRE in the export of unspliced RNA from the nucleus was developed above.

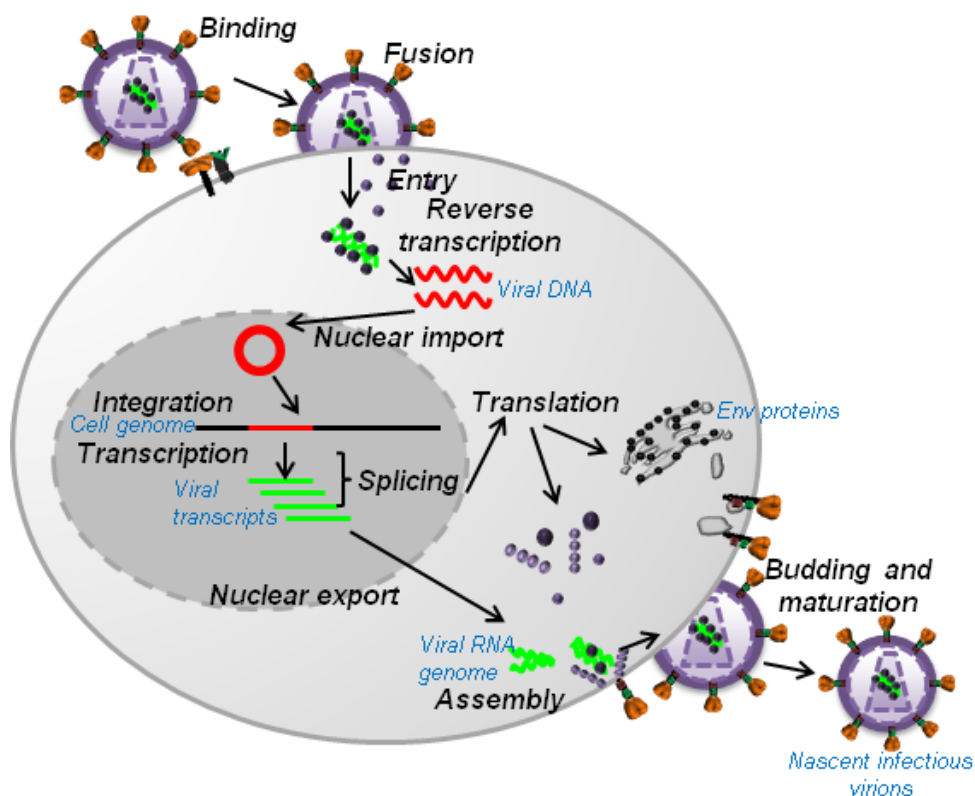
#### 1.1.1.5) A FEW WORDS ON NON-HUMAN LENTIVIRUSES

The non-human lentiviruses used for vectorization are Bovine lentiviruses (Metharom et al. 2000), Equine lentiviruses (Farley et al. 2012), Feline lentiviruses (Mendenhall et al. 2012), Ovine/Caprine lentiviruses (Davey et al. 2012) and the primate Simian immuno-deficiency (SIV) lentiviruses (Negre et al. 2002). Of importance, most of the proteins of these lentiviruses are conserved, except for some variations in accessory proteins (e.g. Vif and Tat). In these viruses, additional genes have been discovered but the function of these other accessory proteins is not always elucidated. However, these accessory proteins are not needed for *in vitro* replication and gene delivery and are thus not included in the lentiviral vector design. These alternative lentiviral vector systems are considered safer by the public because of the fact that they are not derived from a human pathogen, however, this is still under debate. Non-human primate models have been of particular interest for

developing hematopoietic gene therapy strategies in humans because they closely mimic human biology. HIV-based lentiviral gene transfer into macaque cells is inefficient due to the presence of restriction factors in simian cells (e.g. TRIM5 $\alpha$ ) causing an intracellular blocking (Trobridge et al. 2008). Since SIV derived vectors are not subjected to this restriction (Sandrin et al. 2002) latest generation SIN SIV vectors have been developed for preclinical testing in non-human primate models (Verhoeven et al. 2012).

### 1.1.2) LENTIVIRAL LIFE CYCLE

The global mechanisms involved in the HIV-1 infection cycle (Figure 2) are summarized in this paragraph in order to evaluate the essential differences between lentiviruses and lentiviral vectors.



**Figure 2. Schematic representation of the HIV-1 life cycle.**

During cell entry, capsid proteins are uncoated and the two ssRNA viral genomic strands (marked in green) and viral enzymes are released into the cytoplasm. Viral RNA is converted by RT into proviral dsDNA (marked in red) and imported into the nucleus. Then the IN inserts the dsDNA into the host genome. During the early infection phase, only spliced viral mRNAs encoding *tat*, *nef* and *rev* are exported to the cytoplasm. Then, the Rev protein allows the export of unspliced or singly spliced (*vif*, *vpr*, *vpu*, *env*) mRNA. Once every viral component is synthesized, viral genome and proteins are assembled at the plasma membrane and new HIV particles are released from the host cell. Just after the virus budding, the PR induces virus maturation, which renders the viral particles infectious

The natural HIV infection cycle begins by the attachment of the SU (gp120) part of its envelope glycoprotein to its primary receptor CD4 and its co-receptor CXCR4 on T lymphocytes or CCR5 on activated T lymphocytes, monocytes, macrophages and dendritic cells. A conformational change of TM follows this event and leads to viral and host cell membrane fusion. Upon cell entry, capsid proteins are uncoated resulting in the release of the viral RNA genome and the MA, RT, IN and Vpr proteins. The viral RT converts the positive ssRNA into dsDNA, then the proviral DNA is imported into the nucleus and integrated into the host genome by use of its viral IN. During the early phase of infection, only spliced mRNAs encoding for *tat*, *rev* and *nef* can be produced and exported to the cytoplasm. Then, the late phase of infection is initiated by the action of Tat, Rev and accessory protein. Complete viral genome is exported by *rev* into the cytoplasm and proteins are assembled at the plasma membrane. Just after viral budding, the multimerization of Gag and GagPol activates the protease that induces the maturation of the virus by structural rearrangements.

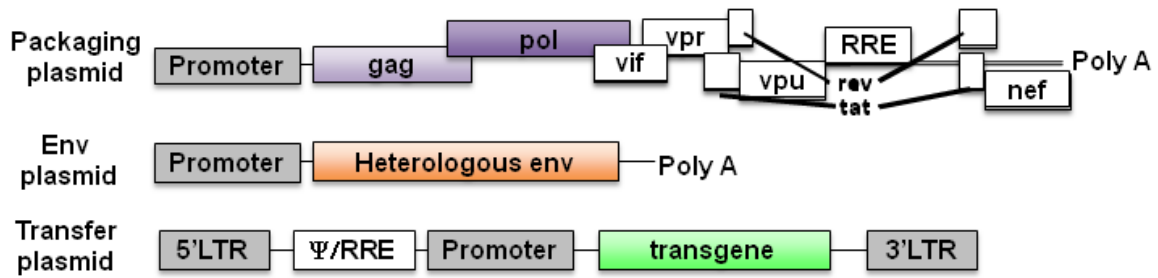
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## 1.2) FROM LENTIVIRUSES TO HIV-1 LENTIVIRAL VECTORS

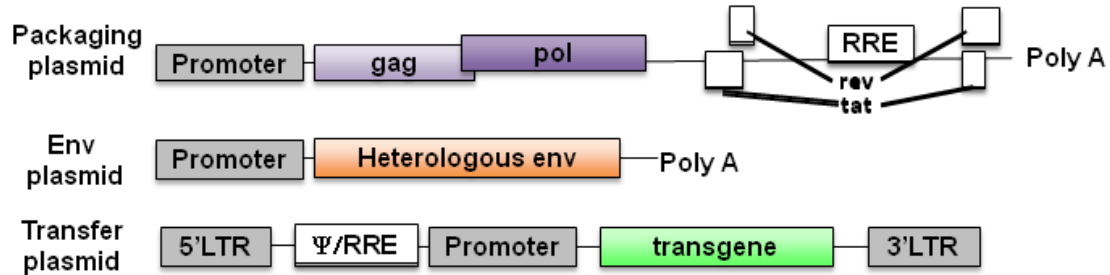
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The first lentiviral vectors (LVs) were developed with the objective to better understand viral replication and they were used as a tool for anti-HIV-1 drug screening. These early HIV-1 LVs were replication competent viruses that carried transgenes. However, the current main goal of LV use is to deliver a gene or a set of genes into a target cell in the absence of replication. Since non-replicating LVs were considered safer their design was achieved by separation of viral sequences on several expression units and identification of the minimal viral components to engineer an efficient gene transfer vector. The first LVs consisted of a plasmid coding for the proviral DNA deleted for the *env* gene and another plasmid encoding the *env* gene. LVs could be produced by transcomplementation without inducing a second round of infection simply because they lacked the *psi* sequence. In these first LVs, transgenes replaced *env* or *nef* sequences and thus their expression was under the control of the promoter in the 5'LTR.

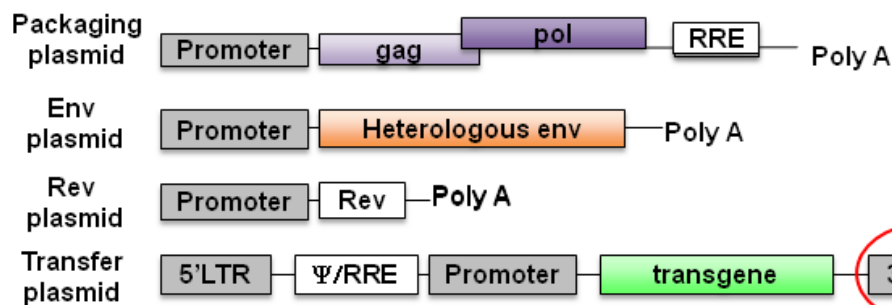
First generation of HIV-1 LVs



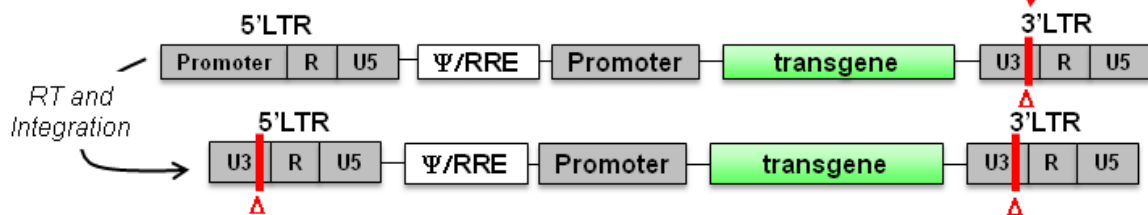
Second generation of HIV-1 LVs



Third generation of HIV-1 LVs



SIN LTR transfer plasmid



**Figure 3. Schematic representations of HIV-1 derived vectors.**

The first generation of HIV-1 vectors is composed of: 1) a packaging plasmid containing all the viral protein encoding genes except for the envelope; 2) an envelope plasmid which encodes a heterologous envelope glycoprotein and; 3) a transfer vector encoding the 5' and 3' LTRs, the packaging signal ( $\Psi$ ) and rev responsive element (RRE) sequences and the transgene driven by an internal promoter. In the second generation of LVs, all the accessory proteins are excluded from the packaging plasmid. In the 3<sup>rd</sup> generation of LVs, regulatory proteins (tat and rev) are excluded from the packaging plasmid and an additional 4<sup>th</sup> plasmid encoding Rev is added. In Self-inactivating (SIN) LTR transfer vectors the 5' LTR U3 subregion is replaced by a strong and constitutive promoter independent from Tat.

### 1.2.1) THE SELF INACTIVATING (SIN) LENTIVIRAL VECTORS, AN IMPORTANT SAFETY MEASURE

---

HIV-derived LVs integrate into the host genome flanked by the two HIV-1 LTRs. This implies that, if replication competent lentiviruses (RCLs) are produced or if transduced cells are co-infected by a wild type virus, new infectious viral particles can be produced. Moreover, cellular genes might be activated by the enhancer and promoter activity of integrated LTR. If this integration occurs near to a proto-oncogene, this can have as dramatic result the induction of oncogenesis. Highly relevant in this context is that if a part of the 3' LTR U3 sub-region is deleted, the same deletion is transferred into the 5' LTR during LTR duplication and these results in elimination of its promoter/enhancer function. As a consequence, the SIN approach was applied to LVs by deletion of the 3' LTR TATA-box, SP1-, NF-kB and NFAT binding sites (Miyoshi et al. 1998) (Zufferey et al. 1998). This SIN modification is still currently maintained in all LV generations because it strongly reduces or even ablates the risk of RCL propagation. It avoids the mobilization of integrated vectors by a wild-type helper virus and it also diminishes insertional activation of cellular oncogenes and transcriptional modifications induced by intact LTRs.

### 1.2.2) 1<sup>ST</sup> GENERATION OF HIV-1 VECTORS

---

In order to reduce the risk of generating RCLs with pathogenic abilities, Naldini et al (1998) introduced a first generation packaging vector with three separated plasmids. The first plasmid contained a packaging construct expressing HIV-1 gag, pol, Tat, Rev and accessory proteins under the control of a strong viral promoter. The Env plasmid encodes a viral glycoprotein that allows the host cell recognition by the LV, virus-cell membrane fusion and LV cell entry. The process of displaying a heterologous viral envelope glycoprotein at the LV surface is called pseudotyping. Several existing strategies for LV pseudotyping will be developed separately in this chapter.

Both packaging and env encoding plasmids are devoid of the packaging signal ( $\Psi$ ) and LTRs in order to prevent their encapsidation into the particle and to reduce RCL production resulting from recombination between homologous sequences on

the different plasmids. The third plasmid also called transfer vector contains the transgene cassette and all the essential *cis*-acting elements such as LTR,  $\Psi$  and RRE without encoding for HIV-1 proteins. An internal promoter was added to control transgene expression in the host cell (Figure 3).

The separation of the different LV functions on three plasmids reduces the risk of RCL formation since this requires at least two recombination events. Moreover, the use of an ectopic envelope glycoprotein rather than the HIV-1 Env by eliminating homologous sequences between the Env and the transfer vector plasmids also decreases the risk of recombination.

### 1.2.3) 2<sup>ND</sup> GENERATION OF HIV-1 VECTORS

---

In order to increase the LV safety, a second generation of LVs was developed by deleting the accessory proteins. Indeed, although these proteins are essential for HIV-1 virulence, they can be deleted without affecting the gene transfer role of LVs. Therefore, in this 2<sup>nd</sup> generation of LVs, the packaging vector encoding plasmid only contained gag, pol, tat and rev and was devoid of all other HIV-1 genes (Sakuma et al. 2012), (Zufferey et al. 1998).

### 1.2.4) 3<sup>RD</sup> GENERATION OF HIV-1 VECTORS

---

This 3<sup>rd</sup> generation of LVs was developed in 1998 in order to increase LV safety by preventing the role of HIV regulatory genes. The independence for Tat regulation was obtained by replacing the 5' LTR U3 subregion with a strong viral promoter from the human cytomegalovirus (CMV). Since then, several other promoters are in use. The envelope encoding plasmid relies on the same design used for the 2<sup>nd</sup> LV generation. Tat and rev sequences have been removed from the packaging plasmid, thus, they carry only the gag and pol sequences. The transfer vector has adapted a SIN configuration and therefore the transgene is driven by an internal heterologous promoter (Figure 3).

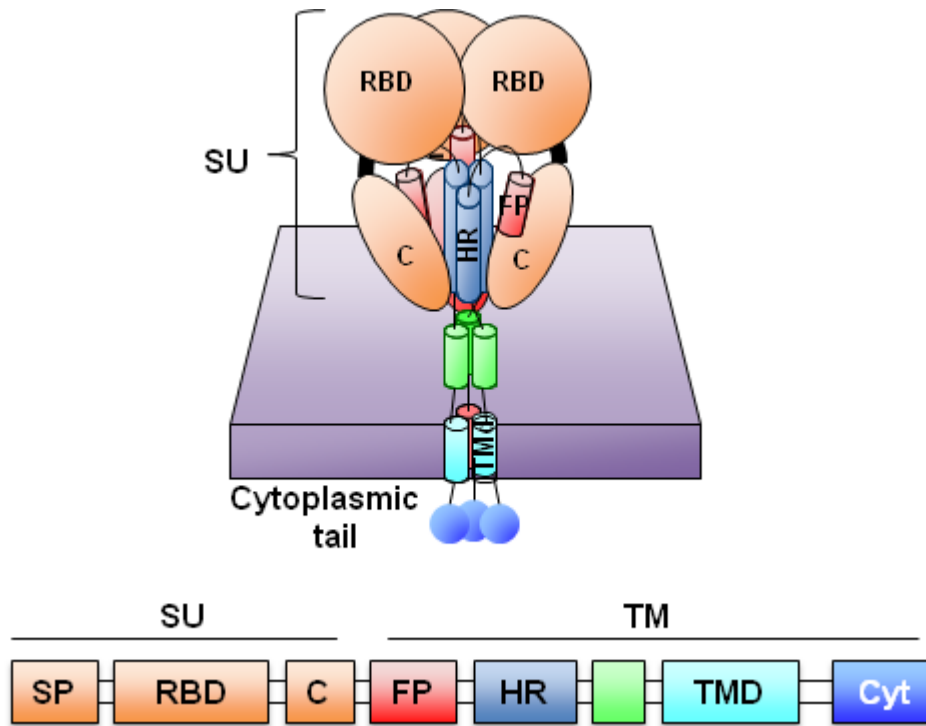
Moreover, a fourth plasmid expressing Rev was introduced to conserve its functions: the nuclear export of unspliced RNAs and the transcript stability. With this

3<sup>rd</sup> generation of LVs, at least three recombination events are necessary to generate an RCL. Even if an RCL would be produced, Tat, accessory proteins and active LTRs would not be present in the resulting viruses. Because of their high infectious titer, high-level transduction and important biosafety features, these 3<sup>rd</sup> generation vectors are currently predominant. Nevertheless, a 4<sup>th</sup> generation of LVs has been developed to still improve biosafety for clinical applications. Several approaches have been evaluated such as the development of Rev independent vectors by replacing the RRE with heterologous viral sequences known to facilitate the export and stability of unspliced viral RNAs. However, the titer decrease induced in these vectors is one of their great disadvantages (Yilmaz et al. 2006). Gag-pol “codon optimization” has been performed in order to eliminate HIV-1 gag-pol homologous regions between the packaging plasmid and the transfer vector. This approach consists in introducing a mutation in the wobble codon position into most of the gag-pol codons in the packaging construct thereby conserving the primary amino acid sequence (Kotsopoulou et al. 2000). Despite of these numerous improvements in terms of biosafety, most laboratories still use the third generation of LVs, except when clinical use is envisaged.

## 2) OPTIMIZATION OF LENTIVIRAL VECTORS TARGETING BY PSEUDOTYPING

---

LV biosafety, change in tropism (more cell-specific or expanded tropism) and stability were obtained by replacing the HIV-1 envelope on the LV surface by a heterologous viral envelope glycoprotein, a process called pseudotyping. The molecular basis of envelope glycoproteins is shown in Figure 4.



**Figure 4. Structure of envelope glycoproteins.**

The sequence encoding the viral envelope glycoprotein is composed of two entities: 1) the SU (surface) subunit contains the Signal peptide (SP), the receptor binding domain (RBD) and the SU carboxy-terminal domain (C); 2) the TM (transmembrane) subunit contains the ectodomain (FP: fusion peptide and HR: heptad repeat), an anchor domain and a cytoplasmic tail containing the fusion inhibitory peptide R.

---

## 2.1) HOW TO OBTAIN LENTIVIRAL VECTORS AT A HIGH INFECTIOUS TITER WITH AN EXPANDED TROPISM?

---

It is clear that the HIV-1 envelope only recognizes the human CD4 molecule on the cell surface, therefore its display on the LV surface will limit gene transfer to very restricted cell types (CD4+ T cell and macrophages).

Many heterologous viral glycoproteins can be incorporated into lentiviral or MLV retroviral particles and mediate infectivity, this technology is named pseudotyping. These include viral surface glycoproteins derived from lyssaviruses (Desmaris et al. 2001), arenaviruses (Miletic et al. 1999), hepadnaviridae (Sung et al. 2002), paramyxoviridae (Frecha et al. 2008), orthomyxoviruses (Hatzioannou et al. 1998), filoviruses (Kobinger et al. 2001) and alphaviruses (Suerth et al. 2012). For instance, human airway targeting is possible by LVs pseudotyped with surface glycoproteins derived from the Ebola or the influenza viruses. In contrast, LVs pseudotyped with glycoproteins from hepatitis C virus (HCV), baculovirus or Sindai



virus can target hepatocytes, while LVs pseudotyped with Rabies glycoproteins target neurons (Frecha et al. 2008) (Table 1). Since it was used for MLV retroviral vector pseudotyping with success, the vesicular stomatitis virus envelope glycoprotein G (VSV-G) was an attractive candidate for LV pseudotyping. The attachment of the VSV-G glycoprotein to the ubiquitously expressed LDL receptor provides the VSV-G pseudotyped LVs a “pan-tropic” cell recognition ability (Finkelshtein et al. 2013).

Likewise, the cat endogenous retroviral glycoprotein (RD114/TR) (Sandrin et al. 2002) was shown to be a very attractive candidate for hematopoietic progenitor cell targeting. LVs have recently been pseudotyped with the hemagglutinin (H) and the fusion protein (F) from the Edmonston vaccinal strain of measles virus (MV-LVs) (Frecha et al. 2008), (Frecha et al. 2009), (Frecha et al. 2011). This particular pseudotype has acquired remarkable properties for immune cell transduction. These newly developed MV-LVs conserved the parental MV tropism since they allowed transduction of human T cells, B cells and dendritic cells, the primary targets of measles virus.

**Table 1. Pseudotyping of Lentiviral vectors with heterologous envelope glycoproteins relying on the natural tropism of these glycoproteins.**

<b>Vector pseudotypes glycoproteins</b>	<b>Evaluated targeted cells</b>	<b>Ref</b>
<b>Rabies virus Rabies-G</b>	Neurons	(Wong et al. 2004), (Mazarakis et al. 2001), (Azzouz et al. 2004), (Watson et al. 2002)
<b>Mokola virus MK-G</b>	Neurons / Retinal pigment epithelium	(Wong et al. 2004), (Watson et al. 2002)
<b>LCMV WE, WE-HP1</b>	Glioma/ Neuronal stem cells	(Miletic et al. 2004), (Stein et al. 2005)
<b>Ross river virus E1, E2</b>	Glial cells	(Kang et al. 2002)
<b>Ebola virus EboZ</b>	Airway epithelium/Skin	(Medina et al. 2003), (Silvertown et al. 2006), (Hachiya et al. 2007)
<b>MuLV 4070A-env</b>	Skin	(Hachiya et al. 2007)
<b>Baculovirus GP64</b>	Hepatocytes	(Kang et al. 2005)
<b>HCV E1, E2</b>	Hepatocytes	(Bartosch et al. 2003)
<b>Sindai virus F protein</b>	Hepatocytes	(Kowolik et al. 2002)

<b>Influenza A HA</b>	Retinal epithelium	(Duisit et al. 2002)
<b>GALV GALV/TR</b>	Hematopoietic cells	(Sandrin et al. 2002), (Christodoulopoulos et al. 2001), (Stitz et al. 2000)
<b>RD114 virus RD114/TR (modified)</b>	Hematopoietic cells	(Sandrin et al. 2002), (Frecha et al. 2012)
<b>Measles virus H and F (truncated or fused with a single chain antibody)</b>	B and T lymphocytes	(Frecha et al. 2008), (Frecha et al. 2009), (Buchholz et al. 2009), (Funke et al. 2008)
<b>BaEV BaEV/TR, BaEVRLess</b>	Hematopoietic cells	(Girard-Gagnepain et al. 2014)

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## 2.2) LENTIVIRAL VECTOR PSEUDOTYPES: PARENTAL VIRUS, TYPE OF ENVELOPE GLYCOPROTEIN AND TROPISM

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### 2.2.1) VSV-G LENTIVIRAL VECTOR PSEUDOTYPE

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Vesicular stomatitis virus is a negative sense RNA virus from *Mononegavirales* and the family of *Rhabdoviridae*. Indiana and New Jersey are the two VSV major serotypes. VSV natural hosts are horses, cattle, pigs and other mammals and insects. It is transmitted by insects or direct contact. Some cases of human infection have been documented causing flu-like symptoms or viral encephalitis. Among, the five genes encoded by its 11 kb genome, the G attachment protein enables infection of a wide range of cells (Hastie et al. 2013). After a search for decades, the low density lipoprotein receptor (LDL-R) has been described as the cell surface receptor that interacts with the G attachment glycoprotein (Finkelshtein et al. 2013, Amirache et al. 2014). The pantropism of VSV is related to the widespread expression of the LDL-R on cell surface. This broad cell tropism added to the viral properties in term of replication, yields, and small size, make VSV a common model virus for basic research. Most importantly, it represented an excellent candidate to confer broad tropism to retroviral vectors.

As the VSV-G murine retroviral vector pseudotypes (Yee et al. 1994), the VSV-G-LV pseudotypes proved stable and could be obtained at higher titers by ultracentrifugation-concentration than obtained with other viral envelopes. Using VSV-G-LVs, numerous cell types have been transduced from human hematopoietic

stem cells to rat neurons and other non-dividing cells. Currently, most of lentiviral vectors are pseudotyped with this envelope, for instance, they can transduce up to 50% of stimulated hematopoietic stem cells at high vector doses. Nevertheless, its sensitivity to the human complement system prevents its potential *in vivo* use. This issue can be resolved by using polyethylene glycol modified VSV-G pseudotypes (Croyle et al. 2004). Moreover, cytotoxicity can be observed with high concentrations of VSV-G pseudotyped LVs. Because of their pan-tropic cell recognition, there are safety concerns for the *in vivo* use of this pseudotype in the clinic. Indeed, it can easily transduce antigen presenting cells (APCs) which might induce a strong immune response in the recipient patient (Humbert et al. 2012).

Because of the above limitations of VSV-G pseudotyped LVs, more selective tropisms were achieved by using glycoproteins from other enveloped viruses characterized by their own tropism.

## 2.2.2) EXAMPLES OF RETROVIRAL PSEUDOTYPES

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### 2.2.2.1) FELINE ENDOGENOUS RETROVIRUS AND RD114 GLYCOPROTEIN

The cat endogenous retrovirus, named RD114 virus was isolated in 1971 from human rhabdomyosarcoma derived cell line after transplantation in fetal cats (McAllister, 1972). It is a recombinant composed of the gag-pol genes from the cat virus FcEV and the *env* gene from the primate baboon endogenous retrovirus (Van Der Kuyl et al. 1999). The presence of a separate *env* gene in the FcEV gammaretrovirus allowed the exchange of this gene and thus the viral recombination.

The RD114 virus envelope glycoprotein recognizes the ASCT2 neutral amino acid transporter (Tailor et al. 1999), (Rasko et al. 1999), (Marin et al. 2003). It was shown that the RD114 receptor is expressed on immature cells from squamous epithelia and some subpopulation of colon, testis, ovary, skeletal muscle and of importance for us in bone marrow (Green et al. 2004). Of importance for gene therapy, RD114/TR pseudotyped LVs have been shown to transduce efficiently hematopoietic stem cells reaching 50% of transduction at low vector doses (Bell et al. 2010), (Di Nunzio et al. 2007), (Jang et al. 2006), (Sandrin et al. 2002). Since the RD114/TR envelope is not cytotoxic and RD114/TR pseudotyped particles are stable

in human sera, this pseudotype offers an alternative to VSV-G –LVs (Bell et al. 2010). Additionally, its resistance to the human complement system is more promising for *in vivo* applications of RD114/TR pseudotyped LVs for HSC transduction as compared to VSV-G-LVs (Frecha et al. 2012).

#### 2.2.2.2) BABOON ENDOGENOUS RETROVIRUS AND BAEV GLYCOPROTEIN

The baboon endogenous retrovirus was isolated from a baboon placenta in 1973 (Benveniste, 1974). The provirus of BaEV is present only in the genomes of the Papionini tribe and in african green monkeys. This virus is able to replicate in diverse species of cultured cells. Like RD114 virus, Baboon endogenous retrovirus is a chimeric retrovirus. It is constituted by the *gag-pol* genes of the PcEV gammaretrovirus and the *env* gene from the first hypothesized ancestor of BaEV, SERV the first endogenous betaretrovirus sequenced from primates (Kato, 1987), (Mang et al. 1999).

As RD114 virus, BaEV recognizes the human ASCT2 receptor. In addition, it also allows binding to the human ASCT1 carrier which presents 57% of homology with ASCT2 and both mediate BaEV infection (Marin et al. 2000), (Marin et al. 2003). Thanks to the multiple receptor recognition of BaEV as compared to RD114 virus, it can be considered as an attractive candidate for lentiviral pseudotyping.

#### 2.2.3) EXAMPLE OF MEASLES VIRUS LENTIVIRAL VECTOR PSEUDOTYPE

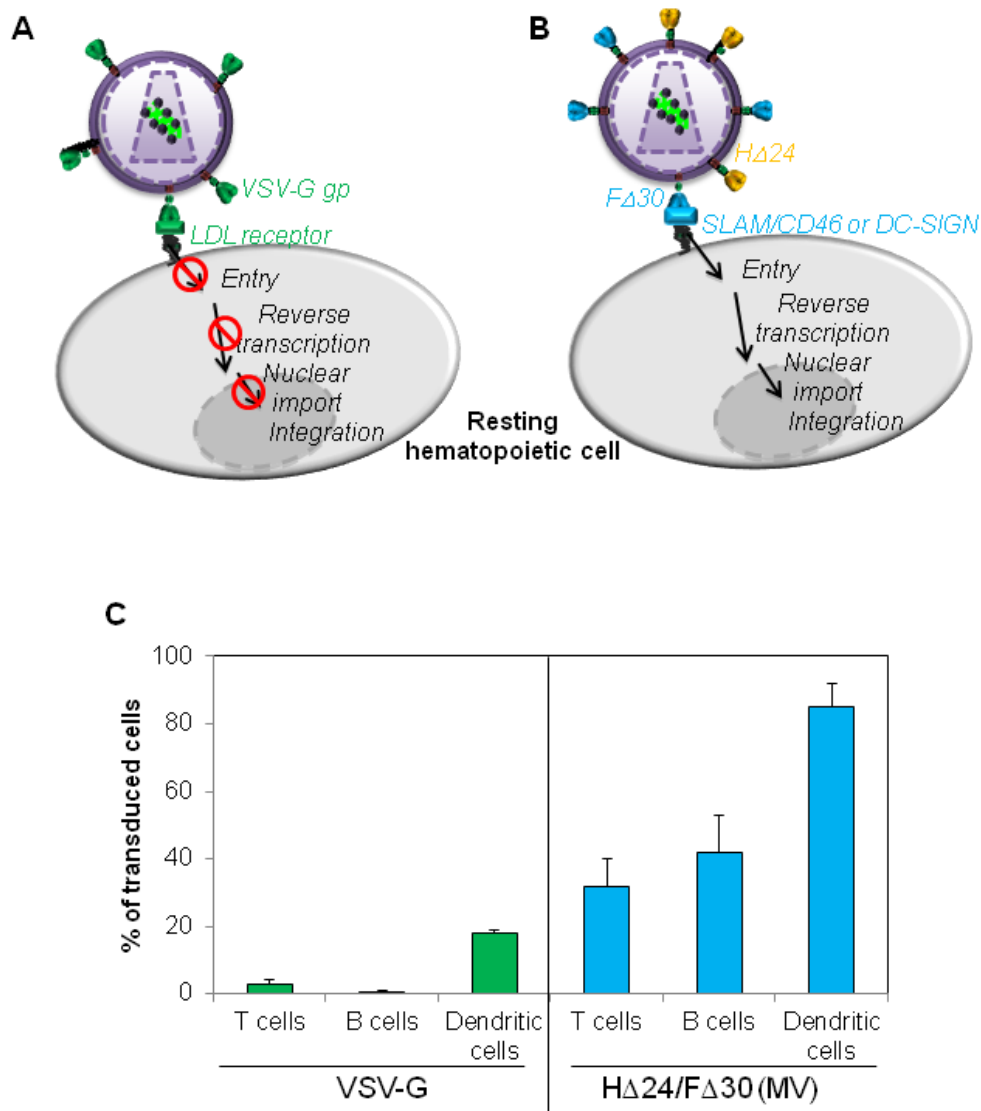
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Measles virus belongs to the Paramyxoviridae family; it is an enveloped single stranded RNA virus. Eight proteins are encoded by MV genome, including two surface glycoproteins: the envelope proteins, Haemagglutinin (H) and the fusion protein F. During MV infection, the virus infects initially dendritic cells and monocytes of the superficial respiratory tract (Lemon et al. 2011). Then, it migrates to the lymph node and infects T and B lymphocytes (De Swart et al. 2007). MV can infect cells thanks to the recognition of several receptors: Signaling lymphocyte activation molecule SLAM/CD150 (Tatsuo et al. 2000), (Yanagi et al. 2006) expressed on some T and B cell subsets and mature dendritic cells (Aversa et al. 1997). The MV receptor, Nectin-4, is present on epithelial cells (Kato et al. 2012), (Muhlebach et al. 2011). MV vaccine strains like Edmonston recognizes both SLAM and CD46 receptors (Leonard

et al. 2008). CD46 is a complement-regulatory molecule expressed on all human nucleated cells (Liszewski et al. 1991).

LVs pseudotyped by the H and F glycoproteins from the Edmonston vaccinal MV strain have been developed (Frecha et al. 2008), (Funke et al. 2008). There were the first LVs to allow efficient transduction of quiescent human T cells (Frecha et al. 2008), healthy (Frecha et al. 2009) and cancer B cells (Lévy et al. 2010) and monocytes derived dendritic cells (Humbert et al. 2012) without inducing entry into the cell cycle. This pseudotype offers great potential for gene therapy and immunotherapy (Frecha et al. 2010), Figure 5 compare H/F-LVs to VSV-G-LVs for transducing resting hematopoietic cells.

In order to allow the retargeting of H/F-LVs, the natural CD46 and signaling lymphocyte activation molecule (SLAM) receptor binding residues of the H protein were mutated and H was fused to targeting ligands such as EGF (Epidermal growth factor) or single chain antibodies specific for antigens expressed on the target cell surface (Funke et al. 2008) (Developed in the discussion part 2.2.2).



**Figure 5. Infectivity of VSV-G and measles virus gp pseudotyped lentiviral vector pseudotypes for resting hematopoietic cells.**

(A) VSV-G-LVs are not able to enter efficiently into quiescent T or B cells through their recently identified low density lipid (LDL) receptor. And even upon entry, the RT activity and nuclear import is inefficient resulting in a very low proviral integration efficiency. These hurdles can only partially be overcome by addition of nucleosides and exogenous ATP. (B) Measles virus gp-LVs manage to enter into quiescent T and B cells and RT activity is detected without activating these cells, followed by efficient nuclear import of the proviral DNA. This results in proviral DNA integration into the resting T and B cell host genome.

(C) Comparison of transduction efficiencies in unstimulated T cells, B cells and dendritic cells (DCs) for VSV-G-LVs and MV-LVs. The transfer vector encodes for the GFP reporter gene, which allows detection of transduction efficiency (% GFP+ cells) in T and B cells and DCs by flow cytometry analysis

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## 2.3) LENTIVIRAL VECTOR PSEUDOTYPE ENGINEERING

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### 2.3.1) PSEUDOTYPE ASSEMBLY

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Two mechanisms are described for assembly of glycoproteins on viral particles. The passive model of glycoprotein incorporation is characterized by the abundance of glycoprotein at the virus budding site (Pickl et al. 2001) and the sterical compatibility of the envelope cytoplasmic tail with virion morphology (Swanstrom et al. 1997). Heterologous glycoproteins with short cytoplasmic tails such as VSV-G probably incorporate on the LV surface by this mechanism. Secondly, the active model of glycoprotein incorporation is based on critical interactions (Cosson et al. 1996), (Murakami et al. 2000), (Vincent et al. 1999), (Wyma et al. 2000) between the cytoplasmic tail of the pseudotyping glycoprotein and components of the viral core. GALV or RD114 envelope glycoprotein incorporation depends on this second mechanism. Indeed, the glycoproteins of some of these retroviruses, have been shown to harbor in their cytoplasmic tail a determinant that restricts incorporation on lentiviral cores (Christodoulopoulos et al. 2001), (Sandrin et al. 2004). The relatively short cytoplasmic tails of mammalian gamma-retroviruses glycoproteins, of about 30-40 amino-acid-long, harbor a 15-20 amino-acid-long carboxy-terminal peptide, named R for murine leukemia viruses. Peptide R cleavage by the homologous viral core protease is required to activate the fusion potential of the glycoprotein (Brody et al. 1994), (Ragheb et al. 1994), (Rein et al. 1994). A possible pathway of incorporation of these glycoproteins on lentiviral cores may involve cleavage of the R peptide by active core proteases at the site of virion assembly, resulting in removal of the cytoplasmic tail determinant that impaired pseudotyping (Bouard et al. 2007), (Sandrin et al. 2004).

### 2.3.2) GLYCOPROTEIN OPTIMIZATION FOR PSEUDOTYPING OF LENTIVIRAL VECTORS

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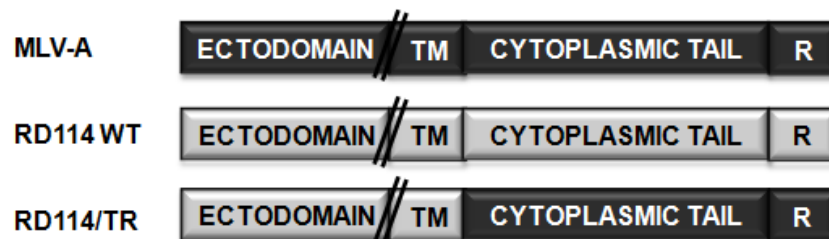
Although the use of the natural tropism of heterologous surface glycoprotein is very interesting for cell targeting, the surface-display of certain wild type glycoproteins on a heterologous LV vector core was very inefficient. Since the

glycoprotein cytoplasmic tail often determines the pseudotyping efficiency, this modification will open new possibilities to increase glycoprotein incorporation on the LV surface.

### 2.3.2.1) EXAMPLE OF RETROVIRAL GLYCOPROTEIN PSEUDOTYPING

When LVs are produced with wild type Gibbon Ape Leukemia virus (GALV) and the RD114 envelope glycoproteins, the resulting viral particles are not highly infectious due to a defect of glycoprotein incorporation on the lentiviral core.

As we previously developed, for pseudotype formation with homologous mammalian gamma-retroviruses viral cores, lack of cleavage of the R peptide by the viral protease alters infectivity of pseudotyped virions but not glycoprotein incorporation (Sandrin et al. 2004), (Bouard et al. 2007), (Brody et al. 1994), (Rein et al. 1994). As a consequence, the cytoplasmic tail of these glycoproteins has been replaced by the one of the MLV glycoprotein that incorporates very well on the lentiviral core (Figure 6). It results in a stronger incorporation on the lentiviral vector surface (Sandrin et al. 2002), (Verhoeyen et al. 2004), (Verhoeyen et al. 2009).



**Figure 6. Optimization of RD114 envelope glycoproteins.**

Mutations of the cytoplasmic tail of the TM subunit can improve envelope glycoprotein incorporation on the lentiviral vector resulting in LV titer increase. The replacement of the cytoplasmic tail of the cat endogenous retrovirus (RD114) wild type glycoprotein by the one of the MLV glycoprotein allowed an important titer increase

### 2.3.2.2) EXAMPLE OF MEASLES VIRUS PSEUDOTYPE

Pseudotyping of LVs with Edmonston MV strain wild type glycoproteins, the hemagglutinin (H) and fusion (F) proteins was inefficient. This was not real surprise since next to the efficient incorporation of both glycoproteins on the LV surface, the H and F complex functionality need to be conserved by a correct stoichiometric



arrangement. Therefore, several combinations of H and F truncated cytoplasmic tails have been tested. The shortest mutants (H $\Delta$ 24 and F $\Delta$ 30) were identified as the more adapted constructs and allowed a better incorporation on LVs and a 100-fold titer increase (Frecha et al. 2008). Importantly, these cytoplasmic tail modifications do not impair the host cell range of the initial viral glycoprotein since the resulting H/F-LVs conserved their tropism for human immune cells.

### 3) ASPECTS OF LENTIVIRAL VECTOR PRODUCTION

Since the theoretical aspects of lenti-vectorology had been discussed above in detail, this part is dedicated to the methods available to produce and characterize LVs. Figure 7 summarizes the LV production process.

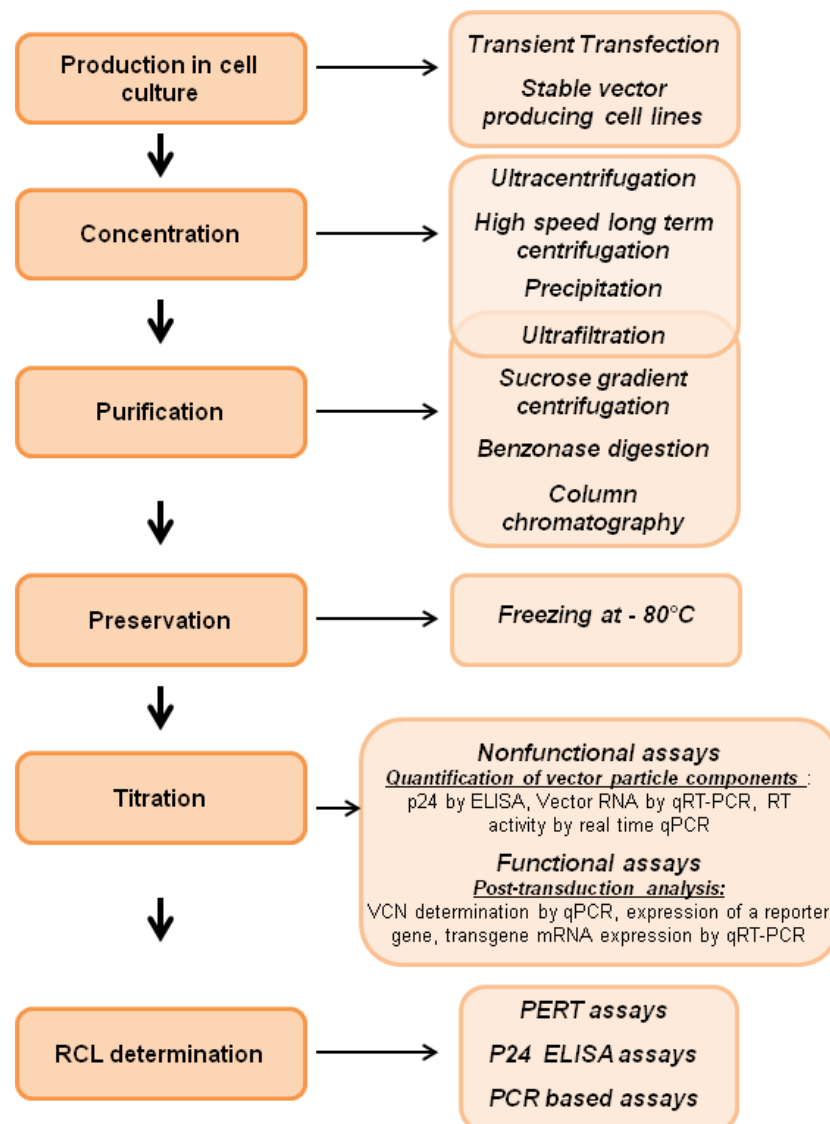


Figure 7. Lentiviral vector production process for clinical applications.

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## 3.1) PRODUCTION OF LENTIVIRAL VECTORS

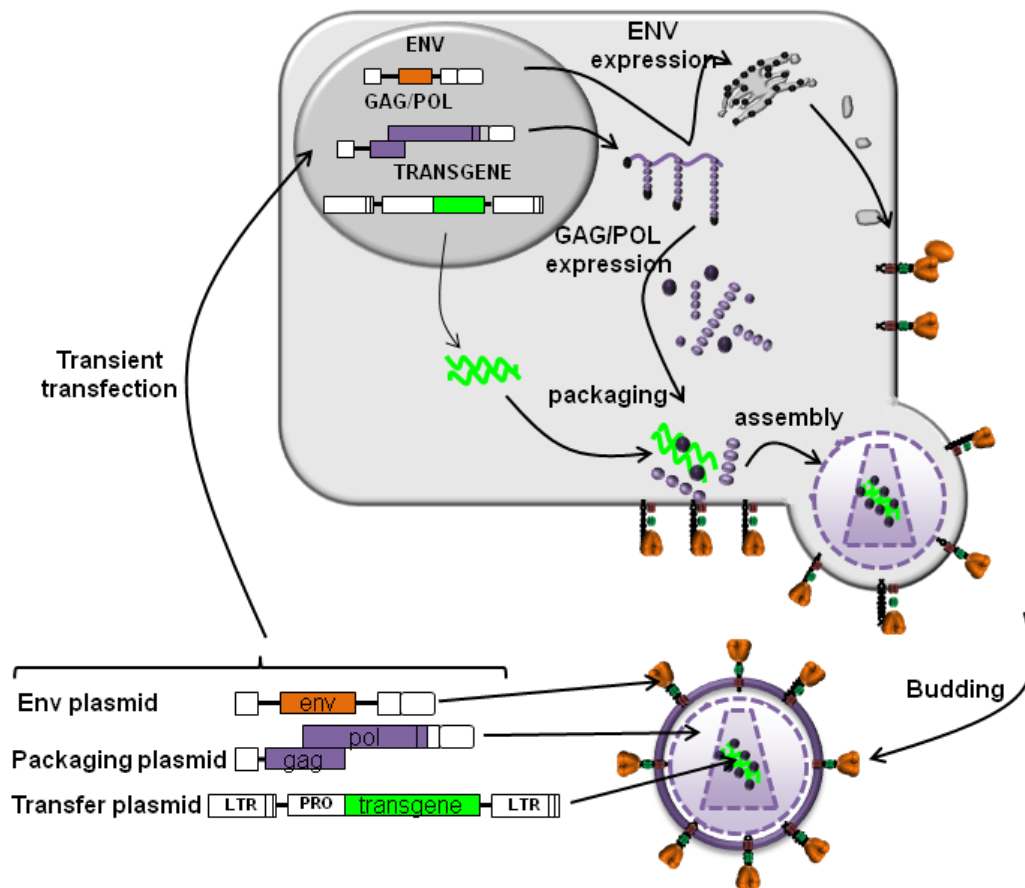
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### 3.1.1) TRANSIENT VECTOR PRODUCTION

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Transient production of LVs is the most commonly used method because it allows to produce LVs quickly with suitable titers for most of the laboratory applications. The HEK (human embryonic kidney) 293T cells or its derived clones are the preferred producing cells. Separate plasmids classically encoding for the packaging sequence, the transfer vector and the envelope (Figure 7) are introduced in the producer 293T cells by different transfection methods. Because of the cytotoxicity of the transfection process during LV production, it became clear that if an excess of plasmids is transfected, the production is inefficient. Therefore, minimal plasmid concentrations need to be determined to allow efficient LV production. Moreover, a silencing of certain tissue-specific promoters can be observed and in this case the choice of the cell line used for titer determination is very important. Once plasmids are in the producer cell, vector RNA and proteins can be produced and assembled followed by budding of the viral particles from the cell (Figure 8).

This production process is transient and possible only during a restricted time (2-3 days) of vector production. Indeed, after this time-point, producer cells suffer from the toxicity of certain viral proteins (e.g. gagpol en VSV-G) and cannot produce LVs efficiently (Sakuma et al. 2012). The transient transfection method is still the most used method for vector production for research purposes and even for clinical trials.



**Figure 8. Transient production of lentiviral vectors.**

For transient transfection, plasmids encoding for the packaging sequence, the transfer vector and the envelope glycoprotein (+/- the rev protein depending on the LV generation) are transfected into 293T cells using a transfection reagent. These 293T cells produce efficiently vector particles at 36 until 72 hours after the transfection. After that a severe drop in infectious titer is detected.

### 3.1.2) PRODUCTION BY STABLE PACKAGING CELL LINES

To better guarantee safe LV production, needed for clinical applications, stable vector producing cell lines (VCL) have been developed. They are engineered to express the different vector components (packaging, transfer and envelope sequences) stably by using transfection or transduction methods to introduce them into the producer cell. In addition, to being a reproducible source of LVs, stable production prevents plasmid DNA contaminations and homologous recombination of transient transfected plasmids, which reduces the risk of RCL formation. Moreover, a stable VCL would reduce enormously the LV production costs and make LV mediated gene therapy accessible to a high number of patients while currently only few patients can be treated with a vector batch. Because of these advantages of stable LV production, many groups have invested in the production of stable VCLs but with moderate success. Although, this stable production is essential for clinical

use, the fusogenicity of viral envelopes such as the VSV-G glycoprotein prevented the development of these stable VCLs or induced a strong titer reduction, too low for clinical use. This required complicated strategies using gag-pol and VSV-G expression under the control of inducible promoters, which only allowed short-term production of LVs.

Moreover, production of a stable cell line is cumbersome since for each therapeutic vector a new cell line needs to be established and before moving it into the clinic an extensive evaluation to prove absence of RCLs is required. The STAR cell line has been developed by the Collins's group (Ikeda et al. 2003), (Strang et al. 2004) by transduction of the necessary coding sequences; this cell line is able to produce high titer non-toxic retrovirus glycoprotein (for example RD114/TR) pseudotyped LVs for more than three months. Although this advance does not solve the VSV-G stable production concerns, it holds great promises for LV stable production notably for RD114/TR envelope pseudotyped LVs. Of note, recently a high titer stable RD114/TR VCL was successfully developed (Stornaiuolo et al. 2013).

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### 3.2) CONCENTRATION AND PURIFICATION OF LENTIVIRAL VECTORS

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After production, there are a lot of protocols that allow concentrating and purifying LVs. The producer cell supernatant is collected and filtrated to remove cellular debris. Subsequently, the vector containing supernatants are concentrated.

The most commonly used method is the ultracentrifugation of the viral supernatant at 1-300,000 g for 1-4 h at 4°C. Then, the viral pellet is re-suspended into an isotonic buffer during several hours. Another step of ultracentrifugation can be applied through a sucrose cushion to remove soluble protein contamination. A 10- to 100-fold titer increase is noted after this concentration and purification step. The purity of these preparations is sufficient for most of laboratory uses. Nevertheless, a lot of LV pseudotypes do not tolerate these high ultracentrifugation forces. In addition, the pelleting of cellular vesicles and debris is a side-effect of this method and is a concern in terms of immune response induction.

As a consequence, other methods have been developed such as high speed long term centrifugation at 10,000 g for 8-12 h at 4°C, precipitation with calcium phosphate or ultrafiltration by using a 100kDa molecular mass cut-off filter (Sakuma et al. 2012).

For the production of LVs used in the clinic, the purification process is well established according to the rules of good manufacturing practice (GMP) (Ausubel et al. 2012), (Merten et al. 2011). Directly following the supernatant harvest, the supernatant is first clarified by filtration. Benzonase nuclease is added in presence of Mg during 6 hours at 37°C in order to degrade most of residual nucleic acids. The clarified and benzonase treated supernatant is ultrafiltered and diafiltered (by a diafiltering (DF) buffer filtration) and centrifugated for 16-20 h at 6000 g. Then, the resulting viral pellet is reconstituted in DF buffer and centrifuged for 1-2 minutes to remove insoluble material. The production of LVs for the clinic involves several steps of chromatography in some laboratories such as Genethon. The majority of the LV production is aliquoted and stored at -80°C but some samples are tested for sterility, titration and undergo quality-safety control. The overall yield of this type of manufacturing production is around 30%.

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### 3.3) TITRATION OF LENTIVIRAL VECTORS

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It is essential to determine the infectious titer of a vector preparation. Many methods have been developed but unfortunately, a standard protocol in titration does not exist (Geraerts et al. 2006). We give an overview of possible methods and their limitations below (Kutner et al. {Acuto, 2003 #450}2009).

#### 3.3.1) TITRATION BY MEASURING THE QUANTITY OF VECTOR PARTICLE COMPONENTS

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This type of titration is based on a non-functional assay by quantifying the content of a vector preparation by measuring the lentiviral capsid antigen (p24 for HIV-1 LVs) by ELISA or the vector genomic RNA content by qRT-PCR. Finally, we can measure the RT activity using real time PCR quantification. The major concern with these methods is that they detect both infectious and non-infectious vector

particles and largely overestimate the LV titer. Moreover, the titer obtained with these methods does not take into account the nature of the vector construct with the addition of *cis*-acting sequences or the use of a tissue-specific promoter, a certain envelope glycoprotein and the targeted cell type, which all can influence the titer.

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### 3.3.2) TITRATION BY FUNCTIONAL ASSAYS

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Different methods of functional titration are available. The first assay consists in the isolation of genomic DNA of the target cells after their transduction. Then the total number of integrated vector copies numbers (VCN) per cell is determined by qPCR, which is normalized to the number of target cells (determined by an endogenous cellular gene used as a reference). However, the VCN does not inform us on the transgene expression in the target cell, which might vary according to the genomic integration sites of the provirus.

When a reporter gene is included in the transfer vector (GFP, antibiotic resistance, surface display of ligands at the target cell surface), the direct measurement of transgene expression by transduced cells appears to be the more suitable method to determine the 'true' infectious LV titer. In absence of reporter elements to measure the transgene expression, a qRT-PCR on transgene mRNA in transduced cells is reported to correlate to titers obtained using reported genes in most cases. Of note, if a tissue- or cell-specific internal promoter is used, the cells using for titering should support expression driven by this specific promoter.

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## 3.4) LENTIVIRAL VECTOR BIOSAFETY DETERMINATION

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### 3.4.1) RCL DETECTION

To enhance biosafety, the detection of RCLs is a necessary step in the testing of LV preparations and many assays for RCL detection have been developed. RCL detection is performed on the supernatant of LV-infected target cells that have been cultured for two weeks to avoid contamination of the original LV used for the infection.

The product-enhanced reverse transcription assay (PERT), a PCR based method is known for its high sensitivity. It consists in using an RNA template as a substrate for RT activity. If a reverse transcript is identified by qPCR, it means that reverse transcriptase is present in a sample contaminated by RCL. Every RCL that present a functional RT can be detected by this method (Rohll et al. 2002), (Pyra et al. 1994). Another frequently used method consists in detecting and measuring CA amounts by detection of p24 using ELISA. Absence of RCLs is shown when serial passaging of supernatant from infected cells is devoid of p24 (Escarpe et al. 2003). However, these methods are based on the principle that RCLs have a usual viral form. As a consequence, there is no current standard for RCL determination and the methods are evaluated case by case in the context of clinical trials. With the 3<sup>rd</sup> or 4<sup>th</sup> LV vector generation, RCLs were not detected using classical methods.

#### 3.4.2) DETECTION OF LVS GENOTOXICITY

Since the concerns raised by the clinical trial that used MLV retroviral vector for X-linked severe combined immunodeficiency (Hacein-Bey-Abina et al. 2003), (Hacein-Bey-Amina et al. 2008), the detection and regulation of transgene integration sites is a major concern in viral vector based therapies. Although this work demonstrated a successful gene therapy in humans, some of the patients developed leukemia due to insertion into a proto-oncogene. Later, it was elucidated that this was caused by the MLV vector integration profile (Hacein-Bey-Abina et al. 2003), (Hacein-Bey-Amina et al. 2008). Today, the knowledge of LV integration tropism has advanced enormously and pointed out the preferential insertion of the provirus in transcriptionally active sites of the cell genome. Indeed, for instance in hematopoietic cells, the insertion pattern of LVs appeared to be quite different than that obtained with MLV retroviral vectors (Modlich et al. 2009), (Cesana et al. 2014).

Nevertheless, SIN LV integration appears to have a genetic impact notably because they may upregulate the expression of genes flanking the integration site (Cesana et al. 2014), induces aberrant splicing or premature termination that involves loss- or gain of function mutations (Montini et al. 2009), (Ranzani et al. 2013), (Moiani et al. 2012), (Cavazzana-Calvo et al. 2010). Therefore, careful analysis of LV genomic integration sites is needed to assess the risk of insertional mutagenesis

when using a specific vector design. Several essays to estimate this risk have been developed and are discussed below.

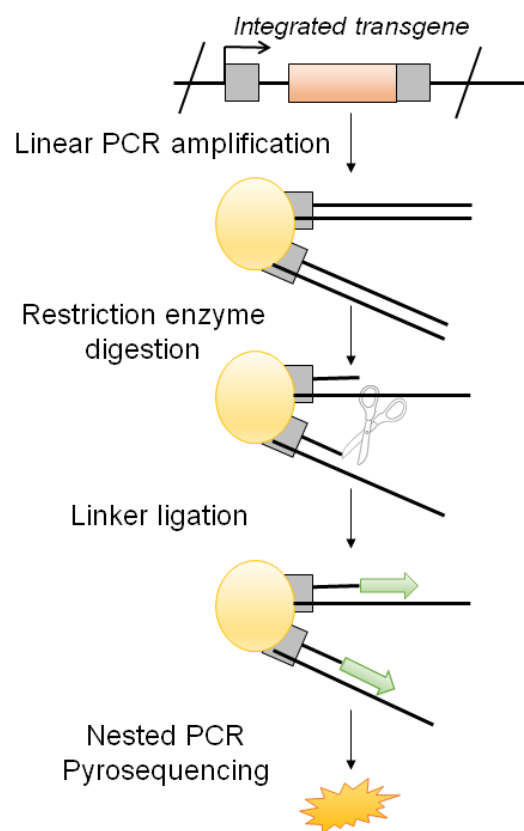
#### 3.4.2.1) *IN VITRO* GENOTOXICITY ASSAY

Insertional mutagenesis can be investigated thanks to a mapping of the genomic integration sites in tumors, called common insertion sites (Montini et al. 2009). cDNA linear amplification mediated PCR (LAM-PCR) was developed in order to identify chimeric LV cellular transcripts from the whole genome of a LV-transduced cell. LAM-PCR consist in amplifying regions of genomic DNA that contains integrated proviral vectors using a LTR specific primer. A magnetic bead capture the DNA fragments and a double strand synthesis is performed followed by restriction enzyme digestion and further ligation of a double stranded linker. Pyrosequencing is realized after a nested PCR stage (Figure 9) (Rae et al. 2013). This method provides qualitative informations on the role of LV integration on aberrant splicing processes but is not sufficient to quantify the frequency of this mechanism. (Ranzani et al. 2013), (Wang et al. 2008). The quantification of aberrant transcripts can be performed on single cell derived clones using RT-qPCR methods (Cesana et al. 2012). LAM-PCR has been described as the most sensitive method to detect LV integration sites allowing for example the detection of miscellaneous vector integration sites (Bartholomae et al. 2012), (Schmidt et al. 2009). This method has been approved for the mutagenesis follow-up of modified cells in preclinical and clinical trials (Ott et al. 2006) (Cartier et al. 2009), (Deichmann et al. 2007).

The combination of LAM-PCR and next generation sequencing (NGS) technologies hold promises for the evaluation of clonogenicity and large scale analysis of vector integration sites (Bartholomae et al. 2012). NGS technologies such as DNA bar coding and pyrosequencing offer a wide characterization of integration sites with high spatial and temporal resolutions (Wang et al. 2008). Briefly, pyrosequencing method is able to generate several hundred thousand sequences with 400-500 base pair read lengths. Amplicons from several samples can be pooled for sequencing and then analyzed separately thanks to DNA bar coding techniques (Wang et al. 2008). More precisely, Lu and colleagues (Lu et al. 2011) have developed the following method: the DNA barcode is a common 6 bp sequence that correspond to a library identifier followed by a random 27bp cellular barcode. LVs are



designed with a barcode sequence in their LTR that they carry to the targeted cells. It is necessary to work on reduced number of cells in order to transfer a unique copy per transduced cell (Lu et al. 2011), (Glimm et al. 2011). *Ex vivo* transduced cells such as hematopoietic stem cells are transplanted in mice (See part 2. 4.2) and replicate containing the barcode sequence. Then, hematopoietic cells are harvested and barcodes are recovered from the genomic DNA using PCR and pyrosequencing; this allows to explore transduced cell clonogenicity in the entire hematopoietic lineage from the stem cell to the myeloid and lymphoid progenitors and end-differentiated cells (Usek et al. 2012).



**Figure 9. Linear amplification mediated PCR (LAM-PCR).**

A LTR (in grey) specific primer allows to amplify integrated proviral vector DNA. Thanks to a magnetic bead capture (in yellow), linear PCR resulting oligos are digested and then linked to a double strand linker (in green). A nested PCR is realized and its products are sequenced by pyrosequencing. This technique allows to explore LV integration sites.

### 3.4.2.2) IN VIVO GENOTOXICITY ASSAYS IN TUMOR PRONE MOUSE MODELS

Although *in vitro* genotoxicity assays give important indications concerning the genotoxic effects of different LV designs, animal models allow to evaluate their effects on *in vivo* hematopoiesis. Tumor prone mice have been described as interesting models to investigate the insertional mutagenesis oncogenic effects (Montini et al. 2009). The currently used tumor prone *cdkn2a* <sup>-/-</sup> model is based on the inactivation of the *Cdkn2a* gene, which controls cell senescence and prevents cell transformation (Sherr et al. 2004). This mouse model has been useful to demonstrate that HIV-1 based LVs are safer than MLV based vectors in term of genotoxicity (Montini et al. 2006). Indeed, transplantation of LV transduced HSCs can be performed in these mice; then, the integration site selection and tumor development in transplanted mice can be determined by previously described *in vitro* insertion site analysis techniques.

Although mouse models are experimentally preferred to larger animals, in preclinical studies dogs and non-human primates are important to follow the long-term risks associated with insertional mutagenesis because of their longer lifespan (Hematti et al. 2004), (Beard et al. 2007), (Kennedy et al. 2011). This is of importance since the insertional mutagenesis in several clinical trials was only revealed several years after engraftment of corrected HSCs.

### 3.4.3) REGULATION OF INTEGRATION OF LVS

Both MLV retroviruses and lentiviruses integrate semi-randomly into the genome, and thus insertional mutagenesis cannot be excluded. This justifies the numerous strategies that have been evaluated to target gene transfer in a specific "safe harbor" in the cell genome and of which some are described below.

Of importance, the SIN vector design in reducing potential risks of insertional mutagenesis and the role of insulators in case of integration in condensed chromatin regions are major advances for clinical applications of LVs. So, in this part we will focus on the other strategies that have been developed in order to decrease the risks induced by random integration of LVs. Many genomic integration regulation strategies have been developed

### 3.4.3.1) INTEGRATION DEFICIENT LVs FOR NON-PROLIFERATING CELLS

For non-dividing target cells, integration deficient lentiviral vectors could represent an alternative approach to reduce insertional mutagenesis. (Philpott et al. 2006), (Wanisch et al. 2009). The product of reverse transcription of LV is a linear dsDNA with LTRs at both ends. Classic LVs with a functional integrase (IN) usually can be integrated into the cell genome as proviruses. But the proviral linear DNA can also be circularized by non homologous end joining that produces 2-LTR circles and by homologous recombination between the LTR or by ligation of RT products that gives 1-LTR circles. In order to exclusively obtain these episomal forms, some mutations are performed in the IN encoding gene. Since IN has a pleiotropic function in the viral cycle such as in virion morphogenesis, in RT, in nuclear translocation and in integration only one type of mutations can specifically affect the cleavage and integration functions. These class I mutations are created by substituting one of the three amino acids of the catalytic triad (D64, D116 and E152 for HIV-1 IN), most authors use the well described D64V mutant. Some groups tried to mute the attachment sites at the end of the provirus but these mutations appeared less efficient than obtained with IN class I mutations. In order to optimize the action of these mutations, a pharmacological treatment against IN or its cofactors is possible by using raltegravir or elvitegravir which are strand-transfer inhibitors (Cornu et al. 2007).

IDLVs are more and more developed for clinical applications such as retinal dystrophy gene therapy with interesting results obtained in rodents and dogs (Yanez-Munoz et al. 2006), (Philippe et al. 2006) or immunization against HIV and tumors.

Although these IDLV vectors appear to be attractive candidates for transgene delivery into quiescent tissues and for gene repair, their interest for longterm transgene expression needs to be confirmed. Indeed, the non-integrated transgene is lower expressed than an integrated one (Matrai et al. 2011). Moreover, the high level of non-replicative episomal molecules in transduced cells could assure a high level of transgene expression while preventing insertional mutagenesis and RCL generation. Many authors are currently trying to develop IDLVs with replicating episomes

(Wanisch et al. 2009) that may provide a stable transgenesis in proliferating tissues while maintaining their biosafety characteristics.

### 3.4.3.2) SITE DIRECTED INTEGRATION OF IDLVS

The targeted integration of LVs can be performed thanks to the use of specific nucleases and non-integrating LVs.

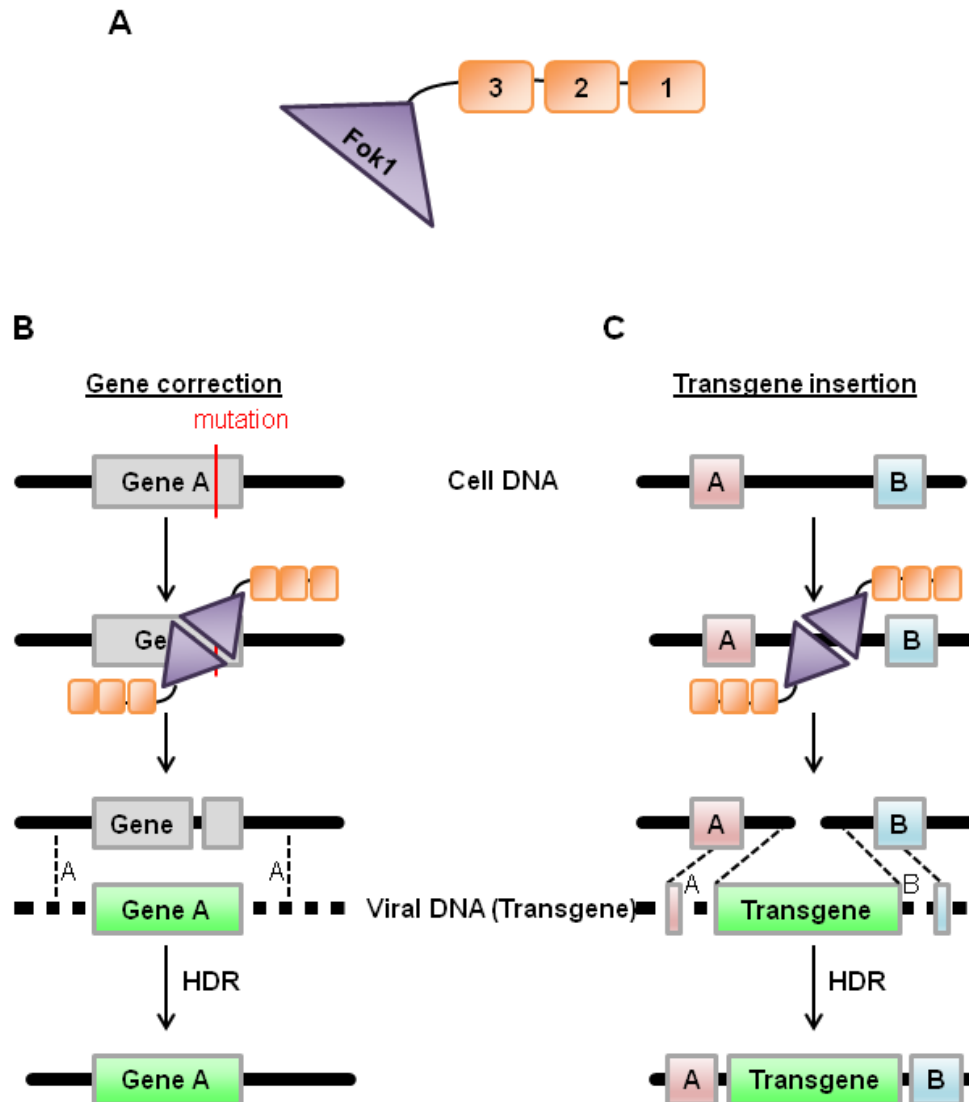
#### 3.4.3.2.1) THE ZINC FINGER NUCLEASE STRATEGY (ZFN)

The Zinc Finger nucleases are hybrid restriction enzymes that link the cleavage domain of FokI to a designed zinc finger protein (ZFP) (Naldini et al. 2011), (Miller et al. 2007), (Lombardo et al. 2007). They are used to modify the eukaryotic genome from simple sequence editions to targeted integration of entire genes. ZFNs introduce a double strand break at the chosen site in the host genome and if an exogenous repair template is available, it may be incorporated into the genome by homology directed repair. The targeting of the cleavage event is mediated by the ZFP domain that can be engineered to recognize a wide range of DNA sequences (Figure 10).

To introduce the ZFN technology into a target cell, Naldini and colleagues used IDLVs successfully (Lombardo et al. 2007). The IDLV episomal accumulating proviral DNA is competent for both transient expression of the ZFNs and a transgene substrate for homologous recombination. A 10- to 100-fold increase in site-specific integration was observed when the ZFNs are co-delivered. In this study, authors managed to correct specifically the IL2RG gene mutation, involved in the X-SCID disease, at its endogenous genomic site.

The choice of the integration locus appears to be very important because it may influence the ZFN accessibility, the permissiveness to homology directed repair (HDR) and the maintenance of transgene expression.

Although these results are very promising and mean a big step forward towards improved biosafety by reducing the risks of insertional mutagenesis, a careful safety assessment is required to validate potential off-target effects of ZFNs.



**Figure 10. Gene editing using designed zinc finger nucleases (ZFN).**

(A) Schematic representation of a ZFN with 3 specific DNA binding domains linked to the FokI nuclease. Targeted endogenous gene correction (B) or transgene addition in a well defined genomic locus (C) which is driven by the activation of the ZFN nucleases encoded by lentiviral vectors by ZFN dimerization, cleavage and then homologous directed repair (HDR) with the corresponding homologous sequences flanking the correcting transgene encoded by a second lentiviral vector.

#### 3.4.3.2.2) THE TALE NUCLEASES STRATEGY

In parallel to ZFNs engineering, many studies are focused on artificial enzymes able to generate double stranded DNA breaks at predefined genomic sequences. Indeed, since the recent discovery of the potential of bacterial transcription activator-like type III effectors (TALEs) to recognize specific DNA sequences, many groups have developed some specific TALEs (Holkers et al. 2013). The DNA binding domain of TALEs displays a tandem array of 15.5 to 19.5 repeat units with 34 residues each. A 2-amino acid hypervariable polymorphism at position 12 and 13 is often the only feature difference among the repeats. The TALE nucleases (TALENs) operate similarly to ZFNs by assembly of a pair of TALENs at a given DNA sequence consisting of two half target sites separated by a spacer sequence.

Nevertheless, the integration seems to be more independent from the chromatin context and the simple DNA binding code used, which suggests that TALENs could bind a wider range of DNA sequence with a more predictable manner than obtained with ZFNs which might reduce the off-target effects.

HIV-1 based LVs or IDLVs bearing TALEN sequences could serve as TALEN delivery platforms and could be safer than the ZFNs ones. However, extensive comparisons between the two systems are needed to make definite conclusions.

Interestingly, another genomic targeting systems made its appearance based on the bacterial CRISPR/CAS system, which is popular because of its easy design.

#### 4) LENTIVIRAL VECTOR APPLICATIONS IN BASIC RESEARCH AND GENE THERAPY

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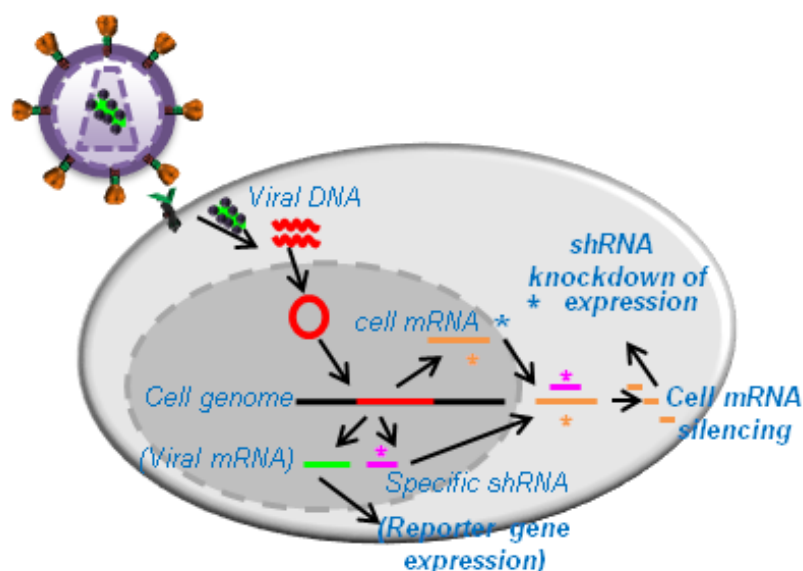
LVs are able to transduce a wide range of host cells including both dividing and non-dividing cells. Their success in preclinical and clinical studies makes them popular tools for gene transfer. Here below several possible applications for LVs in fundamental research and in the clinic will be reported but of course this list is not exhaustive.

## 4.1) LENTIVIRAL VECTOR APPLICATIONS IN FUNDAMENTAL RESEARCH

### 4.1.1) GENE SILENCING VECTORS

RNA interference (RNAi) mediated gene silencing is an interesting technology to study gene functions or to restrict over-expressed or aberrantly expressed genes to specific cell types in some diseases. In mammalian systems, small interfering RNAs (siRNAs) can be expressed endogenously from pol III promoters as short hairpin RNAs (shRNAs) or from pol II promoters as a part of a microRNA molecule (Singer et al. 2008), (Stewart et al. 2003).

Ideally, a gene silencing lentivector would contain both a reporter gene and the shRNA silencing cassette driven by a pol III promoter (e.g. H1 or U6). The interfering RNA target sequence should be ideally 19-23 bases long and several different shRNAs have to be evaluated in order to identify the most effective one (Figure 11). Indeed, if the objective is to knockdown a certain gene, only the sequences showing the highest efficiency should be selected. Since over expression of shRNAs can result in off-target effects, a control shRNA in the context of the same LV backbone and reporter gene should be included. For instance scrambled sequences, non-target sequences or mutated sequences are used as a shRNA control.



**Figure 11. Schematic representation of lentiviral vectors for shRNA silencing.**

The lentiviral transfer vector codes for small hairpin RNAs (shRNA), specific for a gene of interest, under the control of a Pol II promoter. Upon expression the shRNA recognizes a particular sequence

of a host cell mRNA and will silence its expression. To identify the transduced cells these LVs often carry a reporter gene such as GFP.

Since LVs have a large packaging capacity, they can harbor the shRNA combined with a regulated expression system. For instance, a Tet-regulatable shRNA cassette in addition to the TtA sequence has been included in the same LV, and proved functional (Stegmeier et al. 2005).

This gene silencing LV technology can lead to better understanding of cellular biology (e.g. in cancer cells) if it allows to obtain a clear knockdown phenotype for cells and transgenic animals.

#### 4.1.2) LENTIVIRAL VECTOR MEDIATED TRANSGENESIS AND IN VIVO CELL LINEAGE TRACKING

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Many authors have shown that LVs can efficiently transduce embryonic stem (ES) cells. Verma and colleagues (Pfeifer et al. 2002) already showed that in contrast to MLV retroviral vectors, for which expression is silenced in ES cells, LV expression is maintained in human and murine ES cells. Moreover, embryos at morula stage can be transduced and the resulting progeny express the transgene. Likewise, transgenes introduced by LVs in ES cells are transmitted to the germ-line.

Therefore, authors concluded that LVs are very attractive tools for generating transgenic animals. Many transgenic animals have been generated using LVs including mice (Lois et al. 2002), rats (Van den Brandt et al. 2004), cats (Wongsrikeao et al. 2011), rabbits (Hiripi et al. 2010), pigs (Hofmann et al. 2003) and even chickens (McGrew et al. 2004). These transgenic animals either can hold a specific tissue reporter gene such as GFP or can present a specific knock-down in order to study gene function or to produce a disease preclinical model (Tiscornia et al. 2003).

Moreover, LV-mediated stable gene transduction can be exploited *in vivo* in order to follow targeted cells. For instance, Gallo *et al.* (2008) developed a LV for tracking cardiomyocyte differentiation from human ES cells in order to better understand cardiac muscle differentiation.



#### 4.1.3) CELLULAR REPROGRAMMING

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The induced pluripotent stem (IPS) cell generation relies on the introduction of defined pluripotency associated factors such as OCT4, SOX2, KLF4 and cMyc in somatic cells (Takahashi et al. 2007). LVs have been used in order to introduce these multiple factors in fibroblasts, keratinocytes and HSCs in order to reprogram them (Sakuma et al. 2012). After the reprogramming MLV retroviral vector encoding the above factors were spontaneously silenced. Nevertheless, the risks of reactivation of the pluripotency associated factors once integrated into the genome of these IPS cells, led laboratories to develop other technologies such as protein transfer or they privileged integration deficient LVs (IDLVs) for gene transfer of these factors (Mali et al. 2008)

#### 4.1.4) LENTIVIRAL VECTOR MEDIATED IMMUNE MODULATION

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LVs can efficiently transduce APCs including macrophages or dendritic cells. These properties can be used for vaccines against infectious diseases and tumors. The LV-mediated expression of specific antigens in dendritic cells induce both CD4+ and CD8+ T cell responses leading to cellular immunity and humoral response (Breckpot et al. 2008) (Hu et al. 2011). In order to efficiently present transduced antigens on DCs and generate antigen specific immune response, LVs must transduce but also stimulate DCs. Both cell entry and reverse transcription were shown to affect the activation of DCs *in vitro* and *in vivo*. Contrary to adenoviruses, LVs are able to transduce DCs in a persistent, non-toxic and non-immunogenic manner.

Positive results have been obtained in LV transduced DCs for use as immunity boosting vaccines and a lot of researchers are focused on the development of an HIV-1 vaccine using LV immunization. Unfortunately, until now the extreme ability of HIV-1 to escape the immune system prevented the fabrication of an efficient vaccine (De Feo et al. 2012). Of interest, DCs transduced by LVs expressing tumor associated antigens such as melanoma antigens are able to activate cytotoxic T lymphocyte responses and provide protection against tumor challenges. It has been shown that this strategy can efficiently lead to tumor regression (Yang et al. 2011)

An important anti-cancer strategy based on the transfer of tumor-specific T cell receptor (TCR) genes into patient T cells has been proven successful in the clinic. More recently, a radically different strategy consisting of introducing a coding sequence for a chimeric antigen receptor (CAR), allowed to confer the desired specificity for a cancer antigen to T cells. Indeed, ongoing clinical trials have described durable rejection of previously refractory B-cell malignancy in patients after CD19-directed CAR gene therapy of autologous T cells (Scholler et al. 2012).

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#### 4.2) LENTIVIRAL VECTORS IN HEMATOPOIETIC STEM CELL BASED GENE THERAPY, STATE OF THE ART

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For the moment, ex-vivo gene therapy strategies using the correction of autologous target cells *in vitro* followed by re-infusion into the patient are favored (Verma et al. 2013). Although numerous gene therapy trials have been performed using MLV retroviral vectors, the first lentiviral vector based clinical trial was initiated only ten years ago. Although the MLV based retroviral vectors were successful for hematopoietic stem cell based gene therapy, the "safer" LVs are gaining interest in the gene therapy field (Table 2).

**Table 2. Gene therapy clinical trials using Lentiviral Vectors**

Disease	Gene to correct	LV	Nb of treated patients	Benefits for the patients	Ref
<b>X-linked Adrenoleukodystrophy</b>	<i>ABCD1</i>	3rd generation LV  VSV-G HIV-1 pseudotype	2	Cerebral demyelination ceased	(Cartier et al. 2012 et al. 2010 et al. 2009)
<b>β-Thalassemia</b>	<i>β-globin</i>		1	Transfusion independence – Hemoglobin level stabilized (8,5-9 g/dL)	(Bank et al. 2005), (Cavazzana-calvo et al. 2010), (Payen et al. 2012)
<b>Metachromic Leukodystrophy</b>	<i>ARSA</i>		3	Neurodegeneration ceased	(Biffi et al. 2013)
<b>Wiskott-Aldrich syndrome</b>	<i>WAS</i>		3	Protection from bleeding and severe infections – Eczema resolution	(Galy et al. 2008), (Aiuti et al. 2013)

#### 4.2.1) FIRST CLINICAL TRIAL FOR THE GENETIC TREATMENT OF HIV INFECTION

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Levine *et al.* (2006) performed a phase I clinical trial for HIV therapy using a conditional replicating VSV-G pseudotyped LV coding for an antisense sequence complementary to the HIV envelope. Five subjects with chronic HIV-1 infection were enrolled in the trial and autologous CD4+ T cells were transduced with the anti-HIV therapeutic vector and reinfused back into the patients. An increase of CD4+ T cells, normally depleted in AIDS patients, and a decrease of viral load were observed and until now insertional mutagenesis was not detected. Further follow-up is needed to conclude on the safety and the efficacy of this therapy but these first results were promising for further clinical applications of LVs.

#### 4.2.2) X-LINKED ADRENOLEUKODYSTROPHY CLINICAL TRIAL

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Cartier *et al.* (2012 *et al.* 2010 *et al.* 2009) obtained promising results for X-linked adrenoleukodystrophy (ALD) by *ex vivo* correction of autologous HSCs employing LVs encoding for the wild type ABCD1 gene. This disease is indeed characterized by a deficiency in the ALD protein, an adenosine triphosphate binding cassette transporter encoded by the ABCD1 gene. This defect in ALD protein causes a severe brain demyelination in boys and the only currently available treatment to halt the neurodegeneration is an allogeneic HSC transplantation. In this trial, 14-16 months after infusion of autologous corrected cells into the patients, the progressive cerebral demyelination ceased as compared to patient that did not undergo transplant

#### 4.2.3) $\beta$ -THALASSEMIA CLINICAL TRIAL

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$\beta$ -thalassemia major is characterized by absence or strong decrease in  $\beta$ -globin chains of hemoglobin, which triggers the precipitation of unpaired  $\alpha$ -globin chains in erythroid precursor cells. This phenomenon leads to a perturbed erythropoiesis and thus a decreased production of red blood cells and a decrease of the hemoglobin levels.

The phase I/II clinical trial of  $\beta$ -globin gene therapy for  $\beta$ -thalassemia major that was initiated in 2007 also showed very promising results (Bank et al. 2005), (Cavazzana-calvo et al. 2010), (Payen et al. 2012).

In this trial, a SIN-LV containing large elements of the  $\beta$ -globin locus control region (LCR) and chromatin insulators in the LTRs was designed and used to transduce autologous HSCs of one patient. Three years after starting this treatment,  $\beta$ -globin corrected cells are still detected and the patient is transfusion independent and stabilized his blood hemoglobin level. Of note though, a dominant integration clone appeared but this did not lead to leukemia up to now. Indeed, a dominant myeloid cell clone has been evidenced resulting in an activation of the high mobility group AT-hook 2 gene (*HMGA2*) and the expression of a truncated HMGA2 mRNA in the erythroid lineage (Cavazzana-calvo et al. 2010). Cavazzana-Calvo and colleagues hypothesize that this clone dominance may be either the consequence of a stochastic event because of the low number of initially transduced HSCs with HMGA2 erythroid specific expression or explained by a differential proliferation of a myeloid biased lineage induced by a HMGA2 expression dysregulation in early hematopoietic progenitors. Of importance, the overexpression of truncated HMGA2 mRNA seems to induce benign tumours such as lipomas (Cleynen et al. 2008).

#### 4.2.4) METACHROMIC LEUKODYSTROPHY AND WISKOTT-ALDRICH SYNDROME CLINICAL TRIAL

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Very recently, Biffi et al (2013) and Aiuti et al (2013) provide very encouraging results on the HSC-based gene therapy for the correction of metachromatic leukodystrophy and Wiskott-Aldrich syndrome.

Metachromatic leukodystrophy is an autosomal recessive lysosomal storage disease caused by mutations of the ARSA gene encoding for the arylsulfatase enzyme. This enzyme deficiency leads to an accumulation of the enzyme substrate in oligodendrocytes, microglia, Schwann cells and macrophages and in certain neurons of the central nervous system. This results in a widespread demyelination and neurodegeneration. Today, no efficient treatment for this disease exists.

The Wiskott-Aldrich syndrome is caused by a mutation in the WAS gene encoding the WASP protein, which regulates the cytoskeleton architecture. A deficit in WASP induces a X-linked primary immunodeficiency characterized by infections, thrombocytopenia, eczema, autoimmunity and lymphoma. Currently, the only way to treat patients is allogeneic HSC transplantation.

In 2010, clinical trials were initiated using LVs encoding for ARSA (6 patients) and WASP (10 patients). In these clinical trials, no clonal dominance nor preferential integration pattern were observed. Although a long term follow-up of these patients is needed, the first results in three patients for each of these two trials are very encouraging in terms of efficacy.

Since LVs integrate into the host cell genome, they still can perturb endogenous gene expression. Therefore, a careful biosafety assessment needs to be performed. However, their ability to introduce stably a therapeutic gene in multiple different target cells and their favorable integration pattern predicts that they might become powerful gene therapy tools in the near future. In parallel, safer LV vectors are being designed which will extend their applications to other genetic diseases and give hope to many patients suffering from these incurable diseases.

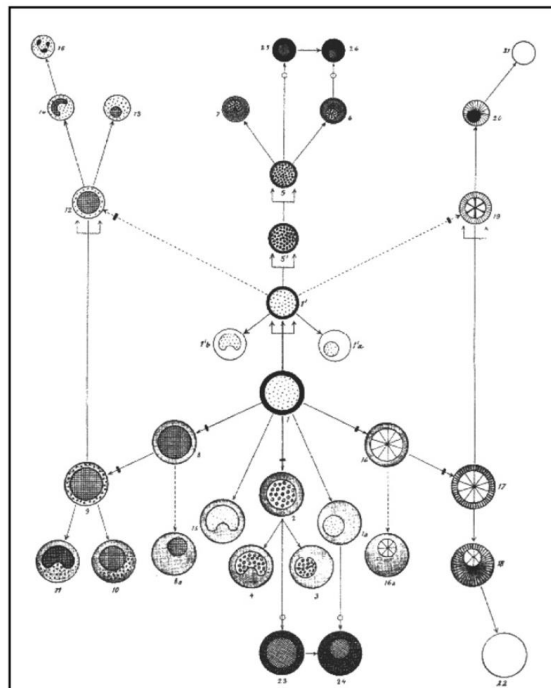
The following part will describe in detail one of the important target cells used in these LV mediated gene therapy trials: the hematopoietic stem cells.

## PART 2 – HEMATOPOIETIC STEM CELLS (HSC)

### 1) IDENTIFICATION AND CHARACTERIZATION OF HSC

#### 1.1) HISTORICAL APPROACH OF HSC

Hematopoietic stem cells (HSCs) are the first identified and the better characterized adult stem cells (Sykes et al. 2013). In 1905, Pappenheim described the “stem cell” fate thanks to the diagram presented in Figure 12. It is striking that his description still approaches the current view on hematopoiesis.



**Figure 12. View of hematopoiesis by Pappenheim in 1905.**

(From Ramalho-Santos et al. 2007). The multipotent stem cell is represented in the middle of the diagram

In 1932, Sabin evoked the presence of undifferentiated hematopoietic stem cells in bone marrow and concluded that irradiation induced impairments in primitive hematopoietic cells and consequently in blood. Some years later (Sabin, 1936), the same authors explored the nature of these stem cells, describing them like small lymphocytes which lack signs of differentiation. Due to limitations in experimental methods, the debate about the existence of a common hematopoietic stem cell continued for several decades.

In 1957, thanks to their work on bone marrow transplantation in dogs, Thomas and colleagues (Thomas, 1957) confirmed that there were specific bone marrow cells which can restore hematopoietic lineages after lethal irradiation.

It was only in the 1960s that Till and McCulloch brought the expected evidence of the existence of HSCs (Ramalho-Santos et al. 2007), (Sykes et al. 2013). They showed that a selected progenitor subpopulation of hematopoietic cells was able to self-renew and to differentiate into mature hematopoietic lineage (Becker, 1963). Indeed, bone marrow cell engraftment to lethally irradiated mice allowed them to survive. Moreover, they found splenic nodules which appeared to be colony forming units (CFU) composed by all hematopoietic populations (Till, 1961). Then, they described the clonally expansion of HSC leading to CFUs (Becker, 1963). These works provided the scientific basis of hematopoiesis and give arguments in favor of hematopoietic stem cell transplantation.

Considering these works, HSCs may be defined as cells that can self-renew and differentiate into the whole hematopoietic lineage.

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## 1.2) CHARACTERIZATION AND ISOLATION OF HUMAN HSC

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Up to now, human HSCs are not characterized by a combination of phenotypic specific markers as it is the case for murine HSCs (Zhang et al. 2005). Indeed, over the last four decades, several methods of human HSC enrichment have been suggested but none is completely satisfying (Wisniewski et al. 2011).

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### 1.2.1) BIOCHEMICAL CHARACTERISTICS OF HSC

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Hoechst 33342 and Rhodamine 123 fluorescent vital dye have been used to enrich population in HSCs in combination of antibody based approaches (Goodel et al. 1996). Although these cells are long term repopulating progenitors, this population is called 'side population' because it does not express CD34 and CD133 markers (see 1.2.2). Moreover, these cells present an impairment in their homing capacity (Matsuzaki et al. 2004) which significantly reduces the interest of this type of isolation for clinical purposes.

Primitive human hematopoietic cells present a high aldehyde deshydrogenase (ALDH) activity (Sladek et al. 1999). The ALDH expressing cell exposition to BODIPY-FI1- labeled amino acetaldehyde (BAAA) induces a selective fluorescence. Indeed, BAAA is converted in fluorescent BAA by ALDH. In order to prevent BAA cell efflux, some ABC transporter inhibitors are needed. The selection of ALDH+ hematopoietic cells allows to enrichment for HSCs (Storms et al. 1999). The use of both BAAA and antigen surface markers staining still increases HSC in the cell population (Christ et al. 2007). Nevertheless, the use of transporter inhibitors and of BAAA cannot be employed for clinical applications because of the induced toxicity of this drug.

### 1.2.2) HSC SURFACE ANTIGENS

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The most commonly known human HSC markers, CD34, has been identified in 1984 by Civin and colleagues (Civin, 1984). CD34 is a member of the sialomucin surface molecule family (Krause et al. 1996) and was suggested to play a role in cell adhesion and differentiation but its role remains not completely understood. Nevertheless, the location of the most primitive fraction of HSCs in the CD34+ subset remains a matter of debate. Several explanations have been developed in order to explain the ability of CD34- cells to engraft and reconstitute the hematopoietic lineage (Bhatia et al. 1998). Some authors hypothesized that CD34- cells could be a more primitive precursor than CD34+ cells (Bhatia et al. 1998) when they demonstrate that CD34- isolated from a CD34+ graft could generate a new CD34+ long term repopulating population, the dynamic interconversion of CD34 on cell surface is proposed (Dao et al. 2003). Of importance, in the CD34- subset, only the CD133+ subpopulation is able to engraft immunodeficient mice (Gallacher et al. 2000). Although the expression of CD34 on HSC surface membrane is still debated, the isolation techniques keep using this marker. Nevertheless, new markers are becoming more generalized (CD133, see below). Therefore, other markers can be used in order to identify the most primitive HSC subpopulation.

The CD133 (AC133) surface marker is expressed on 20 to 60% of the CD34+ isolated cells depending on their origin (Yin et al. 1997). CD133+ CD34+ cell subpopulation presents a high capacity of expansion, bone marrow homing and engraftment (Goussetis et al. 2000), (Wynter et al. 1998). However, CD133 is not a



specific marker, in human it is also found in hepatic (Schmelzer et al. 2007) and neural stem cells (Lee et al. 2005). New isolation kits though are based on the CD133 positive selection since this marker seem to stain more primitive cells than CD34+ cells.

The human CD90 (Thy-1) surface marker has been described by Baum and colleagues (1992) after its first identification in mouse hematopoietic stem cells (Spangrude et al. 1988). Thy-1 is involved in numerous non immunological processes such as cellular adhesion, tumor growth, migration and cell death, its activation has been reported to promote T cell activation (Rege et al. 2006). The CD34+ CD90+ subset presents hematopoietic stem cell properties such as long term repopulation capacity. Both CD90- and CD90+ subsets have self-renewal potential. Nevertheless, the CD90+ subset has been shown to generate more efficiently myeloerythroid lineage and lymphocytes B than obtained with the negative fraction (Baum et al. 1992). Indeed, it was reported that the CD34+CD90+ fraction contains five fold more of HSCs than the total CD34+ population (Notta et al. 2011).

The Vascular endothelial growth factor receptor 2 (VEGFR2) also called Kinase insert domain receptor (KDR) can be added to the list of HSC surface markers. Indeed, the KDR+CD34+ cell fraction (0.1 to 0.5% of the total CD34+ population) was enriched in HSCs (Ziegler et al. 1999). Likewise, the Cub domain protein 1 is co-expressed in the CD34+ CD133+ population only in the HSC subset as compared to mature cells (Conze et al. 2003).

As regards negative markers, the CD34+ CD38- population consists of a highly primitive subset of HSCs. The increase of CD38+ cells is correlated with the HSC cell cycle entry or their differentiation (Terstappen et al. 1991). Likewise, CD45RA- CD34+ cells have been reported to be enriched in HSCs (Lansdorp et al. 1990). HSCs are also negative for lineage markers (lin-) which are presented in Table 3.

A lot of other markers have been described in order to purify the HSC subset, we can quote CD49f (Notta et al. 2011), C1qRp, CD93 (Danet et al. 2002), these markers are not commonly used for the moment.

**Table 3. Hematopoietic lineage markers absent from the surface of HSCs.**  
(Adapted from Calloni et al. 2013)

Hematopoietic cell type	Lineage marker
<b>B lymphocytes</b>	CD10, CD19, CD20, CD24
<b>T lymphocytes</b>	CD2, CD3, CD4, CD5, CD7, CD8
<b>Myeloid cells</b>	CD14, CD15, CD16, CD33, CD41
<b>Erythroid cells</b>	CD71, glycophorin A
<b>Natural killer cell</b>	CD56
<b>Granulocyte</b>	CD66b

To put in a nutshell, the primitive Lin-CD34+CD38-CD90+CD45RA-CD133+ cells should be a very interesting subset very enriched in HSCs (Wisniewski et al. 2011). Nevertheless, as we will explain later, the lack of consensus about HSC population markers lead scientists to develop other techniques in order to demonstrate that they really work on the stem cell subpopulation.

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### 1.2.3) PHYSICAL CHARACTERISTICS OF HSC

The low density of HSCs can be used in addition to surface antigen isolation in order to increase purity of HSC isolation. An iso-osmolar percoll density gradient on CD34+ cells enrich by 5- and 10- to 20-fold peripheral blood and bone marrow CD34+ cells respectively. Furthermore, this technique allows to reduce graft versus host disease thanks to the elimination of the contaminating cytotoxic T cells from the CD34+ fraction (Schriber et al. 1995).

## 2) HSC HOMING AND FATE

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### 2.1) THE STEM CELL NICHE

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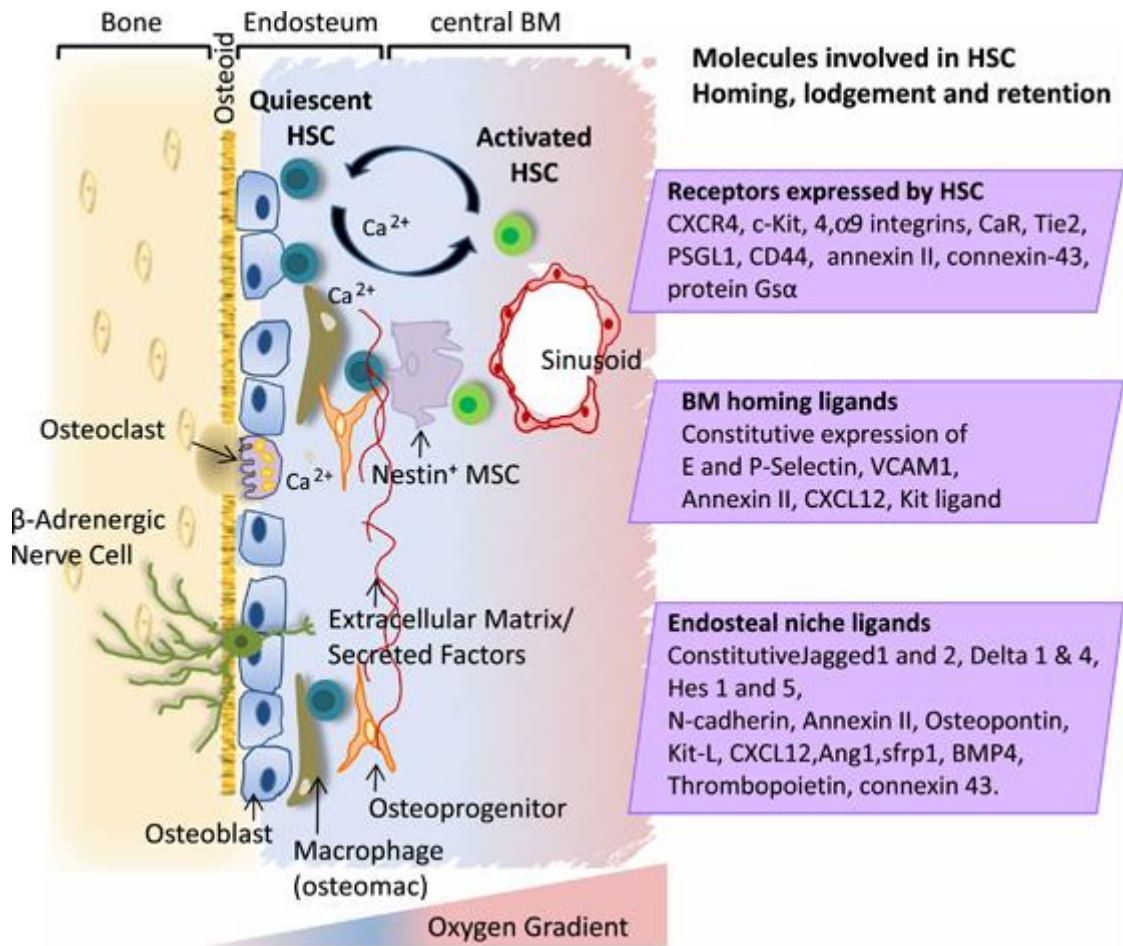
Following Becker and colleagues work (Becker, 1963), Schofield showed that the putative stem cells in the spleen colony forming units were less able to reconstitute hematopoiesis in lethally irradiated animals than bone marrow HSCs

(Schofield, 1978). He hypothesized that a bone marrow niche provides to HSCs a specific environment allowing them to continue their proliferation. In this niche, HSCs could conserve their stem cell properties without differentiating into mature hematopoietic lineages. The HSC niche is composed of osteolineage cells, sinusoidal endothelial cells, mesenchymal stromal and stem cells, sympathetic neurons and extracellular matrix (Krause et al. 2013). This niche controls the quiescence, self-renewal, proliferation, differentiation and migration of HSCs.

At the beginning of the development, HSC niches are in the aorto-gonad-mesonephros region from where they migrate to the placenta, fetal liver and spleen (Morrison et al. 2014). During the second semester of gestation and after birth, HSCs migrate to the bone marrow (Purton et al. 2008). After birth, spleen does not support hematopoiesis in humans, extramedullary hematopoiesis can occur only in case of stress (O'Malley et al. 2005). From birth to the age of five or seven, hematopoiesis takes place in the long bones. In adult, the axial skeleton and the ilium are the major places of hematopoiesis (Purton et al. 2008).

In the bone marrow, HSCs interacts with stroma (adipocytes, reticular cells, macrophages, vascular endothelial cells, smooth muscle cells and mesenchymal stem cells), osteolineage cells and produced cytokines, growth factors and extracellular matrix (Krause et al. 2013) (Figure 13). Each element of the niche microenvironment plays a key role in the HSC niche. Osteolineage cells modulate HSC numbers (Calvi et al. 2003) and maintenance (Krause et al. 2013) and retain them in their niche thanks to calcium sensing (Adams et al. 2006). Mesenchymal stem cells (MSC) are involved in HSC maintenance by secreting CXCL12, SCF, Ang-1, IL-7 and OPN. The MSC nestin expression contributes to retain HSCs in their niches and favors their homing (Mendez-Ferrer et al. 2010). Macrophages contribute to the retention of HSCs by positive regulation of nestin+ MSCs (Chow et al. 2011). The sympathetic nervous system controls the trafficking of HSCs between the niche and the bloodstream (Mendez-Ferrer et al. 2008). The extracellular matrix is a reservoir for growth factors and cytokines that drive cell maturation, trafficking, proliferation and support a lot of HSC functions by interaction with adhesion molecules (Krause et al. 2013). Perivascular cells secrete SCF that support HSC proliferation (Ding et al. 2012).

Of importance, HSCs modulate their niche reciprocally by secreting bone morphogenetic proteins and MSC factors (Krause et al. 2013). Therefore, HSCs are directly involved in their microenvironment development.

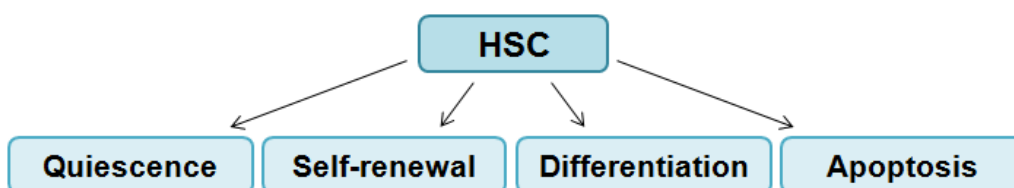


**Figure 13. The bone marrow niche of HSCs.**

(From Lévesque et al. 2010). Schematic representation of the different parts of HSCs niche. In its niche, HSC fate is determined by interactions with endosteal and bone marrow niche cells and specific ligands.

## 2.2) HEMATOPOIETIC STEM CELL FATE

Depending on the hematopoietic conditions, HSCs have several possible fates. They can stay quiescent in their niche, self-renew, differentiate or die through apoptosis (Figure 14).



**Figure 14. HSC cell fate.**

The major part of HSCs stays in a quiescent state in the bone marrow niche. They can also self-renew, differentiate or die through apoptosis.

### 2.2.1) MAJORITY OF HSC ARE IN A QUIESCENT STATE

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On average, up to 70% of HSCs are in the G0 phase of the cell cycle and these cells are responsible for the functional long term repopulation and engraftment in immunodeficient recipient mice (Cheshier et al. 1999) (Passegue et al. 2005). The long term repopulation potential of quiescent HSCs has been demonstrated by serial mice transplantation experiments (Foudi et al. 2009). They act as a reservoir of stem cells mobilized in emergency situations (Wilson et al. 2008). Two models have been designed in order to describe the HSC dynamic. The first one, the clonal succession model implies that only few stem cells would contribute to the blood by successive replacement of quiescent clones. These quiescent cells are sequentially recruited to divide and differentiate and are then replaced by a new set of HSCs. The second one, the clonal maintenance model implies that stem cells contribute indefinitely and stably to the blood (expected to be polyclonal) for the life of the individual (Bystrykh et al. 2012). The correct model is still debated, Bystrykh and colleagues (2012) evocated a large range of possible dynamic scenarios between the two extreme succession and maintenance models depending on the stochastic bone marrow intrinsic and extrinsic signals.

HSC dormance is allowed by specific signals in the bone marrow niche. N-cadherin+ osteoblasts directly induce or support HSC quiescence (Zhang et al. 2003) (Arai et al. 2004). Moreover, multiple stroma cell types and factors have been described to play a role in the induction of HSC quiescence (Zheng et al. 2011).

### 2.2.2) HSC SELF-RENEWAL

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HSC are able of both self-renewal and multipotency. Self-renewal capacity allows to HSC to proliferate without differentiation. On the other hand, differentiation is allowed by the multipotent character of HSCs.

Two different types of cell division can lead to HSC self-renewal: symmetrical and asymmetrical divisions. With symmetrical division, one HSC gives two daughter HSCs, this mechanism expands the HSC compartment. In asymmetrical division, one

daughter is a HSC and the other enters into differentiation, by this mechanism, the HSC pool remains stable (Punzel et al. 2001), (Wu et al. 2007). The major regulation factors for HSC self-renewal have been described (Table 4).

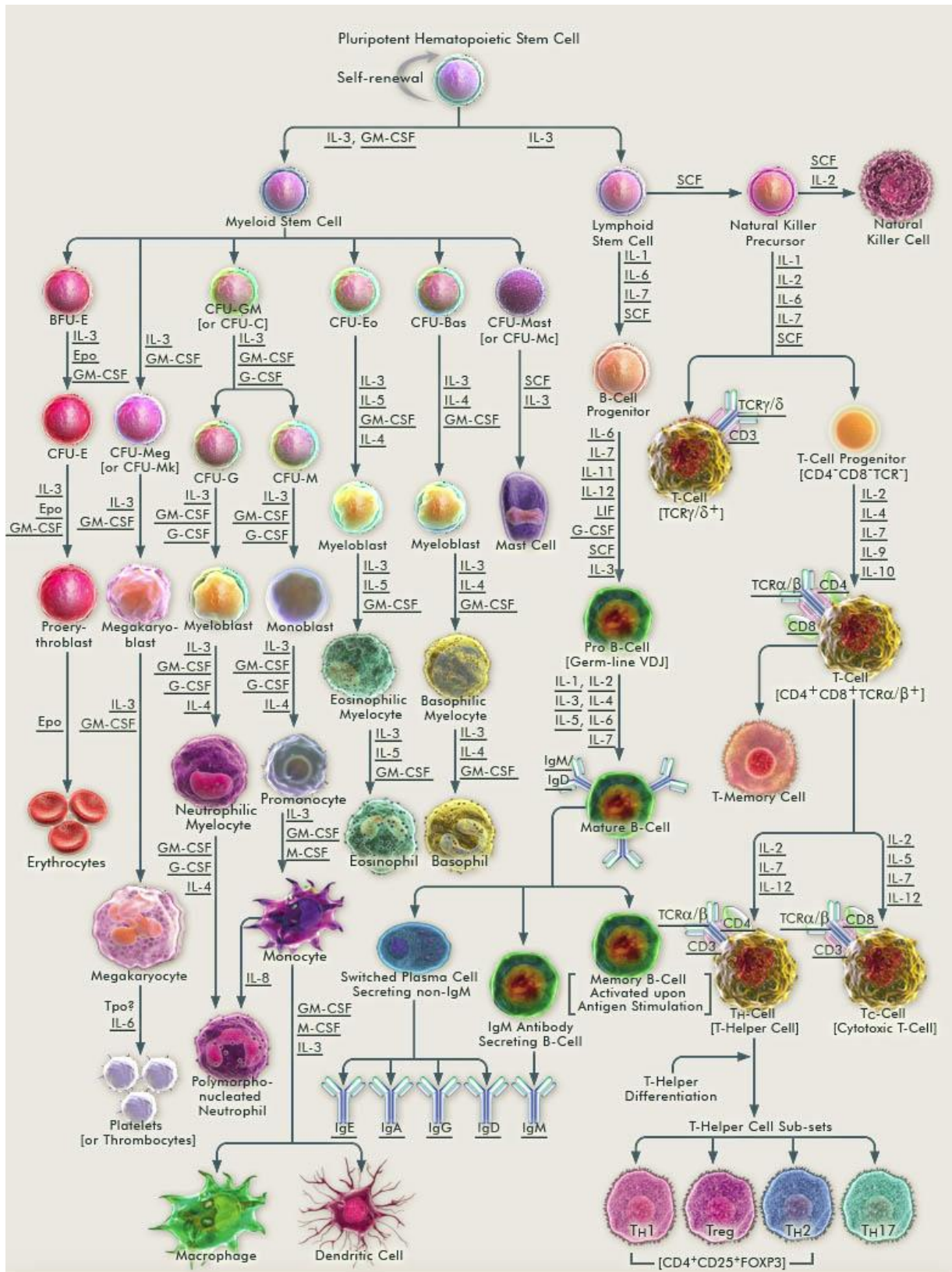
**Table 4. Major regulation factors of HSC self-renewal.**

<b>Regulation factors</b>	<b>Type of regulation</b>	<b>Ref.</b>
<b>Wnt</b>	positive	(Reya et al. 2003)
<b>Notch</b>	positive	(Varnum-Finney et al. 2000)
<b>Hoxb4</b>	positive	(Sauvageau et al. 1995)
<b>Bmi-1</b>	positive	(Park et al. 2003), (Lessard et al. 2003), (Iwama et al. 2004)
<b>Gfi-1</b>	positive	(Hock et al. 2004)
<b>TPO</b>	positive	(Buza-Vidas et al. 2006)
<b>Sonic hedgehog</b>	positive	(Bhardwaj et al. 2001)
<b>Pbx1</b>	positive	(Yamazaki et al. 2009)
<b>CYP26</b>	positive	(Ghiaur et al. 2013)
<b>Retinoic acid</b>	negative	(Ghiaur et al. 2013)
<b>P53</b>	negative	(Liu et al. 2009)
<b>LnK</b>	negative	(Ema et al. 2005)

### 2.2.3) HSC DIFFERENTIATION INTO THE DIFFERENT BLOOD LINEAGES

An average of  $10^{12}$  hematopoietic stem cells is produced every day thanks to the support of the small bone marrow HSC subpopulation (Ogawa et al. 1993). Therefore, an impressive expansion is needed between the quiescent HSCs and the mature non-dividing blood cells. At different stages of HSC differentiation, there are some intermediary progenitors called transiently amplifying cells, which allows to reach the adapted cell number (Diaz-Flores et al. 2006).

When the HSC differentiation process occurs, during the priming phase, the opening of the chromatin structure (Cross et al. 1997) by the way of numerous cell factors induces an upregulation of the lineage specific genes (Table 3) while the transcriptional program of the unselected lineages is down (Enver et al. 1998a), (Orkin et al. 2003). Of importance, cytokines and growth factors are involved in the support of proliferation, maturation and survival of the committed cell rather than they directly influence a certain lineage priming (Enver et al. 1998b). A global view of the HSC differentiation process in the hematopoietic lineages is presented Figure 15 with the major involved cytokines and growth factors.



**Figure 15. Hematopoiesis from HSC.**  
(From Ebioscience manual)

#### 2.2.4) APOPTOSIS

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Apoptosis has been described as an important possible fate for HSCs in regulating their compartment (Domen et al. 2000). Indeed, defects in HSC differentiation or self-renewal can induce hematopoietic insufficiencies and the development of hematopoietic malignancies (Oguro et al. 2007). Cell checkpoint factors, reactive oxygen species, apoptosis specific transcription factors and signaling pathways act in synergy for maintaining the HSC pool (Alenzi et al. 2009).

Of importance, Flt3, a member of the receptor tyrosine kinase family, commonly used in HSC stimulation assays, plays a critical role in HSC homeostasis. Flt3 is required to prevent spontaneous apoptosis and maintain HSC survival. Two other early acting cytokines, SCF and TPO, commonly used for HSC stimulation induce survival but their role in apoptosis prevention remains unclear.

### 3) SOURCES OF HUMAN HSCS AND HSC TRANSPLANTATION

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#### 3.1) BONE MARROW

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CD34+ cells represent 1,1% of the bone marrow mononuclear cells. HSCs from bone marrow present the characteristics described above. Bone marrow stem cell harvesting is possible by puncturing bilateral posterior iliac crests of hipbone on an anesthetized donor,  $2 \cdot 10^8$  total nucleate cells per kg can be harvested (Chen et al. 2013). This technique has been proven safe for more than three decades (Körbling et al. 2001).

Nevertheless, a long recovery time of on average of 15,8 days after donation, 5,9% of acute complications and the induced discomfort for the stem cell donor have been reported (Stroncek et al. 1993). The risk of death related to BM donation is close to 1 in 10000 (Horowitz et al. 2005).



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### 3.2) PERIPHERAL BLOOD

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Peripheral blood is naturally composed of only 0,06% of CD34+ cells, more than 18 times less than in bone marrow (Körbling et al. 2001). The facility of blood harvesting has prompted researchers to develop techniques in order to induce cell migration from marrow to blood. In 1993, the five first syngeneic transplants with peripheral blood mononuclear cells mobilized from bone marrow were reported (Weaver; 1993). Marrow cell mobilization is allowed by 4 to 6 days of granulocyte colony stimulating factor (G-CSF) injections. Likewise G-CSF, Granulocyte macrophage colony stimulating factor (GM-CSF) has been used but it showed less efficiency than obtained with G-CSF (Kopf et al. 2006).

Mobilized peripheral blood stem cells (PBSCs) are collected by a continuous flow cell separator called apheresis (Chen et al. 2013). Two apheresis procedures are possible when respecting a two day interval. G-CSF is commonly well tolerated, however some side effects have been reported such as fatigue, nausea, headache and bone pain (Anderlini et al. 1996). The more serious life threatening reported complication of G-CSF is spontaneous splenic rupture for five healthy donors in the whole literature (Veerappan et al. 2007). The potential risks of CD34+ harvesting and the incidence of acute graft versus host disease (GVHD) after transplantation seems to be comparable with maybe a slight advantage for bone marrow cells regarding chronic GVHD (Cutler et al. 2001). However, the survival rates for cured patients give an advantage to mobilized PBSCs for HSC transplantation (Körbling et al. 2001).

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### 3.3) UMBILICAL CORD BLOOD

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Besides its key role in fetal development, umbilical cord blood (UCB) has been used for numerous clinical applications. Cord blood transplant was first used for treatment in 1972 (Ende, 1972) in a patient suffering lymphoblastic leukemia. UCB has offered an accessible source of HSCs without consequences for the stem cell donor (Broxmeyer et al. 2008). It contains a heterogeneous pool of cells including HSCs, MSCs and embryonic stem cells. UCB has been reported to be enriched in the most primitive subsets, thus it was hypothesized that UCB hosts more immature

HSC population than bone marrow do (McGuckin et al. 2003). Since 2007, the number of UCB transplants exceeded the number of BM transplants. Moreover, HSC transplantation with UCB cells induces less acute and chronic GVHD as compared to bone marrow's HSCs. Although this source of HSCs presents numerous advantages, the limited number of collected cells per UCB unit and the longterm engraftment of these cells needs to be improved in order to consolidate its promising prospects for clinical use.

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### 3.4) EMBRYONIC AND INDUCED PLURIPOTENT STEM CELLS

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Recently, CD34+ hematopoietic precursors have been produced from embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) (Ledran et al. 2008), (Choi et al. 2009). Although the capacity of these cells to differentiate *in vitro* into most of hematopoietic lineages has been demonstrated, their potential use in transplantation and the safety of this procedure need more investigation (Tripura et al. 2013). Nevertheless, iPSCs, derived from blood cells of patients with nonmalignant hematological diseases could be a great advance in the field of HSC transplantation notably in terms of donor compatibility (autologous cells) and increased stem cell numbers through expansion (Ye et al. 2009).

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### 3.5) HSC TRANSPLANTATION (HSCT)

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The first HSC transplantation was performed in 1959 (Thomas, 1959) for the treatment of a patient who suffered from an end-stage leukemia. This patient was treated by an infusion of her twin's marrow after a total irradiation. The identification and typing of HLA system (Human leukocyte antigen) rose the possibility of allogeneic transplantations (Gatti, 1968). Since the 1980s, patient preparations before transplantation evolved with an addition of cyclophosphamide to irradiation protocols or a replacement of this irradiation by busulfan (Thomas, 1977). Adjustments and reductions of these regimens depending on the disease treated and conferred reduced toxicity of this procedure.

As regards HSC sources for transplantation, PBSCs have replaced bone marrow cells for autologous and most of allogeneic transplantations (Copelan et al.

2006). Peripheral blood cells induce more rapid hematopoietic reconstitution despite an increases of the chronic GVHD rates because of the highest proportion of T cell lineage in the progenitor subset (Cutler et al. 2001).

In case of an urgent necessity of HSCT or an absence of suitable donor, banked UCB HSCs are a valid alternative source of HSCs. These UCB HSCs were firstly employed in a Fanconi's anemia patient (Gluckman et al. 1995). UCB HSCT requires less HLA matching; indeed, mismatched UCB cells cause less GVHD while these cells maintain their capacity of struggling against leukemia by the graft versus leukemia effect (Wagner et al. 2002). Combinations of different donors of UCB play a role in engraftment improvement (Barker et al. 2005).

Although HSCT was firstly a last resort treatment, it is now commonly used in the treatment of many diseases as described in Table 5. The consequences of the patient preparation regimens notably regarding the immunodeficiency phase will be treated in a next part.

**Table 5. Major diseases treated by HSCT.**

Type of HSCT	Type of disease	Diseases
<b>Autologous transplantation</b>	<b>Cancers</b>	Multiple myeloma, lymphomas, acute myeloid leukemia, neuroblastoma, germ cell tumors, ewing sarcoma, retinoblastoma, rhabdomyosarcoma, ovarian cancer.
	<b>Other diseases</b>	Autoimmune disorders (systemic lupus erythmatous, systemic sclerosis), amyloidosis.
<b>Allogenic transplantation</b>	<b>Cancers</b>	Acute and chronic myeloid and lymphoblastic leukemias, juvenile chronic myeloid leukemia, multiple myeloma, lymphomas, myeloproliferative syndromes.
	<b>Other diseases</b>	Myelodisplastic disorders, red cell aplasia, aplastic anemia, paroxysmal nocturnal hemoglobinuria, fanconi anemia, thalassemia major, sickle cell anemia, severe combined immunodeficiency, wiskott-aldrich syndrome, hemophagocytic lymphohistiocytosis, metabolism disorders, epidermolysis bullosa, severe congenital neutropenia, Shwachman-Diamond syndrome, Diamond-Blackfan anemia, leukocyte adhesion deficiency, osteogenesis imperfect, chronic granulomatous disease.

## 4) IDENTIFICATION OF MODIFIED HSC

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As we previously explained, surface markers and other HSC purification techniques remains insufficient to prove that a hematopoietic cell is a stem cell (Sato et al. 1999). Indeed, other functional essays are required to ensure that the identified population is most probably a HSC.

### 4.1) IN VITRO ASSAYS

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*In vitro* assays investigate the capacity of hematopoietic progenitor cells to self-renew and to differentiate.

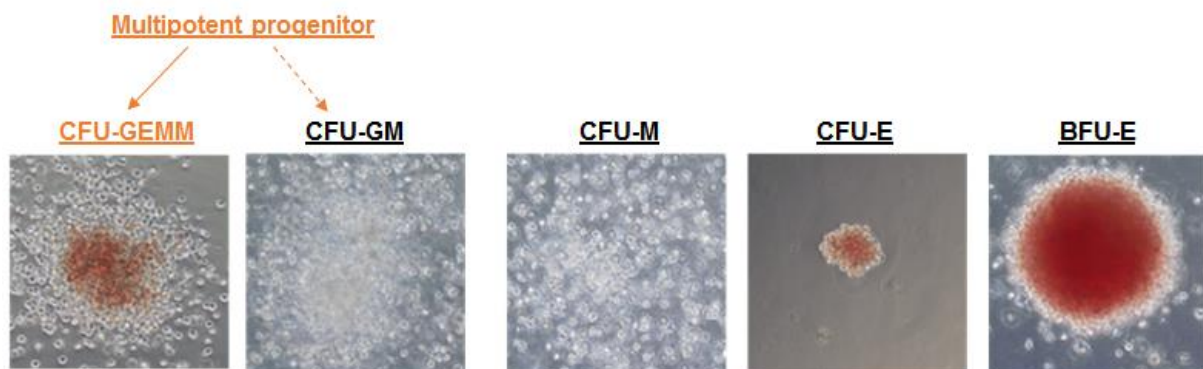
#### 4.1.1) COLONY FORMING CELL ASSAY

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CFC assays can be performed in order to identify hematopoietic progenitors which present limitations in self-renewal or differentiation. A semi-solid medium of methylcellulose supplemented with cytokines and nutrients allows progenitors to produce some various colonies after a two to three weeks culture period (Miller et al. 2008). Myeloid multipotential progenitors are able to give the following colony forming units (CFU):

- CFU-granulocyte, erythroid, monocyte/macrophage, megakaryocyte (CFU-GEMM)
- CFU- granulocyte, monocyte (CFU-GM)

If the studied cells are lineage restricted progenitors, only some of the following colonies will be observed under the microscope: CFU-monocytes (CFU-M), CFU-granulocyte (CFU-G), Burst forming unit erythroid (BFU-E), CFU-erythroid (CFU-E) and CFU-pre B cell. Figure 16 presents major CFUs.



**Figure 16. Colony obtained by a CFC assay.**

(Adapted from Cellular Dynamics international manual) Progenitor cells are cultured 10-12 days in a semi-solid methylcellulose medium supplemented with cytokines and nutrients. If progenitors are multipotents, CFU-GEMM are obtained or at least CFU-GM. If CFU-M, CFU-E, BFU-E or other colonies are preferentially isolated, it means that studied progenitor cells are not multipotents.

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#### 4.1.2) LONG TERM CULTURE INITIATING CELL ASSAY

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The long-term culture initiating cell assay (LTC-IC) allows to estimate the capacity of hematopoietic progenitors to produce myeloid progeny thanks to a 5-12 weeks culture system (Liu et al. 2013). A feeder layer is obtained by coating adherent mouse bone marrow mesenchymal or stromal cells mitotically inactivated by irradiation. This feeder layer is genetically engineered to produce granulocyte-colony stimulating growth factor, human interleukin-3 (IL-3) and human stem cell factor. It provides a bone marrow like environment that secretes stromal substrates (Hogge et al. 1996). The long term colony-initiating cell (LTC-IC) is an immature cell defined by its capacity to produce colony forming cells (CFCs) after a long time of culture superior to five weeks on the above stroma in a minimal cytokine cocktail. During this time, most of the short term CFCs from the initial pool of progenitor cells disappear and the new produced CFCs are inevitably derived from some LTC-ICs.

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#### 4.2) IN VIVO DETERMINATION OF LONG TERM RECONSTITUTION CAPACITY OF TRANSDUCE CELLS

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Despite the practical interests of *in vitro* assays to evaluate differentiation and proliferation of some tested cells independently from their self-renewal capacity, *in vivo* transplantation is a gold standard for assaying stem cell properties. Although competitive repopulation unit assays can be performed by transplanting tested mouse cells in a congenic recipient, this is not possible for evident reasons in humans. Although non-human primates may offer an alternative, for evident ethical and practical reasons, there was a need for small animal models to estimate HSC capacity.

Therefore, xenogenic transplantation models have been developed using different strains of immunodeficient mice. The first hematopoietic progenitor transplantations were developed in severe combined immunodeficiency mice (SCID) (McCune, 1988) and beige/nude/xid (BNX) (Kamel-Reid, 1988) mice, which display defects in their lymphoid lineages.

More specifically, SCID mice derived from the BALB/c C.B.-17 strain (Leblond et al. 1997) carry a DNA dependent protein kinase (DNA PK) mutation that impairs rearrangement of immunoglobulin and T cell antigen receptor genes. In spite of the high level of NK cells in these mice, a part of human hematopoietic cells manages to engraft (Meyerrose et al. 2003). Nevertheless, human T cells derived from human HSC engraftment and hematopoietic humanization remain anergic in mice and thus less interesting for numerous use of this model (Tary-Lehmann et al. 1992).

The BNX mice were obtained by a triple mutation resulting in the reduction of the number of natural killer cells and impaired B and T lymphocytes functions. The 2-year lifespan of this strain and its ability to quickly recover from sublethal radiation are two characteristics of BNX mice, which makes them superior as a model. Nevertheless, they are difficult to breed because of the necessity to use females heterozygous for the nude allele and the variability of the defects in their B cell lineage. Because of these issues, each recipient mouse must be screened before to be transplanted, a procedure that induces important cost and is time consuming.

An improved immunodeficient mouse, the nonobese diabetic SCID strain (NOD/SCID) has been developed and presents a highest capacity of human cell engraftment because of improved defects in NK cells, macrophage function and complement system (Prochazka et al. 1992), (Shultz et al. 1995). Moreover, these mice are easy to breed in contrast to the BNX strain. However, in these mice the human immune system reconstitution of the T-cell lineages is not complete (Meyerrose et al. 2003) and mouse NK cells remain active which eventually leads to rejection of the graft (Doulatov et al. 2012).

As a consequence, during the past decade, NOD/SCID mice with either truncation (NOG) or deletion (NSG) in the IL-2 receptor common gamma chain were developed, resulting in mice with a complete absence of murine B, T and NK lineages. In parallel, Balb/c Rag<sup>-/-</sup>γc<sup>-/-</sup> mice have been developed. A comparison of these recent mouse strains showed an advantage for female NSG mice in case of small number of transplanted HSCs (McDermott et al. 2010).

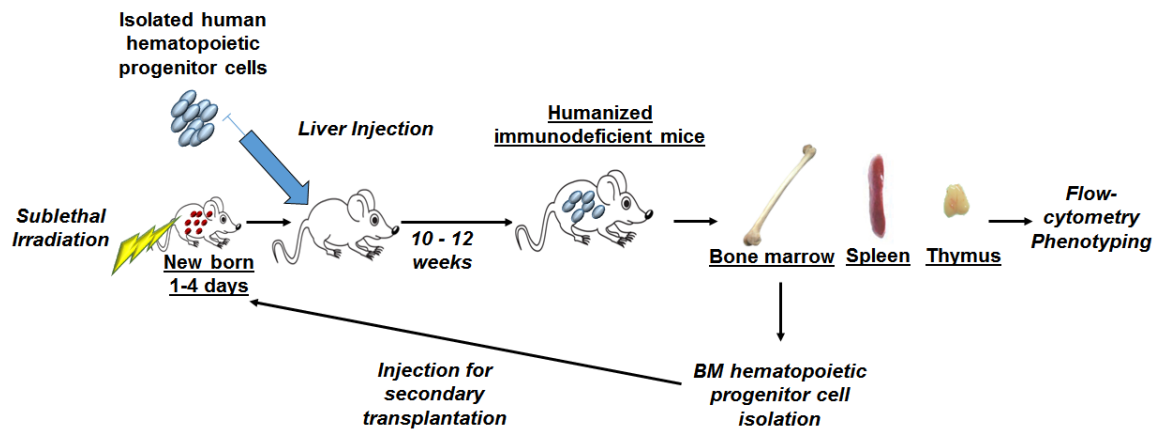
Despite the fact that these models are the standard in hematopoietic progenitor transplantation, it was shown that the diversity/differentiation of human cells and their functionality are still below normal human ranges (Chen et al. 2009), (Shultz et al. 2007). Especially, myeloid differentiation is now very efficient with for instance almost no platelet production.

Thus, new immunodeficient mouse models have been recently developed in order to provide a better human environment that facilitates HSC engraftment and development. Two strategies have been explored: the mouse secretion of human growth factors and the improvement of cell-ligand or cell-cell interactions (Drake et al. 2012). For example, NSG-SGM3 mice able to secrete human SCF, GM-CSF and IL-3 (Billerbeck et al. 2011), Flavell and colleagues reported human immune system reconstitution, diversity and functionality increases by developing knock-in mice for human TPO (Rongvaux et al. 2011), GM-CSF, IL-3 (Willinger et al. 2011) and M-CSF (Rathinam et al. 2011). On the other hand, the engineering of HLA-A2 (Shultz et al. 2010) or NSG HLA-DR4 (Danner et al. 2011) transgenic mice expressing human major histocompatibility complex (MHC) genes have been reported to improve T cell functions in humanized mice.

Summarizing, new immunodeficient mouse models hold promises in the field of humanized models. Until now, the NSG mouse model offers large possibilities to verify stem cell properties of a pool of hematopoietic progenitors.

Figure 17 presents the current protocols applied to identify HSCs which are able to reconstitute the whole human hematopoietic lineage in mice. Immunodeficient new born mice are sub-lethally irradiated at mild radiative doses of three gray (Coulombel et al. 2004) and hematopoietic progenitor cells are injected in their liver (Traggiai et al. 2004). The progeny of long term repopulating cells are evaluated ten to twelve weeks after by identifying human CD45+ cells in the mouse bone marrow, spleen and thymus. The reconstituted hematopoietic lineages are characterized by cytometry in order to demonstrate that injected cells contained long term repopulating cells. Bone marrow isolated hematopoietic progenitor cells can be re-injected in secondary recipient mice to prove the self-renewal capacity of the cells (Broxmeyer

et al. 2008). Thanks to this protocol, the multipotency and the self-renewal of studied cells can be explored in order to confirm their stem cell status.



**Figure 17. Schematic representation of the reconstitution of an immunodeficient mice model with human hematopoietic progenitor cells.**

Newborn immunodeficient mice received sublethal irradiation and were subsequently injected with hematopoietic progenitor cells into the fetal liver. After 10 to 12 weeks of reconstitution, the mice were sacrificed and analyzed for human cell engraftment and human cell phenotyping in the bone marrow, spleen and thymus. At this stage, the multipotency of the hematopoietic progenitor cells is evaluated. Bone marrow hematopoietic progenitors are isolated and re injected in secondary recipient mice in order to explore their self-renewal potential.



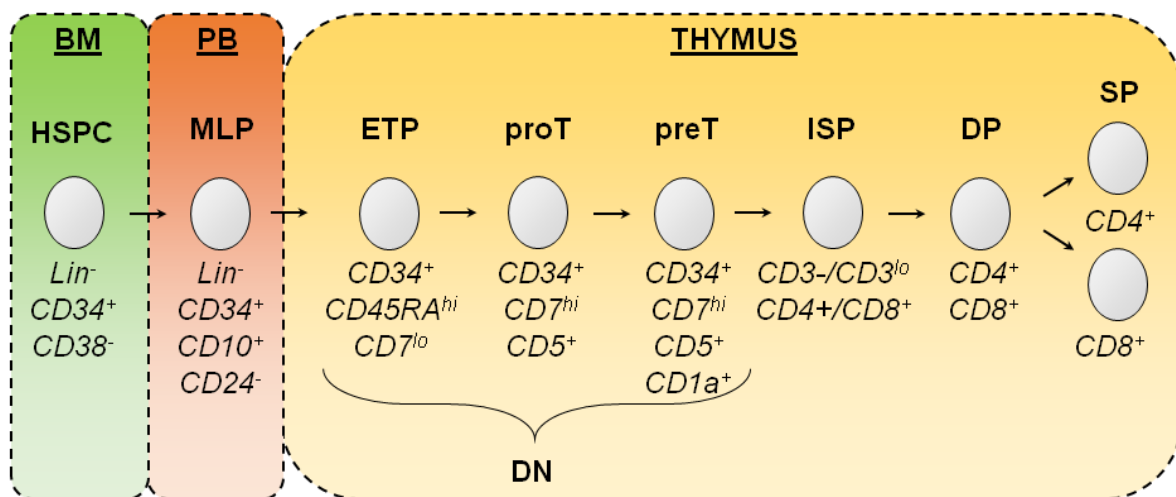


## PART 3 – HUMAN T CELL LINEAGE

### 1) HUMAN T CELL DEVELOPMENT

#### 1.1) EARLY STAGES OF T CELL DEVELOPMENT AND THYMOPOIESIS

The generation of T cell repertoire occurs during thymopoiesis by the differentiation of precursors, which migrate from bone marrow to thymus. The thymus provides a microenvironment that guides the T cell repertoire development stages to yield mature, self-tolerant mature T cells (Shah et al. 2014). Of note, the human thymus is also a host tissue for B cell progenitors (Weerkamp et al. 2005). Likewise HSC, most of our knowledge on T cell development has been obtained from genetically modified mouse. However, human and mouse T cell development display differences regarding the molecular pathway involved, the lineage phenotype and the T cell potentials (Den Braber et al. 2012). Here, we will focus on human T cell development because of our interest in using these cells in a therapeutic setting, Figure 18 describes human thymopoiesis.



**Figure 18. Early stages of T cell development.**

Schematic representation of human stem cell migration to the thymus and thymocyte development. For each differentiation stage phenotypic surface markers are indicated. HSPC= human stem progenitor cells; MLP = multipotent early lymphocyte progenitor, ETP= early thymocyte progenitor, ISP= immature single positive T cell, DP = double positive, SP = mature single positive T cell.

### 1.1.1) T CELL LINEAGE ENGAGEMENT

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Human multipotent early lymphoid progenitors (MLP) are included in the Lin-CD34<sup>+</sup>/CD38<sup>-</sup> (Terstappen et al. 1992), (Gore et al. 1991), (Van Dongen, 1985) bone marrow subpopulation. They preferentially differentiate into lymphoid lineages and are presumed to contain the thymic progenitors. The MLP capacity to differentiate either in T or B lineages depends on the age of the individual. The number of fetal MLPs engaged towards T and B lineage is balanced, whereas their T lymphoid potential decreases after birth and during childhood.

Six and colleagues (Six et al. 2007) proposed a common lymphoid precursor (CLP) population characterized by the Lin-CD34<sup>+</sup>CD10<sup>+</sup>CD24<sup>-</sup> expression. The CD24<sup>+</sup> population has been reported to be B-lineage restricted, whereas the CD10<sup>+</sup>CD24<sup>-</sup> subset presented B, T and NK cell potentials.

The study of human fetal tissue identified a bone marrow CD34<sup>+</sup>CD45RA<sup>hi</sup>CD7<sup>+</sup> population never found in the hematopoietic fetal liver. This population is able to enter thymus parenchyma in *ex vivo* colonization assays (Haddad et al. 2006) and consists in the early T-lineage progenitors (ETP). Human ETPs are comprised in the CD4/CD8 double negative subpopulation. ETP development occurs by upregulation of CD7, CXCR4, CD5 and downregulation of CD10. The proT stage is identified by the following expression profile: CD34<sup>+</sup>/CD45RA<sup>+</sup>/CD7<sup>+</sup>; the progressive expression of CD5 allows the distinction between the CD5<sup>-</sup>proT1 subset and the CD5<sup>+</sup> proT2 subset.

### 1.1.2) T LINEAGE COMMITMENT AND $\beta$ -SELECTION

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At the preT stage, the appearance of CD1a induces the T-cell commitment and the loss of capacity to differentiate into NK cells or DCs. This involves the initiation of T-cell receptor (TCR)  $\beta$ ,  $\gamma$  and  $\delta$  gene rearrangements (Blom et al. 1999). CD1a<sup>+</sup>CD34<sup>+</sup> cells evolve into CD4<sup>+</sup>CD8<sup>+</sup>  $\alpha\beta$ <sup>+</sup> cells firstly by CD4 upregulation, followed by CD8  $\alpha$ <sup>+</sup> and then CD8  $\beta$ <sup>+</sup>. TCR  $\beta$ -selection is reported to occur in the CD4<sup>+</sup>CD8<sup>+</sup>  $\alpha\beta$ <sup>-</sup> and CD4<sup>+</sup>CD8<sup>+</sup>  $\alpha\beta$ <sup>+</sup> double positive (DP) sub-populations (Galy

et al. 1993), (Spits et al. 1998). Some authors hypothesized that the TCR $\beta$  V-D-J recombination can occur in the CD4<sup>+</sup> immature single positive cells (ISP). ISP cells directly follow preT cells expressing CD4 but not yet CD8 (Vicente et al. 2010) and contain precursors for both the  $\alpha\beta$  and  $\gamma\delta$  T-cell lineages (Spits et al. 2002).

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### 1.1.3) POSITIVE SELECTION

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After TCR rearrangements,  $\alpha\beta$  T cells need to interact with low affinity with self-peptides complexed with MHC antigens expressed on thymic epithelial cells to survive. After this positive selection CD3<sup>+</sup>/-CD4<sup>+</sup>CD8<sup>+</sup> double positive (DP) thymocytes differentiate into single positive CD4<sup>+</sup> and CD8<sup>+</sup> cells thanks to CD69 upregulation, RAG1 downregulation (Brandle et al. 1994), (Vanhecke et al. 1995) and other numerous cell marker variations (Spits et al. 2002).

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### 1.1.4) RECENT THYMIC EMIGRANTS

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After functional maturation, CD69 T cell expression decreases and T cells become CD45RO<sup>-</sup>/CD45RA<sup>+</sup> (Vanhecke et al. 1995). Recent thymic emigrants (RTE) can be identified by their expression of CD3-TCR, CD45RA, CD27 and CD62-L (Spits et al. 2002). CD4<sup>+</sup>CD31<sup>+</sup> (Kimmig et al. 2002) and CD8<sup>+</sup>CD103<sup>+</sup> (McFarland et al. 2000) naïve T cells have not yet undergone cell division.

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## 1.2) ROLE OF THE NOTCH1/DL-4 INTERACTION IN THYMOPOIESIS

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Early T cell development depends on the interaction of thymocytes with the thymic microenvironment and essentially relies on the Notch-Ligand signaling pathways. Numerous Notch-Delta like ligands interactions have been reported to regulate and support thymopoiesis. We will focus on Notch-1/DL-4 interactions because of its importance in thymopoiesis, and the fact that recently new *in vitro* protocols were developed relying on Notch-1/DL-4 signaling supporting HSC differentiation into T progenitors (see Part 3 – 4.3)

Notch1 has been reported to be a major factor that induces the choice between T and B cell differentiation of MLPs (Radtke et al. 1999). Notch1 interplays with other

major factors such as IL-7, Wnt and CXCR4 to guide thymopoiesis until  $\beta$ -selection. Members of the Delta-like family are the physiological Notch1 ligands (Jaleco et al. 2001). DL-4 actively participates in the induction and the support of T cell commitment and maturation from double negative to double positive cells exclusively via interaction with Notch1 (Besseyrias et al. 2007). This interaction can promote TCR $\beta$  rearrangement and progression to the double positive stages. The exclusive interaction between DL-4 with Notch 1 is explained by the tissue specific compartmentalization of Delta like family members, this allows specificity in cell fate determination by the Notch signaling pathways (Besseyrias et al. 2007).

## 2) ADULT PERIPHERAL BLOOD T CELL PHENOTYPE, FUNCTION AND SIGNALLING

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After T cell maturation in thymus, mature CD4+ and CD8+ SP T cells migrate to the peripheral blood. The PB T cell pool can be subdivided into different T cell subpopulations.

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### 2.1) PERIPHERAL BLOOD T CELL SUBPOPULATIONS

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Recent thymic emigrant CD4+ and CD8+ single positive T cells migrate from thymus to peripheral blood and lymphoid organs and mature in their respective subsets.

#### 2.1.1) CD4+ T CELL SUBSETS

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CD4+ T cell subsets play an essential role in regulating immune response through cytokine secretions and activation of cells from the innate immune system such as B lymphocytes and cytotoxic T cells. CD4+ T cells also mediate the suppression of immune reaction and inflammation.

##### 2.1.1.1) NAÏVE CD4+ T CELLS

After they interact with antigen-MHC complexes, naïve CD4+ T cells are activated and are induced into differentiation mediated by the cytokine microenvironment. Naïve CD4+T cells can be identified by the following phenotype: CCR7+CD45RA+CD27+CD28+ (Okada et al. 2008).

Next to the well-known T-helper 1 (Th1) and 2 (Th2) cells, T-helper 17 (Th17), T-helper 9 (Th9), follicular helper T cell (Tfh), induced T regulatory cells (iTreg) and regulatory type 1 cells (Tr1) constitute the CD4+ T cell subsets. Differentiation into each of these subtypes depends on cytokine secretion, transcription factors and antigen concentrations (Luckheeram et al. 2012; Table 6).

**Table 6. Differentiation factors and functions of each CD4+ T cell subpopulations.**  
(Jabeen et al. 2012), (Whiteside et al. 2012), (Luckheeram et al. 2012)

CD4+ T cell subset	Differentiation factors	Functions and involvements	Secretion
Th1	IL-12, IFN $\gamma$ , T bet, STAT1/4, Runx 3, Eomesodermin, Hlx	Elimination of intracellular pathogens and organ-specific auto-immunity	IFN $\gamma$ , lymphotoxin $\alpha$ , IL-2
Th2	IL-4, IL-2 GATA3, STAT3/5/6, Gfi-1, c-Maf, IRF4	Immune response against extracellular parasites and development of allergic diseases such as asthma.	IL-4/5/9/13/10/25, amphiregulin
Th17	IL-6, IL-21, IL-23, TGF-b ROR $\gamma$ t, STAT3, ROR $\alpha$ , Runx1, Batf, IRF4, AHR	Immune response against extracellular bacteria and fungi, generation of auto-immune diseases.	IL-17A/17F/21/22
Th9	TGF- $\beta$ , IL-4 IRF4	Pro-inflammatory, Development of allergic diseases such as asthma.	IL-9
Tfh	IL-6, IL-21 Bcl6, STAT3	Humoral immunity mediation, Plasmocytes formation, long live B memory cell development	IFN $\gamma$ , IL-4/10
iTreg	TGF- $\beta$ , IL-2 FOXP3, Smad2/3, STAT5, NFAT	Maintenance of immunologic tolerance to self and foreign antigens, protection against immunopathologies, dual role in cancer.	IL-10/35, TGF- $\beta$ , prostaglandin E2, adenosine
Tr1	IL-27, IL-10 c-Maf, AHR		

### 2.1.1.2) MEMORY CD4+ T CELLS

The CD4+ memory T cell generation begins in secondary lymphoid organs by the binding of the T cell antigen receptor (TCR) on naïve T cells to major

histocompatibility class II (MHCII) on antigen presenting cells (APCs) (Davis et al. 1990). Through CD28 and other costimulatory signaling, naïve CD4<sup>+</sup> T cells divide and become effector cell lymphoblasts (Jenkins et al. 2001), (Swain et al. 1990) as described previously. On average 90% of these effector cells die during a one to two week contraction phase. Surviving cells become long-lived memory cells. The major part of these cells stay quiescent in absence of activation by a MHCII ligand (Seder et al. 2003). Memory CD4<sup>+</sup> T cell is a heterogeneous population that contains at least effector memory (T<sub>EM</sub>) and central memory T cell (T<sub>CM</sub>) subpopulations (Sallusto et al. 2004). These memory T cells can be characterized by the expression of CD45RO. Nevertheless, the initial pathway of activation described by Sallusto and colleagues (Sallusto et al. 2004) (Iezzi et al. 2001): naïve CD4<sup>+</sup> T cell → CD45RO<sup>+</sup> noneffector/T<sub>CM</sub> → effector and T<sub>EM</sub> cells is still discussed, some authors suggesting that this pathway is not unidirectional and that T<sub>EM</sub> could be produced at different time of CD4<sup>+</sup> T cell activation (Song et al. 2005). CD4<sup>+</sup> memory T cell subsets and their characteristics are represented Figure 19.

#### 2.1.1.2.1) CENTRAL MEMORY CD4<sup>+</sup> T CELLS (T<sub>CM</sub>)

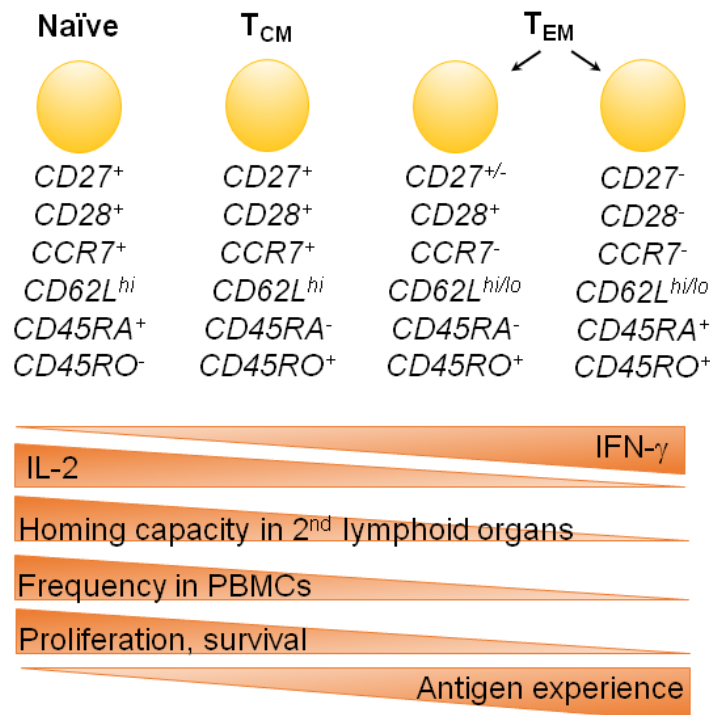
CD4<sup>+</sup> T<sub>CM</sub> cells migrate to the lymph nodes and mucosal lymphoid organs through their expression of CD62-L and CCR7. Indeed, T<sub>CM</sub> CD4<sup>+</sup> cells present the following phenotype: CD28<sup>+</sup>CCR7<sup>+</sup>CCR5<sup>-</sup>. CXCR5, CXCR3 and CCR4 are additionally expressed on non-polarized, preTh1 and preTh2 T<sub>CM</sub> subsets respectively.

CD4<sup>+</sup> T<sub>CM</sub> cells do not produce specific cytokines after TCR stimulation, they secrete IL-2 that allows their expansion and acquire effector lymphokine production at different step of activation (Sallusto et al. 2004), (Song et al. 2005).

#### 2.1.1.2.2) EFFECTOR MEMORY CD4<sup>+</sup> T CELLS (T<sub>EM</sub>)

CD4<sup>+</sup> T<sub>EM</sub> cells can migrate to sites of inflammation by expressing homing receptors on their surface. After being activated by TCR stimulation, they are able to secrete IFN- $\gamma$ , IL-4, IL-5 and IL-17 which are microbicidal or pro-inflammatory cytokines (Pepper et al. 2011).

CD4+ T<sub>EM</sub> cells are characterized by the expression of CD28 and CCR5 and the absence of CCR7 (Pitcher et al. 2002). The more T<sub>EM</sub> cells are antigen experienced, the more they secrete IFN- $\gamma$ , the less they proliferate and survive.



**Figure 19. Phenotypes and characteristics of human CD4+ memory T cell subsets.**

Adapted from (Appay et al. 2008) and (Seder et al. 2003). CD4+ central memory T cells (TCM) express CCR7 and CD45RO markers, they are characterized by a long lifespan, their capacity to migrate until secondary lymphoid organs, their secretion of IL-2 and thus their expansion and frequency in peripheral blood mononuclear cells (PBMCs). Effector memory T cells are CCR7-, they can express both CD45RA and CD45RO, they secrete IFN- $\gamma$  in relation to their antigen experience.

## 2.1.2) CD8+ T CELLS

### 2.1.2.1) FROM CD8+ NAÏVE TO MEMORY T CELLS

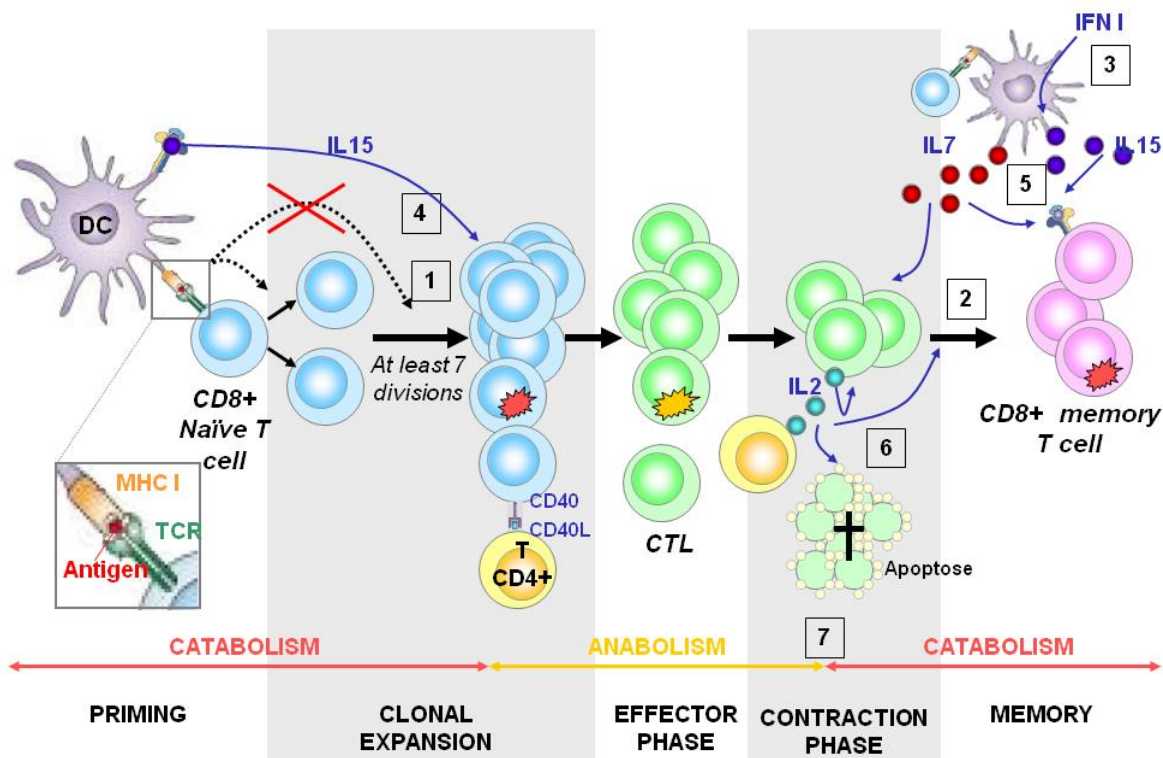
Type 1 immune responses are induced in case of virus or intracellular bacteria infection. During these responses, CD8+ T cells differentiate into cytotoxic lymphocytes (CTLs) that secrete cytokines such as IFN- $\gamma$  and TNF (Tumor necrosis factor). CTLs kill infected cells through perforin and granzyme pathways. CD8+ T cells are an important part of the adaptive immunity against tumors and intracellular pathogens.

Adaptive immune response consists in distinct phases: the recognition of antigen, the activation of lymphocytes with a clonal expansion, the effector phase with the elimination of antigen, the contraction phase with the return to homeostasis



and the memory cell survival (Figure 20). During the infection, a dendritic cell (DC) presents an antigen to naïve T lymphocytes by a MHC class I / TCR interaction. This first exposure triggers the memory T cell priming (Jabbari et al. 2006), (Mempel et al. 2004). The antigen amount affects the number of recruited CD8+ T cell (Prlic et al. 2006), (Prlic et al. 2007) which need to divide at least seven times in order to survive (Kaech et al. 2001). It is an “all or nothing” phenomenon where cells which divide several times initiate a developmental program to differentiate in a first time in cytotoxic T cells (CTLs).

After the CD8+ T cell activation, a transient expression of CD40 has been observed. Similarly to the B cell memory mechanism, CD4+ T cells help CD8+ memory T cell generation thanks to a CD40L/CD40 interaction (Hamilton et al. 2006), (Tanchot et al. 2003). Although a CD8+ T cell priming might be possible without CD4+ T cell help, this help is necessary for a robust secondary response. Indeed, in absence of CD4+ T cell, the pro-apoptotic factor TRAIL is upregulated inducing CD8+ T cells to die (Surh et al. 2006).



**Figure 20. Schematic representation of CD8+ T cell differentiation.**

1) After priming, at least 7 divisions independent from antigen activation are needed for CD8+ T cell survival. 2) CD8+ T cell survival during the contraction phase is allowed by  $\gamma$ c cytokines. 3) Type I IFN induce IL-15 production and secretion by dendritic cells (DCs), 4) IL-15 favors clonal expansion of CD8+T cells and 5) associated to IL-7 it support the CD8+ memory T cell survival. 6) IL-2 regulates the selection of memory CD8+ T cells during the contraction phase.7) Metabolism switch between anabolic and catabolic stages of CD8+ T cells regulate the CD8+ T cell differentiation.

## 2.1.2.2) MEMORY CD8+ T CELLS

### 2.1.2.2.1) MOLECULAR OVERVIEW OF MEMORY CD8+T CELL GENERATION

The generation of CD8+ memory T cells is an area of intense research and are better characterized than CD4+ memory T cells. Figure 20 represents the major signalling pathway involved in CD8+ memory T cell generation.

The number of CD8+ memory T cells generated is correlated with the CTLs number at the immune response peak and CD8+ T cells are able to continue dividing in the absence of a continual antigenic exposure (Kaech et al. 2001), (Jabbari et al. 2006), (Prlic et al. 2007).

Growth factors and soluble inflammatory factors like cytokines are involved in CD8+ memory T cell generation. First, IL15 contributes to the clonal expansion and later allows the maintenance of CD8+ T cell memory thanks to a transpresentation by DCs to CD8+ T cells. It also upregulates Bcl2 which is indispensable for memory maintenance (Villinger et al. 2004), (Burkett et al. 2003). Secondly, IL7 is necessary for the formation of a stable pool of CD8+ memory T cells, it allows survival and maintenance of CD8+ memory T cells (D'Cruz et al. 2009), (Schluns et al. 2003). Finally, the role of IL-2 is still discussed. Indeed, IL-2 is necessary for a robust secondary response but it upregulates pro-apoptotic factors such as Fas, Fas-L and down regulates anti-apoptotic factors like Bcl2, and Bcl-Xl. CD4+ T cells seems to be the main producer of the IL-2 involved in CD8+ memory T cell generation (D'Cruz et al. 2009), (Blachere et al. 2006), (Osborne et al. 2010), (Williams et al. 2006). IFN $\gamma$  works with IL2 in driving the contraction phase. It might regulate the transition between the clonal expansion and the contraction phase by an induction of IL15. Besides, type I IFN (IFN $\alpha/\beta$ ) are potent inducers of IL15 and facilitate memory T cell CD8 generation upregulating the IL15 production and the IL2 mRNA expression in activated DCs (Agarwal et al. 2009), (Xiao et al. 2009).

Interestingly, some authors describe a metabolic switch in CD8+ memory T cell generation. Indeed, naïve cells present a catabolic metabolism. Then, cytokines

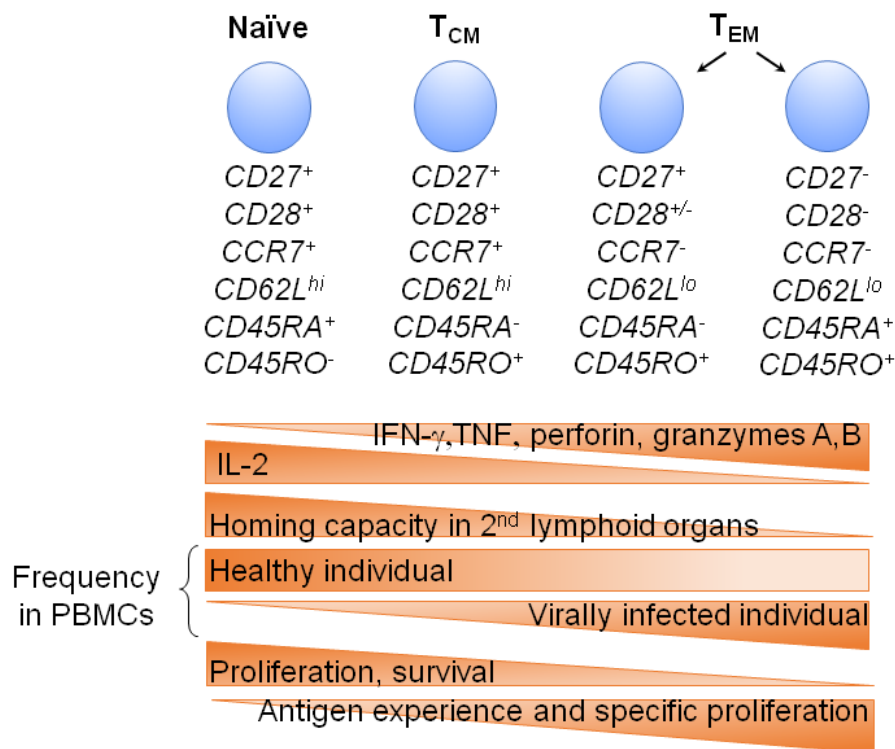
and antigen presentation induce an anabolism activation by a protein-kinase enzyme mTORC1 (mammalian target of rapamycin complex 1) of effector T cells. The reversal switch from anabolism to catabolism takes place during CD8+ memory T cell generation (Araki et al. 2009), (Pearce et al. 2009), (Prlic et al. 2009).

All these molecules involved in CD8+ memory T cell generation could be interesting targets in development of vaccine and immunotherapeutic strategies in order to boost memory T cell function.

#### 2.1.2.2.2) MEMORY CD8+ T CELL SUBSETS

Likewise CD4+ T cells, CD8+ memory T cells are divided in T<sub>CM</sub> and T<sub>EM</sub> cells. A third subset has been identified as the tissue resident memory T (T<sub>RM</sub>) cells. Although CD8+ T<sub>CM</sub> and T<sub>EM</sub> cells display the same phenotypes as CD4+ memory subsets, their frequency in peripheral blood and their ability to proliferate depend on their antigen activation status (Figure 21). Both T<sub>CM</sub> and T<sub>EM</sub> cell subsets circulate in peripheral blood and some authors report that they might interconvert when they pass through lymphoid or non-lymphoid tissues (Kaech et al. 2007), (Jameson et al. 2009). Contrarily to CD4+ memory T cells, both CD8+ T<sub>EM</sub> and T<sub>CM</sub> cells present cytolytic and cytokine production capacity (Appay et al. 2008), (Seder et al. 2003).

T<sub>RM</sub> cells have been reported to reside for a long time in brain and mucosal tissues, they are identified by the CD103<sup>hi</sup>CD69<sup>hi</sup>CD27<sup>lo</sup>CD62L<sup>lo</sup> phenotype and they can produce granzyme B. Thus, they present effector and cytotoxic capacities and they do not proliferate and migrate a lot. Similarly to CD8+ T<sub>EM</sub> cells, T<sub>RM</sub> cells produce less IL-2 than T<sub>CM</sub> cells (Kaech et al. 2012). During a secondary infection, T<sub>EM</sub> and T<sub>RM</sub> cells act in first-line with effector functions against pathogen entry, then, T<sub>CM</sub> generate larger number of secondary effector cells than during primary infection in a shorter time (Kaech et al. 2012).



**Figure 21. Phenotypes and characteristics of major human CD8<sup>+</sup> memory T cell subsets.**

Adapted from (Appay et al. 2008) and (Seder et al. 2003). CD8<sup>+</sup> central memory T cells (T<sub>CM</sub>) express CCR7 and CD45RO markers, they are characterized by a long lifespan, their capacity to migrate until secondary lymphoid organs, their secretion of IL-2 and thus they proliferate better in absence of antigen. Effector memory T cells, T<sub>EM</sub>, are CCR7<sup>-</sup>, they can express both CD45RA and CD45RO, they have cytolytic and cytokine production capacity similar or a little highest than T<sub>CM</sub> depending on the molecules and antigens. They proliferate better in presence of viral antigen.

## 2.2) T CELL ACTIVATION PATHWAYS

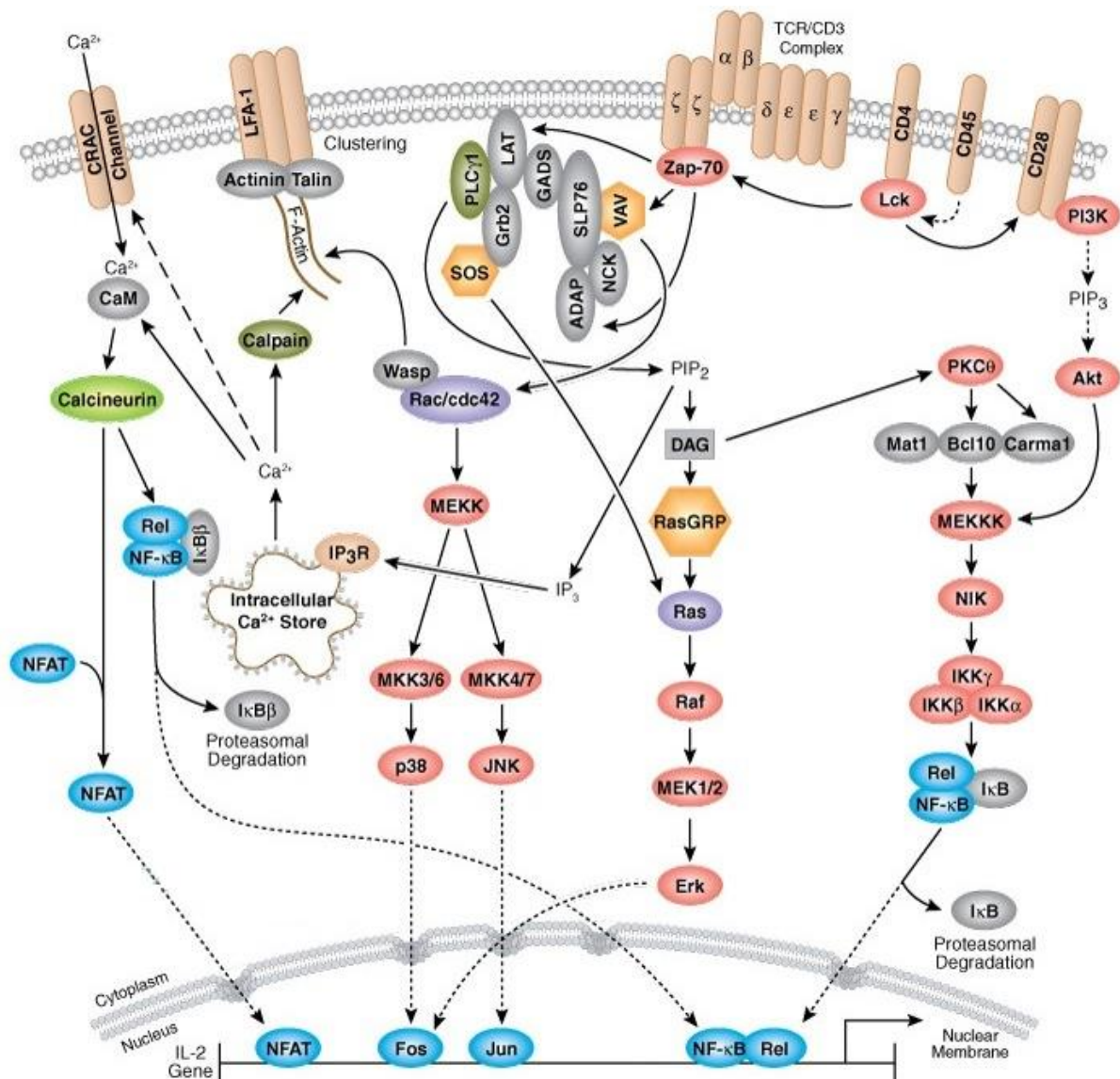
### 2.2.1) TCR ACTIVATION AND *IN VITRO* APPLICATIONS

More than thirty years ago, several groups identified the T cell antigen receptor (Haskins, 1984). Then, the TCR was identified as a complex non-covalently associating the variable  $\alpha\beta$  chain with the CD3 protein (Smith-Garvin et al. 2009). CD3 transduces signals via its immunoreceptor tyrosine-based activation motifs (ITAMs) thanks to their phosphorylation by src protein tyrosine kinases (PTKs).

Activation through the TCR is insufficient in absence of co-stimulation. Indeed, the co-ligation of other cell surface receptors allows T cell activation. CD28 appeared to be one of the most efficient co-ligands promoting T cell proliferation, survival, cytokine production and metabolism (Acuto et al. 2003) via phosphoinositide 3 kinase

(PI3K) activation. Figure 22 presents an overview of the TCR activation pathway where the major Ras, NK-kB and calcium pathways are involved.

An *in vitro* activation protocol based on anti-CD3 and anti-CD28 microbeads and IL-2 activation could be sufficient to obtain rapidly TCR activated T cells. Nevertheless, more natural protocols based on APC layers are needed for instance in case of specific works on TCR activation modalities. Of importance for clinical applications, TCR stimulation induce a severe skewing of T cells that could be dramatic for example in the clinical context of gene transfer protocols (Verhoeven et al. 2003), (Markt et al. 2003).



**Figure 22. Overview of the TCR activation pathway.**  
(From Cell Signaling technology manual).

### 2.2.2) *IN VITRO* STIMULATION OF T CELLS WITH IL-7

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Because of the alteration of the T populations with *in vitro* TCR stimulation of T cells evocated before, the use of survival cytokines such as IL-2 or IL-7 has been developed to stimulate T cells without altering the T cell repertoire functionality (Cavaliere et al. 2003), (Verhoeven et al. 2003), (Ducrey-Rundquist et al. 2002), (Korin et al. 1999). IL-7 have been attractive because of its role in supporting T cell survival and homeostatic proliferation (Fry et al. 2001), (Rathmell et al. 2001). Besides, IL-7 administration might improve immune reconstitution after allogenic bone marrow transplantation in mouse (Alpdogan et al. 2001). As a consequence, IL-7 stimulation of T cells could be useful to accelerate T cell reconstitution in the fields of bone marrow transplantation, chemotherapy and HIV infection, all marked by reduced T cell numbers. Moreover, the IL-7 receptor belongs to the  $\gamma$ -chain receptor family and is present on the whole T lineage from thymocytes to mature naïve and memory CD4+ and CD8+ T cells (Fukui et al. 1997).

Finally, IL-7 treatment of T cells allow their proliferation and survival without inducing their switch between naïve and memory subsets (Webb et al. 1999), (Soares et al. 1998) and only inducing a slight upregulation of CD25, CD98, CD71, CD11a and CD40-L much lower than obtain upon a TCR stimulation (Armitage et al. 1990).

### 3) UMBILICAL CORD BLOOD T CELLS, A NAÏVE ENRICHED T CELL SUBPOPULATION

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Some authors have reported that UCB contains a higher number of T, NK and B cells than adult peripheral blood. Of importance, UCB T cells are essentially naïve and immature and express for the vast majority the CD45RA+/CD45RO-/CD25-/CD69- phenotype. In addition, they proliferate more vigorously and secrete less cytokines as compared to adult peripheral blood (Szabolcs et al. 2003).

In the context of transplantation of cells from UCB, it has been demonstrated that UCB transplants induce less acute and chronic GVHD than bone marrow

transplants (Gluckman et al. 1995), (Rubinstein et al. 1998), (Rocha et al. 2001), (Harris et al. 1994). This might be due to a reduction of cytolytic function of UCB T cells (Muench et al. 1994), (Keever et al. 1995). This UCB property add some arguments in favor of the clinical relevance of UCB T cells for allogeneic transplantation.

Human naïve UCB CD4+T cells can be subdivided into two subsets, the CD31+ subset are suggested to be included in the recent thymic emigrant population whereas the CD31- population might be generate by differentiation of the CD31+ subpopulation after homeostatic cell division (Avezedo et al. 2009), (Kimmig et al. 2002).

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#### 4) T CELL GENE THERAPY AND IMMUNOTHERAPY

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##### 4.1) T CELL GENE TRANSFER FOR EFFECTIVE TREATMENT OF INHERITED OR ACQUIRED DISEASES

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One of the major advantages of using peripheral blood T cells is that they are more accessible for genetic modification than other targets such as HSCs. Moreover, they can be isolated in high amounts. T cells most likely have a lower risk of transformation, as up to now leukemia was not observed in T cell-based gene therapies (Blaese et al. 1995), (Bordignon et al. 1995), (Deeks et al. 2002). Of importance, the naïve T-cell subset, which responds to a novel antigen, has a long-term life span and persists over years in the patients (see part 3). Thus, a longterm-correction can be envisaged by T-cell gene therapy.

Gene transfer into T lymphocytes may allow the treatment of several genetic dysfunctions of the hematopoietic system, such as severe combined immunodeficiency (Blaese et al. 1995), (Bordignon et al. 1995), and the development of novel therapeutic strategies for diseases such as cancers and for acquired diseases such as acquired immunodeficiency infection (AIDS) (Buchsacher et al. 2001).

ADA deficient SCID was the first inherited disease investigated for T cell gene therapy because of a postulated survival advantage for gene corrected T lymphocytes. Aiuti and colleagues showed immune reconstitution in ADA-SCID patients after T-cell gene therapy (Aiuti 2002). Patients received multiple infusions of

autologous retroviral vector transduced peripheral blood lymphocytes (PBLs). Discontinuation of ADA replacement therapy led then to a selective growth of the infused ADA expressing lymphocytes, which eventually replaced the non-transduced T-cell population for nearly a 100%. These ADA corrected T cells were capable of responding to novel antigens and represented a new polyclonal T-cell repertoire. Thus PBL-ADA gene therapy leads to sustained T-cell functions in the absence of enzyme therapy. T cells of Wiskott-Aldrich Syndrome (WAS) patients were also functionally corrected by transduction with lentiviral vector encoding WAS protein (Dupre et al. 2004).

In the treatment of several blood cancers T cell, gene therapy has now proven to correct severe side effects of bone marrow transplantation (Allo-BMT) (Szydlo et al. 1997), (Vigorito et al. 1998). Treatment with allogenic T cells as offers the possibility of cure for patients with chronic myelogenous leukemia (CML) (Horowitz et al. 1990). The specificity of this effect, called graft versus leukemia effect (GVL) is not fully understood. Frequently, but not always, GVL is associated with graft versus host disease (GVHD). The latter is a very severe side effect of allogenic bone marrow transplantation, mediated by allospecific T cells within the graft. A strategy for the prevention of GVHD, now starting to be implemented, is the depletion of the donor T cells *in vivo* following infusion into the recipient in cases where GVDH becomes severe. This can be achieved by gene transfer of a suicide gene such as for example herpes simplex virus thymidine kinase into the T cell before infusion. Should the need for eradication of these cells arise, administration of the drug ganciclovir will induce apoptotic death of transduced T cells. This has already proven to be effective in the treatment of GVHD in clinical trials (Bondanza et al. 2006), (Bonini et al. 1997), (Tiberghien et al. 2001).

An important anti-cancer strategy based on the transfer of tumor-specific T cell receptor (TCR) genes into patient T cells has been proven successful in the clinic (Morgan et al. 2006). More recently, a radically different strategy consisting of introducing a coding sequence for a chimeric antigen receptor (CAR) allowed to confer the desired specificity for a cancer antigen to T cells. Recent case reports from on-going clinical trials have described durable rejection of previously refractory B-cell malignancy in patients after CD19-directed CAR therapy (Kalos et al. 2011), (Kochenderfer et al. 2010). Also for acquired diseases such as AIDS who are in demand of novels therapies, T cell gene therapy might be an important option.



Limitations of Highly Active Anti-Retroviral Therapy (HAART) such as appearance of drug-resistant HIV variants and toxicity show that patients need novel additional immune therapies (Finzi et al. 1997). Therefore, anti-HIV T cell gene therapy might be an option since it would allow protecting the major reservoir, the CD4+ T cells, against HIV infection and at the same time could protect HIV specific memory T cells, which are preferentially attacked by HIV. An HIV-1 based lentiviral vector was engineered expressing an HIV envelope antisense that highly protected T cells from healthy and infected patients against HIV infection (Braun et al. 2005), (Humeau et al. 2004).

These kind of therapies offer a solution for patients either who do not respond any more to anti-retroviral therapy in case of HIV infection or in case of diseases lacking of treatment. Table 7 presents an overview of T cell based gene therapy and immunotherapy.

**Table 7. Overview of T cell based gene therapy and immunotherapy.**  
(Adapted from Frecha et al. 2010) ADA. Adenosine deaminase, WAS. Wiskott Aldrich syndrome.

<b>Disease</b>	<b>Treatment strategy</b>	<b>Ref.</b>
<b>ADA immunodeficiency</b>	ADA cDNA gene transfer	(Aiuti et al. 2002)
<b>WAS immunodeficiency</b>	WAS cDNA gene transfer	(Dupre et al. 2004)
<b>Chronic myeloid leukemia</b>	Suicide gene transfer in allogenic T cells	(Qasim et al. 2007), (Bondanza et al. 2006), (Tiberghien et al. 2001), (Bonini et al. 1997)
<b>Tumors – melanoma</b>	Tumor specific TCR gene transfer in autologous T cells	(Bobisse et al. 2009) (Stanislawski et al. 2001), (Circosta et al. 2009)
<b>AIDS</b>	HIV env glycoprotein antisense RNA transfer / Vectors encoding for several shRNAs directed against HIV genes, for anti-HIV peptide blocking HIV cell entry, for HIV restriction factors	(Levine et al. 2006), (Wang et al. 2009), (Brake et al. 2008), (Egelhofer; 2004), (Hildinger et al. 2001) (Neagu et al. 2009), (Sakuma et al. 2007)

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## 4.2) RESTRICTION IN LV GENE TRANSFER OF T CELLS AND HOW TO OVERCOME THEM

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As mentioned above (See Introduction Part I) a major limitation of the generally used VSVG-LVs is its inability to govern efficient gene transfer into quiescent cells such as primary T cells and B cells, which hampers their application for gene therapy and immunotherapy (Frecha et al. 2008), (Frecha et al. 2009). That the parental virus, HIV-1, can enter into resting T-lymphocytes but does not replicate, has been attributed to multiple post-entry blocks as well as several cellular restriction factors (Strebel et al. 2009). It has been reported that inducing the resting T cells to enter into the G<sub>1b</sub> phase of the cell cycle without triggering cell division could render them permissive to transduction with HIV-1-vectors (Maurice et al. 2002). Our group and others have reported that inducing cell cycle entry into G<sub>1b</sub> via stimulation through the TCR allows efficient transduction of adult naïve T cells by HIV-1-based vectors (Korin et al. 1998), (Maurice et al. 2002). However, TCR-stimulation of T cells alters their half-life and immune-competence, results in an inversion of the CD4/CD8 ratio, and is associated with loss of naïve T cell subsets and a skewed TCR repertoire (Verhoeven et al. 2003), (Markt et al. 2003), (Ferrand et al. 2000).

Of note, up to now T cell gene therapy trials were based on TCR-mediated stimulation of T cells. Protocols were developed allowing LV transduction of T cells in the absence of TCR triggering. It was shown that IL-7, but also IL-2 promoted long-term survival of T lymphocytes (Fry et al. 2001), (Geiselhart et al. 2001). Interestingly, exposure of adult T cells to the cytokines IL-2 and IL-7 renders them permissive to lentiviral transduction in the absence of TCR activation (Cavalieri et al. 2003), (Ducrey-Runquist et al. 2002). These cytokine-treated T cells move out of G<sub>0</sub> into the G<sub>1b</sub> phase of the cell cycle, the phase in which T cells are susceptible to LV transduction, but do not yet proliferate. Clearly, IL-2 and IL-7 stimulation allowing lentiviral T-cell transduction could preserve a functional T-cell repertoire and maintain an appropriate proportion of naïve and memory CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Cavalieri et al. 2003).

Our group developed a strategy for LV mediated targeting by specific vector-mediated target cell activation. Our targeting strategy consists of an interaction of a

ligand displayed on the surface of the vector, with its specific receptor thereby inducing signalling and stimulation of the target cells. A single-chain antibody variable fragment (scFv) derived from the anti-CD3 OKT3 monoclonal antibody, which recognises and activates the T cell receptor, was fused to the amino-terminus of the SU subunit of the MLV envelope glycoprotein. Stimulation by this vector was sufficient to allow gene transfer in up to 48% of the lymphocytes, i.e., 100-fold more than the performance of unmodified lentiviral vectors in non-activated T cells (Maurice et al. 2002). To transduce resting T cells while conserving their phenotype, human IL-7 gene was fused to the amino-terminus of the MLV envelope glycoprotein. IL-7 displaying lenti-vectors allowed efficient transduction, up to 40%, of naïve CD4+ T cells as well as memory CD4+ T cells allowing to maintain the functional characteristics of the naïve T cells (Verhoeyen et al. 2003).

Moreover, our group engineered a novel lentiviral vector pseudotype incorporating measles virus (MV) glycoproteins H and F on their surface (MV-LVs) with the objective to conserve the measles virus tropism for T and B cells. Indeed, these MV-LVs allowed efficient transduction through the MV receptors SLAM and CD46, both present on blood T and B lymphocytes (Frecha et al. 2008a), (Frecha et al. 2008b).

Importantly, a single exposure to H/F-LVs allowed efficient stable gene transfer of quiescent T cells, which are not permissive for classical VSVG-LVs. Indeed, high-level transduction was achieved for resting memory (50%) and naïve T cells (11%) with H/F-LVs. Especially the naïve T cell population is of high interest since these are the long-lived cells in vivo which may induce a long-term gene correction. Unexpectedly, although classical lentiviral transduction demands at least cell-cycle entry into the G<sub>1b</sub> phase of the cell cycle, H/F-LVs did not even induce cell-cycle entry upon transduction in either quiescent T or B cells (Frecha et al. 2008), (Frecha et al. 2009). Importantly, the H/F-LV-mediated gene transfer conserved the naïve and memory phenotypes of transduced resting T cells and B cells.

## 5) ACCELERATION OF IMMUNE RECONSTITUTION AFTER HEMATOPOIETIC STEM CELL TRANSPLANTATION

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As we previously detailed, allogeneic HSCT is an effective treatment for a wide range of malignant and immune diseases.

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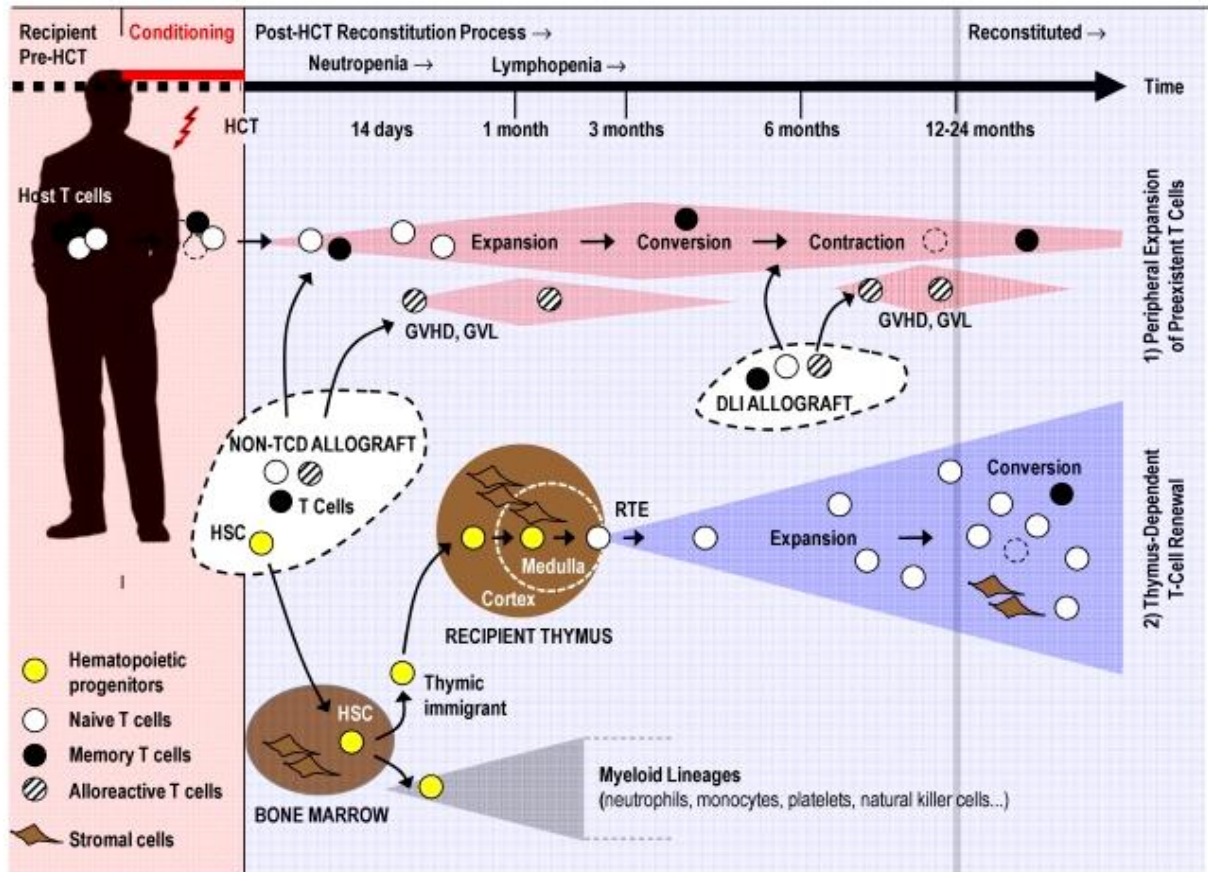
### 5.1) ADAPTIVE IMMUNITY RECONSTITUTION

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HSCT was firstly restricted to patients with HLA-matching donors. Several advances allows to offer some possibilities to patients lacking HLA-identical donor, notably by injecting highly purified CD34+ cells which present partially HLA-mismatches. Although the hematopoiesis restoration is relatively rapid after HSCT, the immune system functionality recovery is much longer. After HSCT, the major concern is to survive during the prolonged immunodeficiency phase, concerning especially slow T cell lineage reconstitution. Opportunistic infections can lead to death in the first hundred days after HSCT (Ruggeri et al. 2011). Indeed, after HSCT, T cell repertoire regeneration is allowed by T cell production from the new thymus derived from donor stem cells (Wils et al. 2007), (Wils et al. 2005). In contrast to other hematopoietic lineages, T lineage recovery could take a long time with an induced reduction of both cell number and activity (Mackall et al. 1995), (Douek et al. 2000), (Storek et al. 2008). Phenotypically mature T cells have been reported to recover in one- to two months, nevertheless T cell neogenesis has been evidenced after six months and normal levels of adult thymic functions were only recovered more than six months after HSCT (Hochberg et al. 2001). Of importance, the number of naïve T cell and T<sub>CM</sub> may never reach pre-transplant levels (Komanduri et al. 2007)

Several factors have been evaluated in order to understand the reasons of this recovery delay. For example, the type of conditioning regimen, GVHD, infectious disease or inflammatory status and the age of patients notably in connection with the thymic involution process might play a role in this delay (Krenger et al. 2011), (Castermans et al. 2011).

Therefore, several strategies to promote and accelerate T-lineage reconstitution are evaluated in order to decrease the long-time of immune system recovery and thus HSCT morbidity. It exists a large number of strategies depending on the T cell recovery pathways presented Figure 23.



**Figure 23. T cell regenerative pathways after allogeneic HSCT.**

(From Krenger et al. 2011). Conditioning regimens reduce the whole T lineage in patients. After HSCT, two mechanisms are involved in T cell regeneration. Initially, two T cell populations can expand in response to host signals or antigens independently from the thymus compartment: residual host T cells during a non T cell depleted (TCD) stem cell allograft and mature donor T cells resulting from a donor lymphocyte infusion (DLI) in association to alloreactive donor T cells that mediate GVHD. These firstly recovered T cell populations have been reported to be altered in their memory conversion and their TCR repertoire. Secondly, a new T cell population can be regenerated in a thymus-dependent pathway during a 12 to 24 month process. The host thymus stromal cells allow the differentiation of thymic immigrants into the mature T lineage and contribute in a major part of the regeneration of long life T cells with a diverse TCR repertoire.

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## 5.2) ENHANCING OF THYMUS INDEPENDENT T CELL RECOVERY

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Two major strategies have been developed to accelerate the thymus independent T cell recovery presented in Figure 23.

Some authors have developed *ex vivo* depletion of alloreactive donor T cells for example by using monoclonal antibodies against activated T cell surface molecules that are upregulated such as CD25, CD69, CD71, CD137 (Samarasinghe et al. 2010), (Hartwig et al. 2006), (Solomon et al. 2005), (Fehse et al. 2000a). This approach has been demonstrated to efficiently prevent GVHD in preclinical models and to accelerate immune recovery after HSCT in numerous preclinical and clinical works (Koh et al. 2002), (Fehse et al. 2000b) (Godfrey et al. 2004), (Solomon et al. 2005), (Amrolia et al. 2006). Albon and colleagues (Albon et al. 2013) have recently developed a close system methodology based on the use of cell differentiation bags and a combined immunomagnetic depletion of alloreactive CD25/CD71 T cells. Nevertheless, these improvements in allo-depletion protocols have not yet been evaluated in children.

On the other hand, the injection of pathogen specific T cells have been investigated in order to reduce the time of immune recovery against specific virus and fungi. *In vitro* expansion of pathogen specific CTLs has been developed in order to perform adoptive immunotherapy. The monitoring of these pathogen specific T cell reconstitution after HSCT can be performed thanks to PBMC intracellular cytokine staining or Enzyme linked immunosorbent spot assay or MHC-multimer staining (Fuji et al. 2013). CMV-, EBV-, anti-aspergillus specific CTLs have become interesting therapeutic options in the prevention and treatment of post-transplant CMV pneumonia (Walter et al. 1995), EBV lymphoproliferative diseases (Rooney et al. 1998) and lung infections caused by fungi (Tramsen et al. 2009).

Numerous clinical trials have demonstrated the efficiency and safety of these adoptive immunotherapies notably in the context of viral diseases (Fuji et al. 2011). Nevertheless, the monospecificity of these *in vitro* generated pathogen specific T cells offers a limitation in patient's immune protection. Indeed, after HSCT patients may suffer from many infectious diseases because of their immunodeficiency; and

since these adoptive immunotherapies are restricted to few pathogens, they do not provide a complete immune system recovery.

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### 5.3) ENHANCING OF THYMUS DEPENDENT T CELL RECOVERY AND ADOPTIVE TRANSFER OF IN VITRO GENERATED T CELLS PRECURSORS

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Strategies designed to accelerate thymus dependent T cell recovery are promising approaches for enhancing the generation of a mature T cell diverse repertoire derived from the donor cells.

First, enhancing of thymic T cell development and the support of thymic tissues is allowed by cytokines, hormones and growth factors.

IL-7 administration after HSCT has been described in mouse models to improve thymopoiesis and homeostatic proliferation of T cells derived from the infused HSCs (Fry et al. 2001), (Alpdogan et al. 2003a). Nevertheless, the immune restorative capacity of IL-7 on thymopoiesis is still discussed (Beq et al. 2006). The major disadvantage of IL-7 therapy in HSCT patients could be a greater risk of acute GVHD because of the proliferation capacity of this cytokine (Dean et al. 2008). In the case of HSCT with HLA-mismatches, Andre-Schmutz and colleagues (Andre-Schmutz et al. 2004) demonstrated that IL-7 administration could have a favourable role on T cell development. According to these studies, IL-7 treatments after HSCT should be limited to T cell-depleted allografts or autologous transplants.

Moreover, the role of numerous hormones in thymic involution has been evidenced. Gonadal steroid suppression could allow to prevent thymic involution. The sex-steroid blocking by LHRH-agonists has been described to accelerate lymphocyte recovery, to improve peripheral T cell survival and function without inducing GVHD and maintaining Graft-versus-leukemia effect (Sutherland et al. 2008). In addition, growth hormone (GH) and Insulin-like growth factor-1 (IGF-1) administration in HSC transplanted patients have been described as interesting factors in restoring thymopoiesis after HSCT (Chu et al. 2008), (Alpdogan et al. 2003b), (Napolitano et al. 2008). Of importance, IGF-1 administration allows a successful thymopoiesis restoration in a child six months after HSCT (Ma et al. 2010), (Wendorff et al. 2010).

Furthermore, IL-22 (Dudakov et al. 2012) and fibroblast growth factor 7 (FGF-7) administration after HSCT have been reported to provide the regeneration of thymic microenvironment after conditioning regimens and enhance thymopoiesis (Stiff et al. 2006).

Alternatively, T cell reconstitution can be enhanced by *in vitro* generation of T cell precursors. Some authors developed Notch based culture system in order to *in vitro* differentiate HSCs in T cell progenitors (Schmitt et al. 2002), (Ohishi et al. 2002), (Reimann et al. 2012). These systems allow the generation of murine and human T cell lineage from cord blood or bone marrow precursors differentiated on a OP-9-Delta-Like 1 cell layer culture system (Reimann et al. 2010), (Ohishi et al. 2002). In 2006, Lefort and colleagues (Lefort et al. 2006) demonstrated that a brief stimulation with Notch ligand Delta-like 4 (DL-4) is sufficient to induce T cell differentiation increasing the T cell potential of human CD34+ cells. It has been demonstrated recently that the culture of human CD34+ CB cells in a DL-4 feeder cell free system allow the *in vitro* differentiation and expansion of large numbers of T cell progenitors. These progenitors display phenotypic markers of early thymic progenitors (ETP) and can lead to the T cell lineage reconstitution. HSCT were performed in the NSG mouse model with a simultaneous injection of DL-4 primed T cell progenitors. Reimann and co-workers showed in this mice model that T cell reconstitution was accelerated and robust (Reimann et al. 2012). Therefore, the co-injection of HSC and DL-4 derived T progenitors from the same autologous HSCs could be an efficient strategy to accelerate T cell reconstitution in HSC transplanted patients. Besides, the basis of this new technique on a feeder cell free protocol appears to be safer for further clinical applications.





## PART 4 – B CELL BASED GENE THERAPY AND IMMUNOTHERAPY

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With this part, we propose a short overview about B cell gene transfer in order to make easier the understanding of the preliminary results presented below.

### 1) MATURE B CELLSUBSETS

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In short, mature B cells are the major targets for gene transfer in gene therapy. The Table 8 summarize the major phenotypes of mature B cell subsets.

**Table 8. Surface phenotype of mature B cell subsets**  
(Adapted from Kamiski et al. 2012)

Subset	Type	Phenotype	Function
<b>Naïve B cells</b>		IgD <sup>+</sup> CD27 <sup>-</sup> CD10 <sup>-</sup> CD24 <sup>+/lo</sup> CD38 <sup>+/lo</sup>	Precursors
<b>Memory B cells</b>	Double negative	IgD <sup>-</sup> CD27 <sup>-</sup>	Recall response
	Non-switched	IgD <sup>+</sup> CD27 <sup>+</sup>	Immunoprotective self antibody? circulating ? MZ-Like? Regulatory?
	IgMonly	IgM <sup>+</sup> IgD <sup>-</sup> CD27 <sup>+</sup>	Immunoprotective self antibody? circulating ? MZ-Like? Regulatory?
	Switched	IgM <sup>-</sup> IgD <sup>-</sup> CD27 <sup>+</sup>	Pathogen protection Autoimmune pathologies
<b>Antibody secreting cell</b>	Plasmablast	IgD <sup>-</sup> CD27 <sup>hi</sup> CD38 <sup>hi</sup> CD138 <sup>-</sup>	Antibody secretion
	Plasma cell	IgD <sup>-</sup> CD27 <sup>hi</sup> CD38 <sup>hi</sup> CD138 <sup>+</sup>	

### 2) B LYMPHOCYTES ARE A TARGET OF INTEREST IN GENE THERAPY AND IMMUNOTHERAPY

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B-cell gene therapy or immunotherapy has been strongly hampered due to the lack of an efficient LV ensuring stable longterm transgene expression in these cells but, as reported below, the recently developed H/F-LVs now allow many applications.

Transgene expression in B cells is of particular interest as B cells have the potential to induce specific immune activation and tolerance, which could improve genetic vaccination against cancer or autoimmune diseases (Lei et al. 2005), (Melo et al. 2002), (Stripecke et al. 2000). One of the major goals of cancer immunotherapy is to increase the immunogenicity of tumor cells. Although several

surface molecules can mediate T-B cell collaborations, the role of the CD40 receptor appears to be crucial (Aruffo et al. 1993), (Fuleihan et al. 1993). Thus, to overcome the immunological defect in malignant B cells, several groups induced forced ectopic expression of CD40L or other stimulatory factors (Stripecke et al. 2000), (Cantwell et al. 1997), (Cantwell et al. 1996) . Thus, vaccination strategies using autologous tumor cells manipulated *ex vivo* might be considered as a new approach for patients with B-cell malignancies, especially for those patients who are not responding to current treatment regimens (Moskowitz et al. 2006)

Autoimmune diseases represent failure of self-tolerance in populations of circulating B and T cells (Melo et al. 2002), (Lei et al. 2005). Current treatments include immunosuppression and immune deviation, but they are accompanied by deleterious effects. Thus novel approaches that can induce tolerance are required. B-cell gene therapy is an important option for induction of potent tolerogenic APCs. Scott and colleagues (1979) have shown that peptide-IgG fusion proteins, delivered via retroviral vectors into mouse B cells render these cells highly tolerogenic for the epitopes present in the peptide associated with the IgG *in vitro* and *in vivo* (El Amine et al. 2000), (Gourley et al. 2002), (Xu et al. 2004). Mouse B cells can in this way efficiently express multiple antigenic epitopes presented in a tolerogenic manner on class II MHC, inducing tolerance in naive and already primed immunocompetent animals. The clinical relevance of this B-cell gene therapy approach has been demonstrated by tolerance induction for targeted antigens in experimental models of several autoimmune disease (Xu et al 2004), (Agarwal et al. 2000), (Song et al. 2004). In these models, this gene therapy induced protection, delayed the onset of the disease or even reversed the ongoing clinical course.

Of importance, primary B cells are the most potent antibody producing cells. Indeed, the programming of B cells to make a predefined protective antibody would provide a continuous supply of antibodies *in vivo* that might reduce the viral load in patients (Luo et al. 2009). Recently, a B cell based anti-HIV strategy was proposed. LVs were used to introduce an anti-HIV antibody coding regions into HSCs and differentiation of the transduced cells into antibody-secreting autologous B cells was achieved with success (Luo et al. 2009).

Alternatively, lentiviral transduction and reinfusion of autologous B cells would allow a quick and continuous supply of HIV neutralizing antibodies *in vivo*. Of note, in the context of B-cell immunotherapy, the traditional methods used for generating human antibodies include screening Epstein-Bar virus (EBV-) transformed human B-cell clones (Cole et al. 1984), (Liao et al. 2009) or antibody phage display libraries. These methods are often time-consuming and can give low yields of pathogen-specific antibodies. A novel strategy requiring efficient B-cell gene transfer may facilitate production and the identification of monoclonal neutralizing antibodies by allowing efficient transduction of primary patient memory B cells with BCL6 and BCL-XL genes inducing immortalization (Kwakkenbo et al. 2010).

Stable gene transfer in human B cells may thus allow the engineering of improved genetic vaccination strategies against cancer, infectious or autoimmune diseases (Li et al. 2006), (White et al. 2000).

### 3) RESTRICTIONS OF LV-MEDIATED GENE TRANSFER IN HUMAN B CELLS

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Several studies have now established the capacity of VSVG-pseudotyped HIV-1-derived vectors to transduce various types of non-proliferating cells both in vitro and in vivo (Vigna et al. 2000). However, some cell types that are important gene therapy targets are refractory to gene transfer with classical lentiviral vectors.

Until recently, achieving long-term gene transfer into primary human B cells has been notoriously difficult (Bovia et al. 2003), (Janssens et al. 2003), (Serafini et al. 2004) and LV transduction of truly quiescent B cells had not yet been reported. Serafini et al. (2004) showed that conventional VSVG-LVs only achieved low transduction efficiency in anti-CD40-triggered B cells . It has been demonstrated that VSVG-LVs can indeed enter into a very small subset of B cells, undergo reverse transcription (RT) and that proviral integration takes place into the B-cell genome. It is worth pointing out though, that VSV-G-LV cell entry, RT and nuclear entry occur in proliferating B cells but are very inefficient (Serafini et al. 2004). Our group recently demonstrated that the VSV-G receptor (LDL-R) is not expressed on resting B cells and is not a lot expressed when B cells are activated (Amirache et al. 2014).

Moreover, no B-specific putative blocking factors have been yet identified which makes difficult to overcome these obstacles in B cell lentiviral gene transfer.

#### 4) CO-CULTURE CONDITIONS THAT OVERCOMES LENTIVIRAL VECTOR RESTRICTIONS FOR GENE TRANSFER INTO PRIMARY RESTING B CELLS

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Different B-cell activation/stimulation protocols have been set up in order to achieve stable gene transfer into human B cells. One way of inducing B-cell activation is by the addition of antiCD40-crosslinking antibodies and IL-4 that induces proliferation and entry into S/G<sub>2</sub>/M phase of the cell cycle. Under these conditions VSVG-LVs allow only very poor transduction of B cells. This was reported in three independent studies (Bovia et al. 2003), (Janssens et al. 2003), (Serafini et al. 2004). As reported by Bovia and colleagues (2003), VSVG-LVs allow efficient transduction of human B cells only upon their activation and proliferation in a co-culture system using murine thymoma cells as helper T cells in the presence of a cocktail of cytokines. Additionally, infection of B cells by Epstein-Barr Virus (EBV) or stimulation of cells with CpG DNA and cytokines can lead to efficient transduction (Kvell et al. 2005).

More sophisticated approaches use LVs that display an anti-CD20 scFv to target gene transfer to B cells (Funke et al. 2008), (Yang et al. 2006), (Ziegler et al. 2008) (See Introduction Part1 2.2.3). However, all these protocols that render the B cells permissive to LV transduction have a serious drawback: and induce B cell differentiation (Buchholz et al. 2009), (Fecteau et al. 2009).

It is of the utmost importance for many applications and fundamental studies that the primitive characteristics of the target B cell remain as intact as possible upon transduction, and that the vector does not induce itself any activation and/or differentiation as a secondary effect.

## 5) LENTIVIRAL VECTOR PSEUDOTYPED WITH MEASLES VIRUS GLYCOPROTEINS ALLOWS QUIESCENT B- LYMPHOCYTE TRANSDUCTION WITHOUT AFFECTING THE CELL CYCLE STATUS

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As mentioned above, a major limitation of current LVs such as the generally used VSVG-LVs is their inability to govern efficient gene transfer into quiescent cells such as B cells, which hampers their application for gene therapy and immunotherapy.

To overcome this limitation, our group engineered a lentiviral vector pseudotype incorporating measles virus (MV) glycoproteins H and F on their surface (H/F-LVs) with the objective to conserve the measles virus tropism for T and B cells.

Of interest, B cells were transduced up to 50% in their resting state by H/F-LVs whereas VSVG-LVs remained refractory (Frecha et al. 2009). Unexpectedly, although classical lentiviral transduction demands at least cell-cycle entry into the G<sub>1b</sub> phase of the cell cycle, H/F-LVs did not even induce cell-cycle entry upon transduction in quiescent B cells. Importantly, the MV-LV-mediated gene transfer conserved the naive and memory phenotypes of transduced resting B cells.

Of utmost importance, the H/F-LVs have been the first tools to allow stable transduction of B-CLL cells, one of the most prominent leukemic B-cell cancers arrested in the G<sub>0</sub>/G<sub>1a</sub> cell cycle phase (Frecha et al. 2009), (Levy et al. 2010). It was also demonstrated that H/F-LVs were in addition able to efficiently transduce unstimulated marginal zone lymphoma (MZL) B cells, another less recurrent B-cell malignancy (Levy et al. 2010).



## RESULTS

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## PART 1 – IMPROVING GENE DELIVERY INTO HEMATOPOIETIC STEM CELLS

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### 1) BAEV- AND H/F-LVS, TWO NEW TOOLS FOR GENE TRANSFER INTO RESTING AND STIMULATED HSCS

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Up to now, none lentiviral vector (LV) pseudotype was totally satisfactory for gene transfer in resting and stimulated human CD34+ cells. For a long time, VSV-G-LVs have been the reference for transducing CD34+ cells. Indeed, VSV-G LVs can be produced at high infectious titers, stably concentrated by ultracentrifugation and above all they recognize the LDL receptor expressed on the stimulated CD34+ cells (Amirache et al. 2014). However, there are disadvantages in using VSV-G-LVs. Toxicity is associated with long-term expression of VSV-G glycoprotein which makes generation of stable cell lines difficult. In addition, only high VSV-G-LVs doses (multiplicity of infection = MOI of 50-100) allow efficient hCD34+ cell transduction (up to 50%) increasing the risk of multicopy integration and thus genotoxicity (Di Nunzio et al. 2007). Furthermore, VSV-G-LVs are sensitive to human complement (Sandrin et al. 2002), which makes them unsuited for *in vivo* use. All these evidences limit *ex vivo* and *in vivo* use of VSV-G-LVs.

Consequently, during the last decade, they have been searching in the field for a new alternative envelope glycoprotein able to transduce efficiently HSCs at low vector doses and that renders LVs unsensitive to the human complement allowing their use *in vivo*. The RD114/TR envelope glycoprotein appeared to be a good alternative envelope. Indeed, this pseudotype is not sensitive to the human complement system which makes it attractive for *in vivo* applications (Sandrin et al. 2002). Moreover, its receptor, the human ASCT2 amino acid carrier (Rasko et al. 1999), is represented on human activated CD34+ cells (Green et al. 2004) and the chimeric RD114/TR glycoprotein gives high infectious titers (about  $6 \cdot 10^7$  IU/ml) when it is presented on LVs as compared to the wild type RD114 glycoprotein. The low toxicity of RD114/TR glycoprotein expressed in producer cells has lead to the development of stable cell lines (Ikeda et al. 2003), (Strang et al. 2004), (Stornaiuolo

et al. 2013). However, the transduction rates of CD34+ cells obtained using RD114/TR-LVs remains low for clinical applications (below 50%).

Of importance, a major limitation of these conventional LVs hampers their application for HSC gene therapy, as they do not allow efficient gene transfer into a subpopulation of quiescent HSCs (Sutton et al. 1999), (Verhoeyen et al. 2005). To overcome these limitations, many studies using LVs for hCD34+ cell transduction employed a high vector input and the presence of very strong cytokine cocktails (TPO, SCF, Flt3-L, IL-6, IL-3) in order to induce HSC cycle entry (Amsellem et al. 2002). However, an undesirable effect of extended cytokine stimulation is a decrease of the multipotentiality and longterm engraftment of human HSCs (Ahmed et al. 2004), (Uchida et al. 2011). IL-3 prestimulation is involved in CD34+ cell differentiation and although it increases the transduction levels, its application drops the repopulation potentiality of CD34+ cells (Liu et al. 2008), (Mazurier et al. 2004), (Millington et al. 2009). Moreover, high vector doses or multiple administrations of vector were applied to achieve high gene transfer levels into HSCs (Case et al. 1999), (Di Nunzio et al. 2007), (Millington et al. 2009). Nevertheless, a too high vector dose poses the risk for multicopy integration, under these conditions, insertional mutagenesis cannot be neglected (Baum et al. 2003), (Cavazzana-Calvo et al. 2004).

Here, in a first article recently published in the Blood journal, we report for the first time that LVs incorporating modified baboon endogenous retrovirus glycoproteins, BaEV/TR and BaEVRless on their surface are easy to produce and resistant to the human complement system. Mutant BaEV/TR glycoprotein is not cytotoxic and both BaEV-LVs can be used at low vector doses. They can transduce 30% of quiescent CD34+ cells and up to 80% of mild stimulated hCD34+ very early progenitors. Both quiescent and stimulated transduced CD34+ cells are able to engraft primary and secondary NSG mice. Of importance, the two fold increase of the proportion of GFP + CD45+ cells in secondary transplanted mice with unstimulated transduced CD34+ cells highly suggests that BaEV-LVs preferentially transduce the HSC subset in the resting CD34+ population. This demonstrates that BaEV-LVs highly transduce the HSC subset in the CD34+ cell population even if they are quiescent.

In parallel, we evaluated H/F-LVs which have been previously developed in our group (Frecha et al. 2008), (Frecha et al. 2009). We demonstrate that H/F-LVs succeed in transducing next to 100% of mild cytokine stimulated CD34+ cells and up to 70% of resting CD34+ cells. Contrarily to T and B cells, we show that H/F-LVs bind and enter into resting and stimulated CD34+ cells through the CD46 receptor while SLAM and Nectin 4 MV receptors appear to be barely expressed on the CD34+ surface. Primary and secondary transplantation of transduced CD34+ cells in NSG mice confirm that H/F-LVs allow gene transfer into HSCs.

Both BaEV- and H/F-LVs hold promises in the field of HSC gene delivery. BaEV/TR-LV would be useful for further clinical prospects because it can be produced at high infectious titers, it is not cytotoxic that make easier the production of a stable vector producing cell line and BaEV glycoprotein is resistant to the human complement system for potential *in vivo* applications. On the other hand, H/F-LVs would be a powerful tool for future basic research in hematopoiesis since at the moment it cannot be easily be produced for the clinic. Indeed, H/F-LVs remains difficult to produce at very high infectious titers, H and F glycoproteins are cytotoxic that prevent an immediate stable production and it need to be optimized to escape to MV neutralization for *in vivo* prospects in the large number of MV vaccinated people.



2) BABOON ENVELOPE PSEUDOTYPED LENTIVIRAL VECTORS  
OUTPERFORM VSV-G PSEUDOTYPED LENTIVIRAL VECTORS FOR  
GENE TRANSFER INTO CYTOKINE-STIMULATED AND RESTING HSCS



## GENE THERAPY

### Baboon envelope pseudotyped LVs outperform VSV-G-LVs for gene transfer into early-cytokine-stimulated and resting HSCs

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#### Key Points

- New LVs allow transduction of unstimulated hematopoietic stem cells.

Hematopoietic stem cell (HSC)-based gene therapy holds promise for the cure of many diseases. The field is now moving toward the use of lentiviral vectors (LVs) as evidenced by 4 successful clinical trials. These trials used vesicular-stomatitis-virus-G protein (VSV-G)-LVs at high doses combined with strong cytokine-cocktail stimulation to obtain therapeutically relevant transduction levels; however, they might compromise the HSC character. Summarizing all these disadvantages, alternatives to VSV-G-LVs are urgently

needed. We generated here high-titer LVs pseudotyped with a baboon retroviral envelope glycoprotein (BaEV-LVs), resistant to human complement. Under mild cytokine prestimulation to preserve the HSC characteristics, a single BaEV-LV application at a low dose, resulted in up to 90% of hCD34<sup>+</sup> cell transduction. Even more striking was that these new BaEV-LVs allowed, at low doses, efficient transduction of up to 30% of quiescent hCD34<sup>+</sup> cells, whereas high-dose VSV-G-LVs were insufficient. Importantly, reconstitution of NOD/Lt-SCID/ $\gamma$ c<sup>-/-</sup> (NSG) mice with BaEV-LV-transduced hCD34<sup>+</sup> cells maintained these high transduction levels in all myeloid and lymphoid lineages, including early progenitors. This transduction pattern was confirmed or even increased in secondary NSG recipient mice. This suggests that BaEV-LVs efficiently transduce true HSCs and could improve HSC-based gene therapy, for which high-level HSC correction is needed for life-long cure. (*Blood*. 2014;124(8):1221-1231)

#### Introduction

Although the murine leukemia virus (MLV)-based retroviral vectors were successful for hematopoietic stem cell (HSC)-based gene therapy, some clinical trials unfortunately led to severe genotoxic side effects.<sup>1-5</sup> Therefore, the lentiviral vectors (LVs), conferring a safer integration profile,<sup>6</sup> are gaining interest in the field. Cartier et al reported the successful treatment of patients suffering from X-linked adrenoleukodystrophy by ex vivo correction of autologous HSCs using LVs.<sup>7</sup> In the clinical trial of  $\beta$ -globin gene therapy for  $\beta$ -thalassemia, initiated in 2007, the single treated patient is still transfusion independent; however, clonal expansion was detected.<sup>8</sup> In 2013, Biffi et al<sup>9</sup> and Aiuti et al<sup>10</sup> provided very encouraging results for HSC-based gene therapy for the correction of metachromatic leukodystrophy or Wiskott-Aldrich syndrome. Moreover, new trials for X-linked severe combined immunodeficiency and Fanconi anemia, 2 other severe monogenetic defects in children, are being initiated.<sup>11</sup>

Importantly, for the correction of all these defects in the hematopoietic system, the therapeutic gene must be delivered to cells able both to self-renew and to differentiate into all hematopoietic lineages.

Because HSCs meet these criteria, they represent the attractive candidates for gene therapy. Although classical vesicular-stomatitis-virus-G protein (VSV-G)-LVs can transduce nondividing cells, fully quiescent G<sub>0</sub> cells are poorly transduced.<sup>12-15</sup> Hence, HSCs are poorly permissive for classical VSV-G-LV transduction because 75% of them reside in G<sub>0</sub>.<sup>14</sup> Moreover, we confirmed a very low expression of the VSV receptor, the low density lipid-receptor (LDL-R)<sup>16</sup> in unstimulated CD34<sup>+</sup> cells, perfectly coinciding with their poor VSV-G-LV-mediated transduction.<sup>17</sup> Only early-acting cytokine stimulation of hCD34<sup>+</sup> cells, up-regulating the LDL-R, permitted high-level VSV-G-LV transduction.<sup>17</sup> However, intense exposure of HSCs to cytokines might affect their homing and extravasation ability and promote differentiation rather than expansion of the HSC pool.<sup>18,19</sup> Moreover, when combining high vector doses with strong cytokine stimulation, the risk for multicopy integration and insertional mutagenesis cannot be neglected.<sup>3,4</sup> In an effort to achieve high-level transduction of HSCs under mild transient cytokine stimulation to preserve stem cells fitness,<sup>20-22</sup> we engineered LVs displaying stem

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cell factor (SCF) at their surface, able to efficiently transduce HSCs in vivo.<sup>22-24</sup> Therefore, mild cytokine stimulation allowing efficient HSC transduction is an important objective.

Clearly, to reach this goal, alternatives to VSV-G-LVs are urgently needed. As a good alternative, we identified the RD114 feline retrovirus envelope glycoprotein.<sup>25</sup> In contrast to VSV-Ggp, RD114gp is resistant to degradation by human complement, an attractive characteristic for in vivo applications.<sup>23,25</sup> An RD114 mutant glycoprotein (RDTR) allows efficient pseudotyping of LVs, which confers efficient transduction of hCD34<sup>+</sup> HSCs.<sup>24-30</sup> RD114 belongs to the betaretroviruses, also including the baboon endogenous retrovirus (BaEV). Both of these viruses use the neutral amino acid (aa) transporter 2 (ASCT-2), present on hCD34<sup>+</sup> cells.<sup>31-33</sup> Interestingly, BaEV also recognizes another aa transporter, ASCT-1, not recognized by RD114.<sup>31,34</sup> The ASCT-1 and -2 receptors, 57% identical in sequence, are differentially expressed in cells and are transporters of an overlapping but nonidentical set of neutral aa.<sup>34</sup> Because BaEV uses ASCT-1 in addition to ASCT-2, it demonstrated a broader tropism than RD114.<sup>35</sup> Therefore, we hypothesized that LVs displaying the BaEV envelope at their surface might permit an increase in hCD34<sup>+</sup> cell transduction.

Here, we generated for the first time LVs that incorporated the BaEVgp at their surface, which efficiently and stably transduced macaque and human hCD34<sup>+</sup> cells up to 90% at low vector doses and on mild cytokine stimulation, outperforming by far VSV-G- and RDTR-LVs. Moreover, these new pseudotypes were highly superior over VSV-G and RDTR-LVs for efficient gene transfer into hCD34<sup>+</sup> cells in the absence of any exogenous stimuli. Remarkably, these BaEV-LVs transduced up to 30% quiescent hCD34<sup>+</sup> cells (vs 5% for VSV-G-LV), with NOD/Lt-SCID/ $\gamma$ c<sup>-/-</sup> (NSG) long-term repopulation capacity of high importance for gene therapy.

## Materials and methods

### Envelope construction

RDTR is a mutated form of the feline retroviral glycoprotein (gp), RD114, described previously.<sup>25</sup> By polymerase chain reaction amplification, the cytoplasmic tail of wild-type (WT) BaEV (BaEVwt) was replaced by MLV-A, resulting in BaEVTR-gp. The BaEVRless-gp was constructed by deletion of the R peptide sequence from BaEVwt-gp (Figure 1A; supplemental Figure 1, available on the *Blood* Web site).

All chimeric envelope gps were expressed in the phCMV-G expression plasmid.<sup>36</sup>

### Production and titration of LVs

Self-inactivating HIV-1-derived vectors encoding green fluorescent protein (GFP) under the control of a spleen focus foamy virus promoter were generated by transfection of 293T cells.<sup>37</sup> For display of RDTR, BaEVTR, and BaEVRless-gps on LVs, 7  $\mu$ g of the respective envelope gp-encoding plasmids was transfected with gagpol and the self-inactivating (SIN) transfer vector; 3  $\mu$ g of VSV-Ggp encoding plasmid was used for VSV-G-LV production. Titering of the LVs was performed on 293T cells (supplemental Methods).

### Western blot analysis

Western blot analysis was performed as previously described.<sup>38</sup> For the detection of the BaEV gps, a goat anti-BaEV polyclonal antibody (ViroMed Biosafety Labs) was used. p24 HIV-1 capsid was detected by anti-HIVp24 antibody to assess equivalent loading of purified vectors.

### Sample collection and isolation of CD34<sup>+</sup> cells

Cord blood (CB) samples and mobilized blood were collected in sterile tubes containing the anticoagulant citrate dextrose (Sigma-Aldrich) after informed

consent and approval was obtained by the institutional review board (Centre International d'Infectiologie, Lyon, France) according to the Declaration of Helsinki. Human and macaque CD34<sup>+</sup> cells were isolated as previously described.<sup>22,23</sup>

### Stability of pseudotyped vectors in human/macaque serum

For details, see supplemental Methods.

### Transduction of human and macaque CD34<sup>+</sup> cells

Human and macaque CD34<sup>+</sup> cells were incubated for 14 or 18 to 24 hours (as indicated) in 24-well plates in serum-free medium (CellGro; CellGenix) supplemented with human recombinant cytokines (as indicated): SCF (100 ng/mL), thrombopoietin (TPO) (20 ng/mL), Fms-related tyrosine kinase 3 ligand (Flt3-L) (100 ng/mL), interleukin (IL)-6 (20 ng/mL), and IL-3 (20 ng/mL) (Preprotech); 5  $\times$  10<sup>4</sup> prestimulated CD34<sup>+</sup> cells were transduced in 48-well plates with concentrated LVs at indicated multiplicities of infection (MOIs) in serum-free medium. Cells were replenished with cytokines every 3 days. Three, 6, and 14 days after transduction, the percentage of GFP<sup>+</sup> cells was determined by fluorescence-activated cell sorter (FACS). Alternatively, CD34<sup>+</sup> cells were freshly isolated and transduced immediately in the absence of cytokines. Where indicated, transductions were performed on RetroNectin-coated plates according to the manufacturer's instructions (Takara). For myeloid differentiation of CD34<sup>+</sup> cells, see supplemental Methods.

### Conditioning and reconstitution of NSG mice

NSG mice used were housed in our animal facility (Plateau de Biologie Expérimentale de la Souris, Lyon, France). Experiments were performed in accordance with the European Union guidelines on approval of the protocols by the local ethical committee (Authorization Agreement C2EA-15; Comité d'Evaluation Commun au Centre Léon Bérard, à l'Animalerie de transit de l'ENS, au PBES et au laboratoire P4, Lyon, France).

Two- to 3-day-old newborn NSG mice were subjected to 1-Gy irradiation, and 2  $\times$  10<sup>5</sup> transduced prestimulated CB CD34<sup>+</sup> cells or 5  $\times$  10<sup>5</sup> transduced prestimulated mobilized CD34<sup>+</sup> cells were injected intrahepatically. Unstimulated freshly isolated CD34<sup>+</sup> cells were transduced for 24 hours, and 3  $\times$  10<sup>5</sup> transduced CD34<sup>+</sup> cells were injected into NSG mice as above.

After 12 weeks of reconstitution, extensive cell phenotyping in the different hematopoietic tissues (bone marrow [BM], spleen, thymus, and blood) of these mice was performed (supplemental Methods).

For secondary engraftments, the CD34<sup>+</sup> cells were isolated from the BM of primary engrafted mice as above, and 4 to 5  $\times$  10<sup>5</sup> isolated CD34<sup>+</sup> cells were injected into newborn NSG mice following the same procedure and phenotype analysis as above.

### DNA extraction and quantitative polymerase chain reaction

For details, see supplemental Methods.

### Cell cycle analysis

Cell cycle analysis was performed by staining DNA and RNA with 7-amino-actinomycin-D (7AAD) and pyronin-Y.<sup>39</sup>

### Statistical analysis

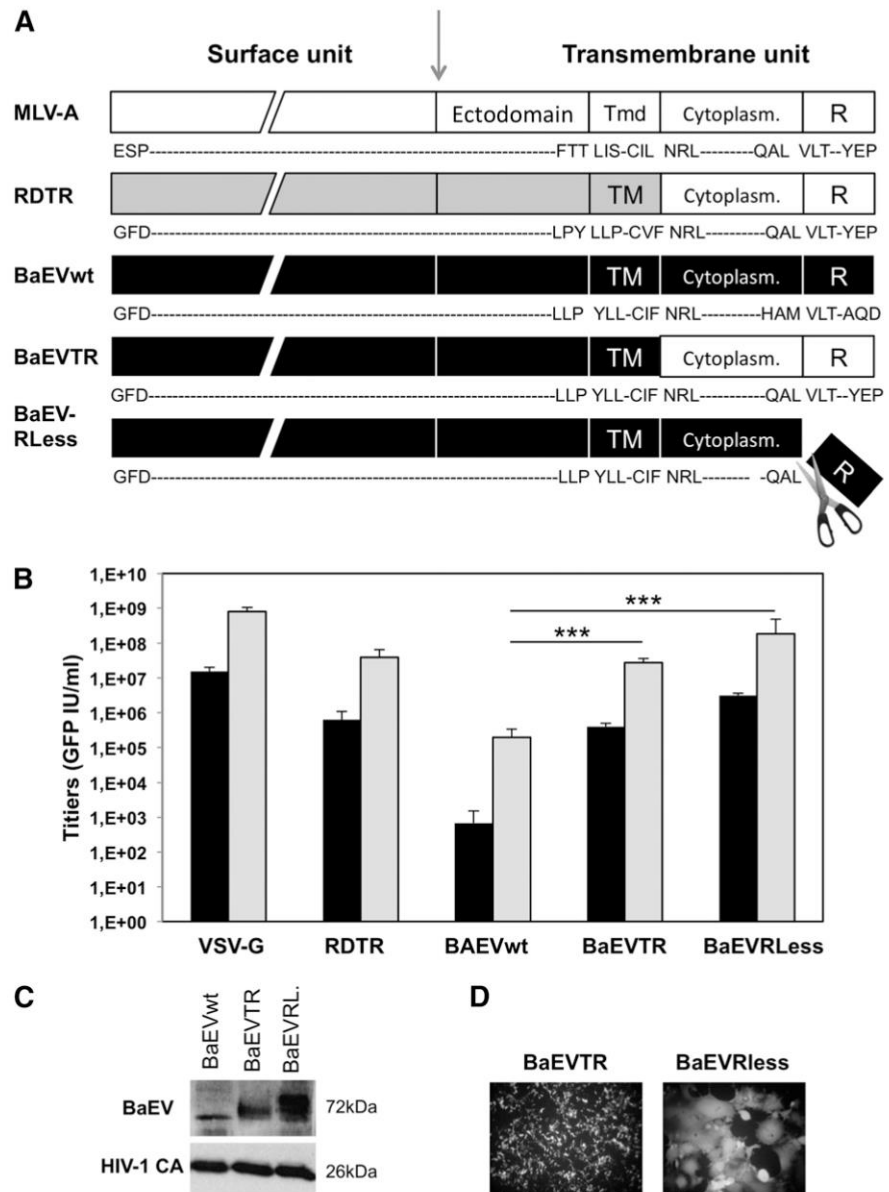
Statistical analysis was performed using a paired Student *t* test.

## Results

### Mutants of the baboon envelope glycoproteins, BaEVTR and BaEVRless, efficiently pseudotype LVs

Pseudotyping of LVs with WT BaEV gps (Figure 1A) was not efficient because it resulted in fresh and 100-fold concentrated titers

**Figure 1. Modification of the cytoplasmic tails of BaEV glycoproteins allows efficient pseudotyping of lentiviral vectors.** (A) Schematic representation of the WT (BaEVwt) and mutant BaEV (BaEVTR, BaEVRLess), RDTR- and MLV-A gps. The 17 aa long cytoplasmic domain of the cat and baboon retroviral gps, RD114 and BaEVwt, respectively, was exchanged for one of the MLV-A glycoproteins resulting in the chimeric RDTR- and BaEVTR-gps, respectively. The R peptide of the cytoplasmic tail of BaEVwt was deleted, resulting in the BaEVRLess mutant gps. Amino acid sequences and different domains for these gps are shown in supplemental Figure 1. (B) Titers of the different pseudotyped LVs encoding GFP, obtained by infection of HEK293T cells with serial dilutions of fresh or 100-fold concentrated vector preparations (infectious units/mL). The percentage of GFP<sup>+</sup> cells was determined 3 days after infection by FACS (means ± standard deviation [SD], n = 8; \*\*\*P < .005). (C) Immunoblots of LV particles displaying the different BaEV-gps at their surface. LVs were purified over a sucrose cushion by ultracentrifugation. The upper part shows staining with antibodies against the surface domain of BaEVwt. The corresponding HIV-1 capsid was revealed to verify equal loading. The positions of the BaEVgps and the HIV-1 capsid (HIV1-CA) are indicated. (D) Pictures show 293T cells 48 hours after transfection with BaEVTR- and BaEVRLess-gps.



of  $6 \times 10^3$  and  $2 \times 10^5$  infectious units (IU)/mL, respectively (Figure 1B). Therefore, a chimeric BaEVTR-gp was engineered using the same strategy used to create the RD114 mutant gp, RDTR, by exchange of its cytoplasmic domain for the murine leukemia virus gp (MLV-A-gp; Figure 1A).<sup>25</sup> A second mutant was engineered by the deletion of the fusion inhibitory R peptide resulting in the BaEVRLess-gp (Figure 1A; supplemental Figure 1). We compared these different mutant BaEV-gps to VSV-G- and RDTR-gps for their ability to pseudotype LVs.

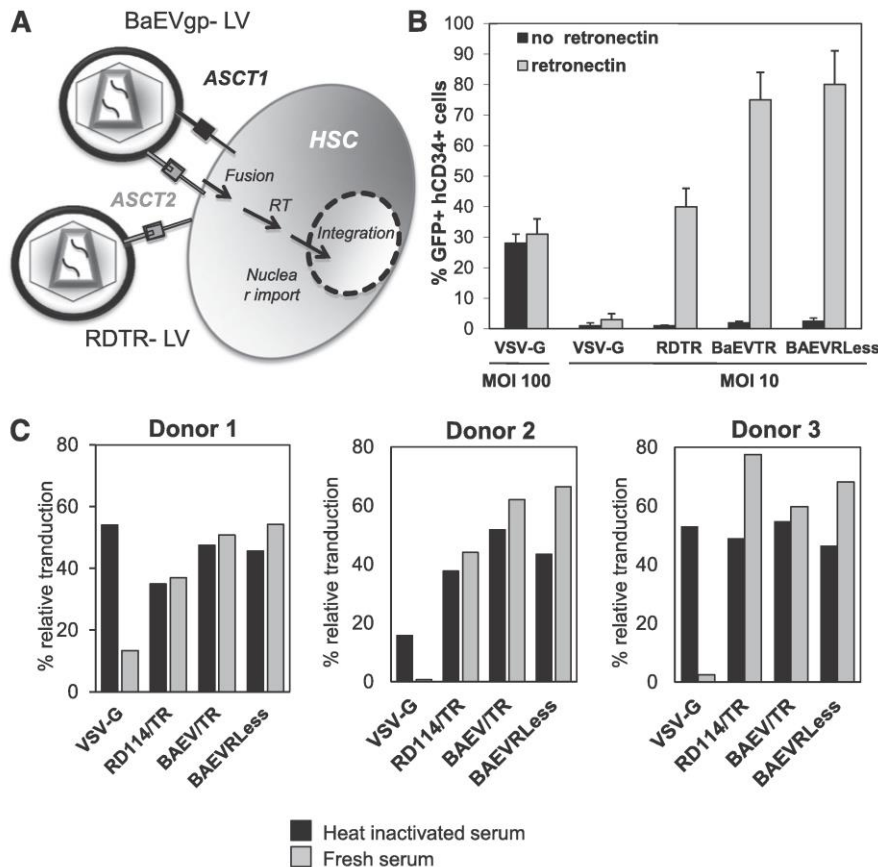
RDTR, BaEVTR, BaEVRLess, and VSV-G pseudotyped SIN LVs encoding GFP were generated by transient transfection in 293T cells and concentrated 100-fold. Average concentrated titers of  $5 \times 10^7$  IU/mL were obtained on 293T cells for BaEVTR- and  $2 \times 10^8$  IU/mL for BaEVRLess-LVs resulting in a 250- and 1000-fold titer increase, respectively, compared with BAEVwt-LVs (Figure 1B; supplemental Table 1). This was in agreement with increased incorporation of BaEVTR- and BaEVRLess-gps on LV particles compared with BaEVwt-gp (Figure 1C). RDTR- and VSV-G-LVs gave titers of  $6 \times 10^7$  and  $1 \times 10^9$  IU/mL as expected

(Figure 1B).<sup>24,25</sup> Physical particle content was similar for the different pseudotypes (supplemental Table 2). Of note, in contrast to BaEVRLess-gp, which induces huge syncytia formation, BaEVTR-gp did not induce any fusion or toxicity in 293T cells (Figure 1D).

The high titers obtained for the BaEVTR- and BaEVRLess-LVs incited us to evaluate them for hCD34<sup>+</sup> early progenitor transduction.

**BaEVgp pseudotyped LVs outperform VSV-G- and RDTR-LVs for the transduction of immature hCD34<sup>+</sup> cells**

HSCs are important gene therapy targets because, on correction, they can reconstitute and correct the entire hematopoietic system.<sup>7-10</sup> As depicted in Figure 2A, RDTR-LVs only bind to the hASCT2 receptor, whereas both BaEVTR- and BaEVRLess-LVs (BaEV-LVs) bind additionally to amino acid transporter, hASCT1, expressed on the cell membrane (Figure 2A). We prestimulated hCD34<sup>+</sup> cells with multiple cytokines (SCF/TPO/Flt3-L) and transduced them with VSV-G-, RDTR-, BaEVTR-, and BaEVRLess-LVs at a MOI of 10 in the presence or absence of the transduction facilitating agent, RetroNectin.



**Figure 2. BaEV-LVs are superior for the transduction of human CD34<sup>+</sup> cells over VSVG-LVs and RDTR-LVs.** (A) Schematic representation of LVs pseudotyped by BaEV-gps (BaEVTR or BaEVRLess) that bind to the amino acid transporters, hASCT1 and hASCT2, on the HSC surface. RDTR-LVs only bind to the hASCT2 receptor. Once the LVs fuse with the cell membrane of HSCs, reverse transcription of the viral RNA into proviral DNA occurs followed by its nuclear import and integration into the host genome. (B) Cord blood hCD34<sup>+</sup> cells were prestimulated with SCF, TPO, and Flt3-L for 14 hours and transduced with the indicated LV pseudotypes in the presence and absence of RetroNectin at an MOI of 10. VSVG-LV transductions were performed at an MOI of 10 or 100. GFP expression of the cells was analyzed 6 days after transduction by FACS (means  $\pm$  SD, n = 5). \*\**P* < .05 when comparing BaEVgps with other pseudotypes in the presence of RetroNectin; \*\*\**P* < .005 when comparing BaEVgps with or without RetroNectin. (C) Percentage of transduction of HEK293T cells by the indicated LV pseudotypes on their incubation at 37°C in heat complement-inactivated or fresh human sera. Data for 3 different serum donors are shown. The stability of virions was calculated as the relative percentage of infectivity of macaque/human serum-treated viruses vs viruses treated with fetal calf serum set to 100%.

The BaEV-LVs outperformed the other pseudotypes for the transduction of hCD34<sup>+</sup> cells in the presence of RetroNectin (Figure 2B). Importantly, under these conditions, up to 90% of the hCD34<sup>+</sup> cells were transduced by a single exposure to the BaEVTR- or BaEVRLess-LVs. Because it is difficult to compare different pseudotypes using their infectious titers, we also transduced stimulated CD34<sup>+</sup> cells with equivalent amounts of physical particles (HIV p24). Significantly higher transduction of CD34<sup>+</sup> cells for BaEVgp-LVs compared with VSVG-LVs was confirmed (supplemental Figure 2A).

In view of clinical in vivo application, it would be an advantage if the BaEV-LVs resist inactivation by the human complement system. Therefore, transduction efficiencies of LVs incubated in fresh or heat-inactivated human sera were compared. Residual transduction efficiencies obtained are shown in Figure 2C. As expected, the VSVG-LVs were inactivated by the 3 different sera. In contrast, BaEVTR- and BaEVRLess-LVs were resistant to human complement to the same extent as RDTR-LVs (Figure 2C).<sup>25</sup>

Of importance for their preclinical evaluation, the BAEV-LVs transduced efficiently cynomolgus and macaque BM hCD34<sup>+</sup> cells and resisted inactivation by the macaque complement system (supplemental Figure 3A-B).

Taken together, BaEV-LVs are excellent candidates for hCD34<sup>+</sup> cell transduction and are better suited than VSVG-LVs for in vivo applications.

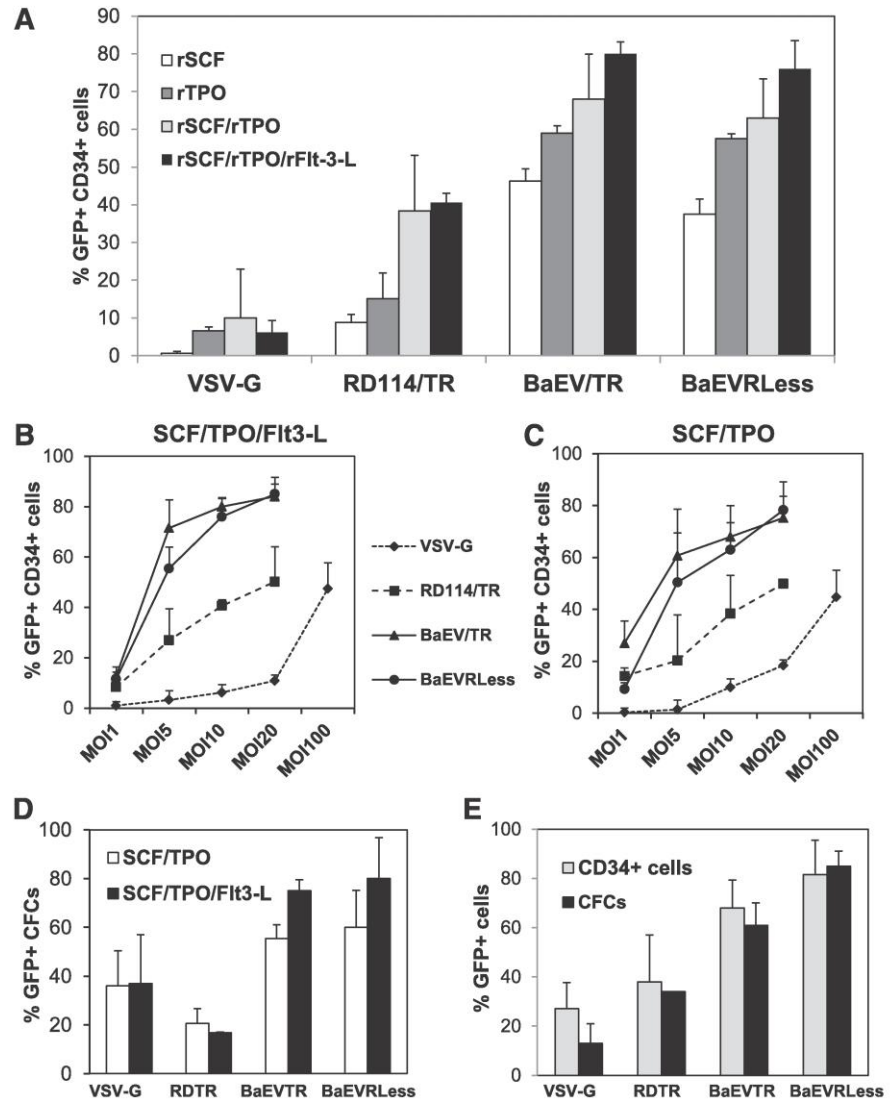
#### BaEV-LVs allow high-level transduction of hCD34<sup>+</sup> cells on mild cytokine prestimulation and at low vector doses

Strong stimulation with a cytokine cocktail can induce HSC differentiation and loss of self-renewal capacity. Thus, the less the cells are prestimulated, the more they keep their stem cell

characteristics.<sup>18,19,22,23,40</sup> Therefore, we performed LV transductions of hCD34<sup>+</sup> cells using different cytokine prestimulation conditions: (1) stimulation with a single cytokine (rSCF or rTPO); (2) rSCF+rTPO; and (3) rSCF+rTPO+rFlt3-L (Figure 3A). Even the strongest cytokine prestimulation used here was milder than cytokine cocktails currently used in a clinical setting. Remarkably, TPO stimulation is sufficient to transduce hCD34<sup>+</sup> cells up to 60% using BaEV-LVs, whereas VSVG-LVs and RDTR-LVs transduced only 8% to 20% of the hCD34<sup>+</sup> cells (*P* < .05). A single SCF prestimulation permitted up to 35% to 50% BaEV-LV-mediated transduction of hCD34<sup>+</sup> cells, whereas VSVG-LVs and RDTR-LVs reached maximum transduction levels of 10% (*P* < .05). A combination of TPO+SCF prestimulation and a single BaEV-LV transduction resulted in up to 60% to 80% hCD34<sup>+</sup> cell transduction, whereas RDTR- and VSVG-LVs, at the same MOI, achieved on average 40% and 10% transduction, respectively (*P* < .01 for BaEV-LVs vs VSVG-LVs). The triple cytokine cocktail (TPO+SCF+Flt3L) resulted in a gain of transduction levels for the BaEV-LVs (80-90%), whereas significantly lower transduction was obtained for the other pseudotypes (*P* < .005; Figure 3A).

In gene therapy, insertional mutagenesis must be limited to avoid side effects. Thus, a lower vector copy number/cell might reduce the risk of genotoxicity. Therefore, different vector doses were tested under rSCF+rTPO and rSCF+rTPO+rFlt3-L stimulation conditions. BaEVTR-LVs and BaEVRLess-LVs transduced significantly higher levels of hCD34<sup>+</sup> cells at low vector doses than the other LV pseudotypes (*P* < .05). They reached 60% (rSCF+rTPO) and 70% (rSCF+rTPO+rFlt3-L) transduction at an MOI of 5, whereas VSVG-LVs did not even reach 5% transduction (Figure 3B-C). Only at an MOI of 100, the VSVG-LVs allowed efficient hCD34<sup>+</sup> cell transduction rates, which remained far below that of BaEV-LVs

**Figure 3. BaEV-LVs allow high-level transduction of hCD34<sup>+</sup> cells following mild cytokine prestimulation.** (A) CB hCD34<sup>+</sup> cells were prestimulated overnight (18-24 hours) with SCF, TPO, SCF+TPO, or SCF+TPO+Flt3-L and transduced in the presence of RetroNectin by the indicated LV pseudotypes at an MOI of 10. Analysis for the percentage GFP<sup>+</sup> cells was performed 6 days after transduction (means ± SD, n = 4). hCD34<sup>+</sup> cells were prestimulated with (B) rSCF+rTPO+rFlt3-L or (C) rSCF+rTPO and transduced by the different LV pseudotypes at different MOIs (1, 5, 10, and 20); for VSV-G-LVs, an additional MOI of 100 was applied. Analysis of percentage GFP<sup>+</sup> cells was performed 6 days after transduction by FACS (means ± SD, n = 3). (D) CB hCD34<sup>+</sup> cells were prestimulated with rSCF+rTPO or rSCF+rTPO+rFlt3-L and subsequently transduced with the indicated LV pseudotypes. An MOI of 10 was applied except for VSV-G LVs (MOI = 100). Three days after transduction, the cells were seeded in myeloid differentiation medium for 14 days. The percentage of GFP<sup>+</sup> CFCs is shown for both stimulation conditions (means ± SD, n = 3). (E) Mobilized hCD34<sup>+</sup> cells were prestimulated with SCF+TPO+Flt3-L+IL-3 and transduced with the indicated LV pseudotypes at the same vector doses as in D. Myeloid differentiation and transduction analysis was performed as in D. The percentage of GFP<sup>+</sup>hCD34<sup>+</sup> cells and CFCs are shown (means ± SD, n = 3). All transduction were performed in the presence of RetroNectin, and *P* < .05 when comparing CFC transduction levels of BaEV-LVs with the other pseudotypes.



(Figure 3B-C). For the strongest cytokine stimulation, BaEVTR- and BaEVRLess-LV transduction reached a plateau at 80% to 90%. Nevertheless, a low vector copy number per cell was revealed (supplemental Table 3). Stable transduction was demonstrated by differentiation of the transduced hCD34<sup>+</sup> cells into myeloid lineages (Figure 3D).

Mobilized hCD34<sup>+</sup> cells are readily accessible and thus valuable target cells for gene therapy in the clinic. Similar to CB hCD34<sup>+</sup> cells, BaEVRLess-LVs allowed up to 90% transduction of mobilized hCD34<sup>+</sup> cells, and this transduction level persisted in colony forming cells (CFCs) derived from these cells, indicating that short-term progenitors were stably transduced (Figure 3E).

Concluding, BaEV-LVs outperformed VSV-G- and RDTR-LVs by far for hCD34<sup>+</sup> cells transduction even when using low cytokine stimulation combined with low vector doses.

**BaEV-LVs promote high-level transduction of prestimulated HSCs reconstituting primary and secondary recipient NSG mice**

Because of its potential to support high-level engraftment of human cells, we chose the NSG mouse<sup>41,42</sup> model to evaluate the long-term reconstitution capacity of BaEV-LV-transduced hCD34<sup>+</sup> cells in vivo. To address this question, we transplanted prestimulated

CB hCD34<sup>+</sup> cells transduced with the different LVs into irradiated NSG mice (Table 1). A comparable high level and variability of engraftment with human cells was detected for all pseudotypes (Table 1; supplemental Figure 4A), indicating that the migration and engraftment of these transduced early progenitors was not impaired.

Overall, VSVG-LVs and RDTR-LVs allowed a much lower transduction of SCID repopulating cells (SRCs) compared with BaEV-LVs (Table 1). BaEVTR-LVs- and BaEVRLess-LVs-transduced hCD34<sup>+</sup> cells resulted in the highest transduction levels of engrafted human cells in the BM (up to 95% CD45<sup>+</sup>GFP<sup>+</sup> cells). These high transduction levels were sustained in the primary recipient mice in all hematopoietic tissues (BM, spleen, and thymus; Table 1). Detailed flow cytometry analysis of the BM revealed equivalent high level transduction for the different B-cell subpopulations (CD19<sup>+</sup> pre-B/pro-B, pro-B, and immature/mature B cells), CD13<sup>+</sup> myeloid progenitors, and CD14<sup>+</sup> monocytes (Figure 4A; supplemental Table 4). For the BaEV-LVs, equivalent high levels of GFP<sup>+</sup> early progenitors (CD34<sup>+</sup>CD19<sup>-</sup>CD10<sup>-</sup>) were detected. In contrast, we observed for VSV-G-LVs and RDTR-LVs a high transduction variability between different tissues and the different cell lineages in the BM (Figure 4A; supplemental Table 4). Consistent with these results, detailed phenotyping of the splenocytes revealed a similar transduction

**Table 1. BaEV-LVs allow high rate transduction of NSG repopulating cells**

Pseudo-type	BM (%hCD45 <sup>+</sup> )	BM (%GFP <sup>+</sup> cells/total lin <sup>+</sup> cells)					Spleen (% GFP)	Thymus (% GFP)
		hCD45	hCD34	hCD19	hCD13	hCD14	hCD45	hCD45
<b>Cytokine-prestimulated hCD34 cells</b>								
VSV-G								
1	22	32	13	34	20	25	24	20
2	57	6	18	13	22	19	7	2
3	33	13	9	7	11	15	13	10
4	43	2	2	0.6	0	0	3	0.6
5	74	36	43	30	39	48	26	14
RDTR								
1	13	37	31	36	41	46	38	27
2	16	27	33	26	28	25	40	17
3	26	69	62	78	57	45	38	40
4	25	12	14	10	11	5	20	16
5	82	34	41	31	49	40	23	4
6	25	52	40	56	25	17	52	35
BaEVTR								
1	66	16	19	16	20	16	22	28
2	53	29	35	24	44	41	38	35
3	27	92	90	92	82	73	82	83
4	32	69	57	62	56	71	65	66
5	46	75	81	72	82	69	69	63
6	8	88	82	88	80	67	83	66
7	67	70	73	69	86	80	87	96
BaEVrless								
1	20	40	30	24	44	41	42	42
2	61	52	56	52	61	60	53	50
3	89	87	84	87	83	84	70	77
4	6	95	87	97	75	80	71	84
5	56	46	54	47	57	47	48	73
6	72	58	57	58	57	59	53	78
7	18	72	73	72	69	75	67	70
<b>Unstimulated hCD34 cells</b>								
BaEVTR								
1	71	8	7	6	18	19	19	15
2	73	8	2	7	6	6	14	1
3	14	6	8	5	6	6	8	2
4	81	16	19	16	27	12	19	12
5	90	14	13	10	15	12	16	8
6	81	35	27	34	40	42	35	62
7	61	26	32	35	27	26	18	18
BaEVrless								
1	70	22	20	23	25	20	16	22
2	75	22	23	27	21	14	26	10
3	29	11	6	13	11	13	13	58
4	72	19	19	18	23	19	20	10
5	64	11	15	12	11	10	23	11
6	77	36	40	35	39	32	30	54
7	85	50	55	49	52	56	52	48

Two- to 3-day-old newborn NOD/SCID  $\gamma c^{-/-}$  mice were subjected to a sublethal irradiation of 1 Gy.  $2 \times 10^5$  hCB-CD34<sup>+</sup> cells, transduced at an MOI of 10 with the indicated LV pseudotypes, were injected intrahepatically into the newborns. After 12 weeks of reconstitution, the humanized mice were killed, and the BM, spleen, and thymus were harvested and assessed for levels of GFP<sup>+</sup> human cell engraftment (hCD45<sup>+</sup> GFP<sup>+</sup>). Multilineage engraftment in the BM was demonstrated by FACS analysis of GFP<sup>+</sup> lineage-positive cells. Independent experiments were performed with different hCD34<sup>+</sup> cell samples and different batches of each vector.

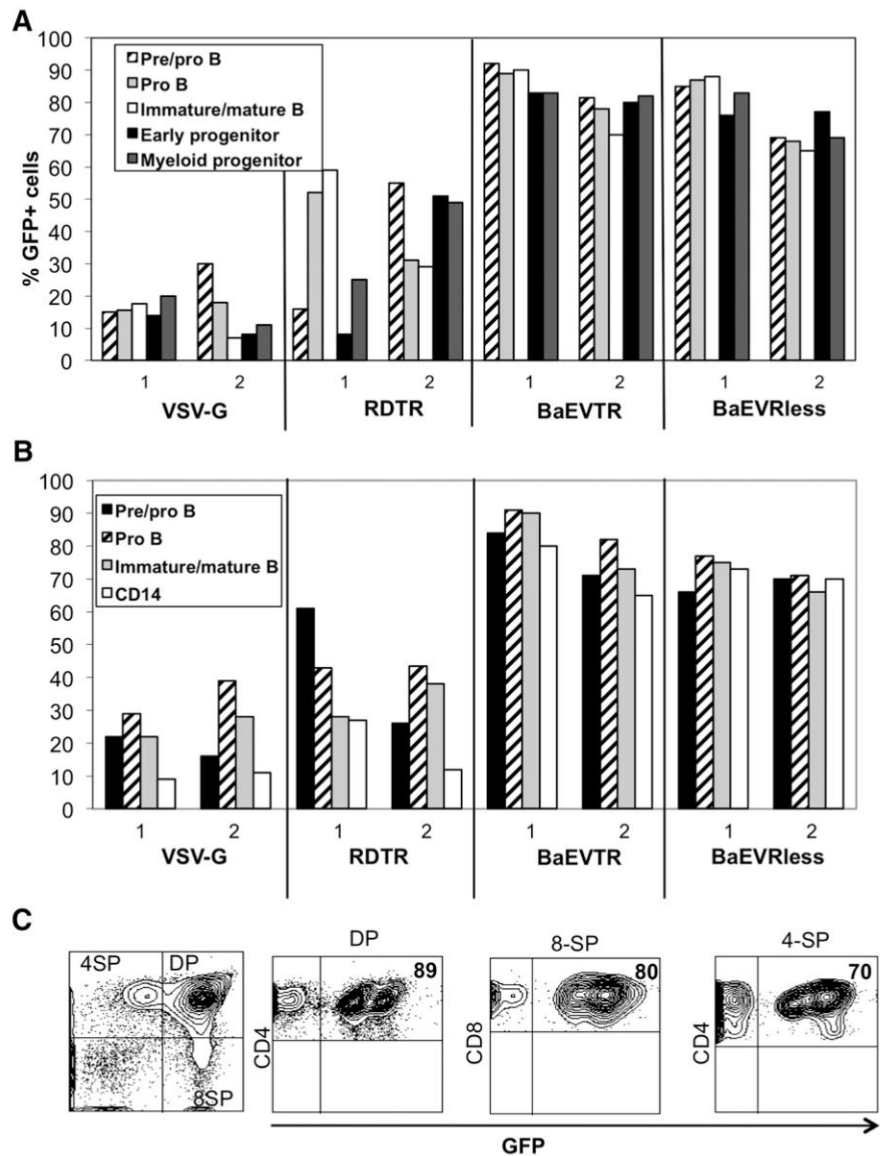
profile (Figure 4B). Importantly, the thymocytes (Table 1) and all thymic subpopulations were transduced to the same extent (Figure 4C).

As shown above, BaEVrless-LVs allowed up to 90% transduction of mobilized hCD34<sup>+</sup> cells (Figure 3E). These cells allowed high-level engraftment of NSG mice, and the transduction level was sustained in the BM immature progenitors, B cells and myeloid cells, splenocytes, and thymocytes. Detailed phenotyping confirmed that very early progenitor CD34<sup>+</sup> cells, as well as B-cell progenitors, myeloid progenitors, and monocytes, in the BM were transduced to the same extent. An identical picture was found for T and B cells and

monocytes in the spleen. The different thymic subpopulations showed high-level transduction (supplemental Figure 5).

To confirm that true HSCs were gene modified, secondary reconstitutions of NSG mice were performed with hCD34<sup>+</sup> cells isolated from primary NSG mice previously reconstituted with BaEVTR- and BaEVrless-LV-transduced prestimulated hCD34<sup>+</sup> cells. To improve engraftment into secondary recipients, the hCD34<sup>+</sup> cells from the BM of primary NSG mice were cultured, before injection, in SCF and IL-6 to maintain their homing capacity.<sup>43</sup> At 12 weeks after transplantation, the BM and spleen of the secondary recipient

**Figure 4. BaEV-LVs efficiently transduce NSG repopulating hCD34<sup>+</sup> cells.** CB hCD34<sup>+</sup> cells were prestimulated for 24 hours with a cytokine-cocktail (TPO+SCF+Flk-3L) and transduced the presence of RetroNectin with RDTR-LVs, BaEVTR-LVs, or BaEVrless-LVs at an MOI of 10 for 36 hours. VSV-G-LV transductions were performed at an MOI = 10. The cells were then injected into the liver of newborn NSG mice. On reconstitution for 12 weeks, the different hematopoietic tissues (BM, spleen, and thymus) of these engrafted mice were analyzed for human cell engraftment by anti-hCD45 staining (Table 1). (A) Transduction levels of the different cell lineages in the BM of NSG reconstituted mice. The percentage of GFP<sup>+</sup> immature early progenitor cells (CD34<sup>+</sup>CD19<sup>-</sup>CD10<sup>-</sup>), pre/pro-B cells (CD34<sup>+</sup>CD19<sup>+</sup>CD10<sup>-</sup>), pro-B cells (CD34<sup>-</sup>CD19<sup>+</sup>CD10<sup>-</sup>), immature and mature B cells (CD34<sup>-</sup>CD19<sup>+</sup>CD10<sup>+</sup>), and myeloid progenitors (CD13<sup>+</sup>) are shown. Data for 2 representative mice from Table 1 for each vector are shown. (B) Transduction levels of different cell lineages in the spleen of NSG reconstituted mice. The percentage of GFP<sup>+</sup> pre/pro-B cells (CD34<sup>+</sup>CD19<sup>+</sup>CD10<sup>-</sup>), pro-B cells (CD34<sup>-</sup>CD19<sup>+</sup>CD10<sup>-</sup>), immature and mature B cells (CD34<sup>-</sup>CD19<sup>+</sup>CD10<sup>+</sup>), monocytes, and granulocytes (CD14<sup>+</sup>) are shown. Data shown correspond to the mice represented in A. (C) Transduction levels of human thymocyte subpopulations of NSG mice reconstituted with hCD34<sup>+</sup> cells transduced with BaEVrless-LVs. The percentage of GFP<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup> (DP), CD4<sup>+</sup>CD8<sup>-</sup> (4-SP), and CD4<sup>-</sup>CD8<sup>+</sup> (8-SP) is indicated in the upper right quadrant. A representative blot of the BaEVrless mice in Table 1 is shown.



mice were analyzed for the percentage of hCD45<sup>+</sup>GFP<sup>+</sup> cells (Figure 5A). In the spleen and BM, the percentage of CD45<sup>+</sup>GFP<sup>+</sup> cells was maintained or even increased in the secondary NSG recipients. Importantly, for both BAEVTR- and BAEVrless-LVs, high levels of CD34<sup>+</sup>GFP<sup>+</sup> early progenitors, lymphoid (CD19<sup>+</sup>), and myeloid (CD13<sup>+</sup>) cells were detected in the BM of these secondary recipient mice, and equivalent percentages of GFP<sup>+</sup> cells were detected in the different lymphoid and myeloid lineages in BM and other hematopoietic tissues (supplemental Figure 6A).

Overall, these data strongly suggest that BaEV-LVs allowed genetic modification of very early progenitor cells, hHSCs, allowing long-term engraftment and differentiation into all the different cell lineages in vivo.

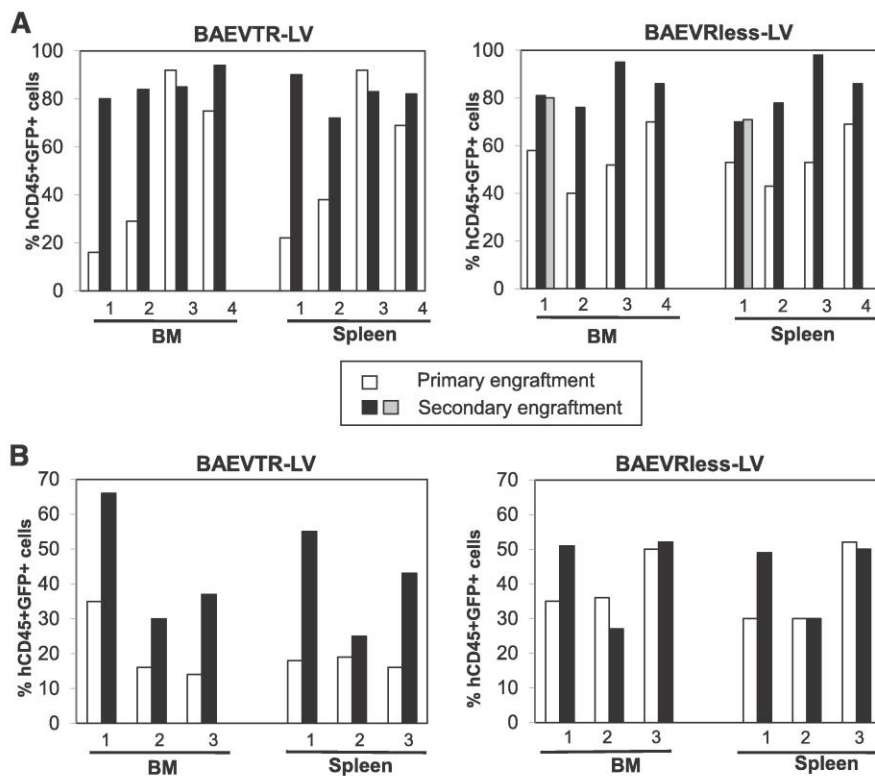
**BaEVgp-LV pseudotypes permit efficient transduction of resting hCD34<sup>+</sup> cells without inducing cell cycle progression**

Because BaEVgp-LVs permitted efficient transduction of hCD34<sup>+</sup> cells on stimulation with a single cytokine (eg, TPO or SCF; Figure 3A), we hypothesized that their binding and signaling through both aa transporters, ASCT-1 and ASCT-2, might be sufficient to

transduce unstimulated hCD34<sup>+</sup> cells with the objective to avoid their differentiation and loss of the HSC subpopulation.<sup>18,19</sup>

Freshly isolated hCD34<sup>+</sup> cells were therefore transduced with BaEVTR-, BaEVrless-, RDTR- and VSV-G-LVs in the absence of exogenous stimuli. Importantly, the BaEVTR- and BaEVrless-LVs were able to efficiently transduce resting hCD34<sup>+</sup> cells (15-30%) in the absence of any stimulus following a single exposure at low vector doses (MOI = 10; Figure 6A). In agreement with our previous reports,<sup>17,24</sup> the VSV-G-LVs were unable to transduce efficiently resting hCD34<sup>+</sup> cells, even when applied at high vector doses (Figure 6A; supplemental Figure 2B). Interestingly, the RDTR-LVs, which bind only to the ASCT-2 receptor, were not able to transduce resting hCD34<sup>+</sup> cells efficiently (<5%; Figure 6A). These BaEVgp-LV transduction levels persisted in CFCs derived from the hCD34<sup>+</sup> cells, confirming that short-term progenitors were stably transduced (Figure 6B). Important for safety, a low vector copy number per cell was revealed for the BAEV-LVs (supplemental Table 3).

As efficient transduction of hCD34<sup>+</sup> cells with classical VSV-G-LVs requires cytokine prestimulation,<sup>17</sup> which leads to cell cycle entry (Figure 6C) and probably differentiation, we wanted to exclude that BaEVgp-LV binding to ASCT-1- and -2-induced cell cycle



**Figure 5. BaEV-LVs transduce efficiently long-term reconstituting HSCs.** Transduced cells were injected into the liver of irradiated newborn NSG mice. On reconstitution, the BM and spleen of these primary engrafted mice (first transplantation) were analyzed for transduced human cell engraftment (GFP<sup>+</sup>CD45<sup>+</sup>) in the BM and the spleen. Subsequently, the hCD34<sup>+</sup> cells, isolated from the BM of these mice, were injected following the same procedure as for the primary NSG engraftments. Ten weeks after engraftment, these secondary recipient mice were analyzed for the percentage of transduced human cells (GFP<sup>+</sup>CD45<sup>+</sup>) in the BM and spleen. (A) Analysis of engraftment of transduced human cells (GFP<sup>+</sup>CD45<sup>+</sup>) in the BM and spleen of primary transplanted mice reconstituted with prestimulated (TPO+SCF+Flt-3L) hCD34<sup>+</sup> cells and transduced with BaEVTR-LVs or BAEVRless-LVs in the presence of RetroNectin and of secondary recipient NSG mice. (B) Analysis of engraftment of transduced human cells (GFP<sup>+</sup>CD45<sup>+</sup>) in the BM and spleen of primary and secondary transplanted mice reconstituted with unstimulated hCD34<sup>+</sup> cells transduced with BaEVTR-LVs or BAEVRless-LVs in the presence of RetroNectin and of secondary recipient NSG mice.

entry. Using a method that allows simultaneous visualization of DNA and RNA,<sup>15</sup> we evaluated cell cycle entry on transduction with the different pseudotypes (Figure 6C). As controls, hCD34<sup>+</sup> cells were incubated without vector in the absence or presence of cytokine cocktail stimulation. Although BaEVTR- and BAEVRless-LVs resulted in high-level hCD34<sup>+</sup> cell transduction, these cells did not actively enter into the cell cycle but resided in the G<sub>0</sub>/G<sub>1</sub> cell cycle phase. This is essential because the majority of the hCD34<sup>+</sup> cells with SCID repopulating potential reside in the G<sub>0</sub> phase of the cell cycle.<sup>44,45</sup>

#### BaEV-LVs promote high-level transduction of resting HSCs

To evaluate whether BaEV-LVs transduced long-term reconstituting HSCs in the resting hCD34<sup>+</sup> cell population, we applied a short transduction protocol (18-24 hours) at low vector doses (MOI = 10) without any addition of cytokines before injection into NSG newborn mice. High-level reconstitution with human cells was detected for BaEV-LV-transduced G<sub>0</sub>hCD34<sup>+</sup> cells, indicating that the homing, engraftment, and differentiation capacity of these cells was not affected (Table 1; supplemental Figure 4). Moreover, the human cell engraftment for BaEV-LV-transduced G<sub>0</sub>hCD34<sup>+</sup> cells was significantly higher than for their prestimulated counterparts (supplemental Figure 4; *P* < .01). Interestingly, for the low (8-20%) and high (>30%) transduction efficiencies, equivalent transduction rates in all the different lineages in the BM and spleen were revealed (Table 1; Figure 6D; supplemental Table 5).

Importantly, both ASCT-1 and ASCT-2 mRNA levels are up-regulated on cytokine stimulation (supplemental Figure 7A-B), which might strongly increase the affinity of BaEV-LVs to hCD34<sup>+</sup> cells and explain higher transduction levels in these stimulated cells compared with their resting counterparts.

Secondary reconstitutions of NSG mice were performed as for the prestimulated hCD34<sup>+</sup> cells. The percentage of CD45<sup>+</sup>GFP<sup>+</sup> cells in the BM and spleen was maintained or even increased by 1.5- to

threefold in the secondary recipient mice (Figure 5B). Importantly, for both BaEV-LVs, high levels of transduced CD34<sup>+</sup> early progenitors and lymphoid (CD19<sup>+</sup>) and myeloid (CD13<sup>+</sup>) cells were detected in the BM of these secondary recipient mice, and equivalent percentages of GFP<sup>+</sup> cells were detected in the other hematopoietic tissues (supplemental Figure 6B).

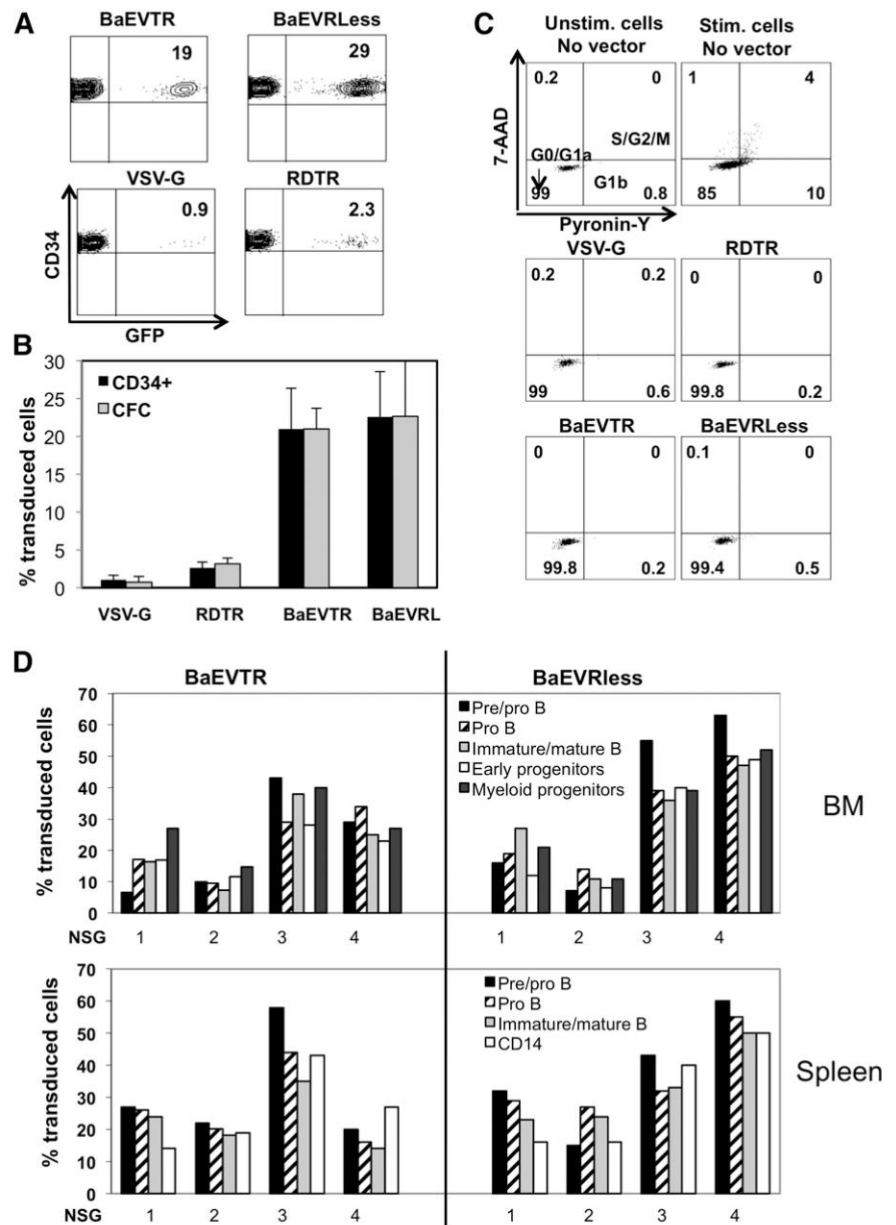
In summary, these BaEVgp-LVs allowed genetic modification of very early progenitor repopulating cells, so-called hHSCs in the unstimulated hCD34<sup>+</sup> cell population, able to home, engraft, and differentiate into all the different lineages in primary and secondary NSG mice.

## Discussion

We describe for the first time efficient pseudotyping of LVs with the envelope glycoprotein BaEV, derived from the baboon endogenous virus. Like RDTR-LV pseudotypes,<sup>25</sup> they are stable in human and macaque sera, which offers potential clinical perspectives for in vivo gene therapy. These BaEV-LVs did not only allow up to 90% transduction of prestimulated hCD34<sup>+</sup> SRCs at low vector doses but also high transduction levels of quiescent hCD34<sup>+</sup> SRCs, relevant for clinical application, whereas RDTR- or VSV-G-LVs performed very poorly in this latter condition. Moreover, in using BaEV-LVs, we would be able to significantly decrease the vector doses without a significant loss of gene transfer efficiency, which could make this treatment accessible to a higher number of patients.

During engraftment, transplanted HSCs home to the BM niche, where they initiate both differentiation and self-renewal. There, they reside in a hypoxic niche, where they acquire dormancy and maintain stemness.<sup>42,46</sup> It might be, therefore, crucial to not overexpose the hCD34<sup>+</sup> HSCs to cytokines, currently required for high-level transduction with VSV-G-LVs<sup>18,19</sup> Indeed, recent clinical trials<sup>7-10</sup> still use strong cytokine cocktails, although this can affect the cell

**Figure 6. BaEV-LVs are highly superior over RDTR-LVs and VSV-G-LVs for the transduction of unstimulated hCD34<sup>+</sup> cells.** Freshly isolated CB hCD34<sup>+</sup> cells were directly transduced by the indicated LV pseudotypes at an MOI of 10. For VSV-G-LVs, an MOI of 100 was used. Analysis for the percentage GFP<sup>+</sup> hCD34<sup>+</sup> cells was performed 3 days after transduction by FACS. A representative dot blot is shown for each pseudotype in A and the average transduction levels are indicated in B (means ± SD, n = 6). (B) Three days after transduction, the hCD34<sup>+</sup> cells were seeded in myeloid differentiation medium. The percentage of GFP<sup>+</sup> CFCs is presented (means ± SD, n = 4; P < .001 when comparing BaEV-LVs with VSV-G- and RDTR-LVs). (C) Cell cycle progression was monitored 3 days after transduction of the hCD34<sup>+</sup> cells with the different pseudotypes by simultaneously visualizing RNA (Pyronein-Y) and DNA (7-AAD) content of the cells. As controls, unstimulated and stimulated (SCF+TPO+Flt3-L) hCD34<sup>+</sup> cells without exposure to vector (no vector) were included. The percentages of cells in the G<sub>0</sub>/G<sub>1</sub>a, G<sub>1</sub>b, and S/G<sub>2</sub>/M phase of the cell cycle are indicated in the dot blots. (D) Freshly isolated CB hCD34<sup>+</sup> cells were transduced with BaEVTR-LVs and BaEVRLess-LVs at an MOI = 10 for 24 hours. The cells were then injected into the liver of newborn NSG mice. On reconstitution for 12 weeks, the different hematopoietic tissues (BM, spleen, and thymus) of these engrafted mice were analyzed for human cell engraftment by anti-hCD45 staining (Table 1). Transduction levels of the different cell lineages in the BM of NSG reconstituted mice are presented (upper histograms). GFP<sup>+</sup> immature early progenitor cells (CD34<sup>+</sup>CD19<sup>-</sup>CD10<sup>-</sup>), pre/pro-B cells (CD34<sup>+</sup>CD19<sup>+</sup>CD10<sup>-</sup>), pro-B cells (CD34<sup>+</sup>CD19<sup>+</sup>CD10<sup>+</sup>), immature and mature B cells (CD34<sup>-</sup>CD19<sup>+</sup>CD10<sup>+</sup>), and myeloid progenitors (CD13<sup>+</sup>) are shown. Transduction levels of different cell lineages in the spleen of NSG reconstituted mice (lower histogram). The percentage of GFP<sup>+</sup> pre/pro-B cells (CD34<sup>+</sup>CD19<sup>+</sup>CD10<sup>-</sup>), pro-B cells (CD34<sup>+</sup>CD19<sup>+</sup>CD10<sup>+</sup>), immature and mature B cells (CD34<sup>-</sup>CD19<sup>+</sup>CD10<sup>+</sup>), monocytes, and granulocytes (CD14<sup>+</sup>) are shown. Data for 4 representative mice from Table 1 for each vector type are shown. All transductions were performed in the presence of RetroNectin.



cycle status and the integrity of the HSC pool.<sup>47-49</sup> Importantly, compared with hCD34<sup>+</sup> cells in G<sub>0</sub>, cells in G<sub>1</sub> or S/G<sub>2</sub>/M phases of the cell cycle demonstrated diminished engraftment.<sup>44,48,50,51</sup> Here, we show that BaEV-LVs achieve high transduction rates (60-90%) of hCD34<sup>+</sup> cells when they are prestimulated briefly with rSCF, rTPO, or a combination of rSCF and rTPO. These mild cytokine stimulation protocols might strongly reduce the risk of HSC differentiation, cell cycle entry, and homing impairment and better conserve HSC function. In agreement, we previously confirmed that LVs displaying only the survival cytokine SCF at their surface transduced long-term SRCs.<sup>22-24</sup> Remarkably, our results indicated that BaEVgp-LVs can transduce up to 30% of unstimulated hCD34<sup>+</sup> cells, whereas VSV-G-LVs and RDTR-LVs remained inefficient. Thus, the former vectors might improve HSC transduction and conserve HSC potential, a combination needed for successful treatment of diseases in which gene-corrected repopulating cells have no selective advantage (eg, hemoglobinopathies). Although a higher level of HSC correction and conservation is of benefit for all monogenic diseases, patients with Fanconi anemia would in particular

benefit from resting hCD34<sup>+</sup> cell transduction. Indeed, the Fanconi anemia genetic defect causes fragility of the hCD34<sup>+</sup> cells, which are strongly reduced in number in these patients.<sup>52</sup> Moreover, cytokine stimulation induces Fanconi anemia hCD34<sup>+</sup> cells into apoptosis. Therefore, minimizing hCD34<sup>+</sup> cell stimulation combined with efficient gene transfer would be invaluable for Fanconi anemia gene therapy.<sup>11,53,54</sup>

That the transduction performance of BaEV-LVs is highly superior to the ones obtained by VSV-G-LVs, especially in quiescent hCD34<sup>+</sup> cells, is not surprising because we recently solved this mystery by demonstrating that unstimulated hCD34<sup>+</sup> cells do not express the VSV receptor LDL-R.<sup>16</sup> Even after a short-term cytokine stimulation, only a subpopulation up-regulated the LDL-R, limiting VSV-G-LV transduction.<sup>17</sup> The receptor for RD114, ASCT-2,<sup>31,32,55</sup> is up-regulated in cytokine-prestimulated hCD34<sup>+</sup> cells compared with unstimulated cells<sup>56</sup> (supplemental Figure 7), which might explain the low RDTR-LV transduction levels in quiescent hCD34<sup>+</sup> cells, although some ASCT-2 expression is detected.<sup>57,58</sup> The RDTR-LV transduction levels obtained for cytokine-stimulated hCD34<sup>+</sup>



cells reached a plateau at 50% in agreement with many other reports.<sup>25,56,59-62</sup> Remarkably, the BaEV-LVs allowed hCD34<sup>+</sup> cell transduction levels to significantly increase (80-90%) cytokine stimulation compared with RDTR-LVs, and they even achieved efficient transduction of these cells without any stimulation. Most probably, this increase must be attributed to the fact that, in contrast to RD114, BaEV recognizes the ASCT-1 receptor in addition to ASCT-2 on the hCD34<sup>+</sup> cell population.<sup>34</sup> Indeed, both receptor mRNA levels are up-regulated cytokine stimulation (supplemental Figure 7), which might increase the affinity of BaEV-LV binding to hCD34<sup>+</sup> cells.

Currently, LVs for gene therapy are produced by transient transfection. To date, one important instrument missing is a stable LV packaging cell line. This proved more difficult than expected because some viral proteins are toxic for 293T-HEK producer cells such as the VSV-G envelope gp.<sup>63</sup> Several stable cell lines were engineered; however, they still are not used in the clinic.<sup>64-68</sup> Importantly, 2 stable cell lines for high-titer RDTR-LV production were engineered.<sup>30,69</sup> This points toward BaEVTR-gp as an important candidate for establishing a stable LV packaging cell line because its long-term expression is not cytotoxic to 293T cells (Figure 1D), and these vectors allow high-level HSC transduction. Of note, we confirmed that these BaEV-LVs also allow high-level gene transfer in human T and B cells (A.G.-G. and E.V., unpublished data, June 2013).

Summarizing, the new BaEV-LVs allowed improvement of HSC gene transfer levels, accompanied by conservation of HSC function. They transduce unstimulated hCD34<sup>+</sup> cells with NSG long-term repopulating capacity at high levels that are relevant for gene therapy. Therefore, these new pseudotypes can improve current clinical protocols for HSC-based gene therapy to assure lifelong cure.

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## Authorship

Contribution: E.V. coordinated the project, designed and performed experiments, analyzed and discussed the data, and wrote the manuscript; A.G.-G. and F.A. designed and performed experiments, analyzed the data, discussed results; C.L., C.F., C.C., F.F., and D.N. performed experiments; D.L. provided reagents, critical discussion, and technical advice; and F.-L.C. provided critical discussion and reading of the manuscript.

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#### **MLV-A**

**MAARSTLSKPPQDKINPWKPLIVMGVLLGVGMA**ESPHQVFNVTWRVTNLMTGRNANATSLTGGTVQDAFPKLYFDL  
CDLVGEEWDPSPDQEPYVGYGCKYPAGRQRTRTFDFYVCPGHTVKSGCGGPGEGYCGKWGCETTQQAYWPKPTS  
SWDLISLKRGNTPWDTGCSKVACGPCYDLSKVSNSFQGATRGGRCNPLVLEFTDAGKKANWDGPKSWGLRLYRT  
GTDPTMFLSTRQVLNVGPRVPIGNPVLDPQRLPSSPIEIVPAPQPPSPLNTSYPPSTTSTPSTSPSPVQPPPPG  
TGDRLALVKGAYQALNLTNPKTQECWLCVSGPPYEGVAVVGTYNHSTAPANCTATSQHKLTLSEVTGGQLC  
MGAVPKTHQALCNTTQSAGSGSYLAAPAGTMWACSTGLTPCLSTTVLNLTTDYCVLVLELWPRVIYHSPDYMYGQ  
LEQRTKYKREPVSLLTALLLGGTMMGGIAAGIGTGTTALIKTQQFEQLHAAIQTDLNEVEKSITNLEKSLTSLSEVVLQ  
NRRGLDLLFLKEGGLCAALKECCFYADHTGLVRDSMAKLRERLNQRQKLFETGGQWFEGLFNRSWFTTLLSTI  
**MGPLVLLLLLLFGPCILNRLVQFVKDRISVVQALVLTQQYHQLKPLEYEP**

#### **RDTR**

**MKLPTGMVILCSLIIVR**AGFDDPRKAIALVQKQHGKPCCECSGGQVSEAPPNSIQVTCPGKTAAYLMTNQKWKCRVT  
PKISPSGGELQNCPCNTFQDSMHSSCYEYRQCRRINKTYTATLLKIRSGSLNEVQILQNPQLLQSPCRGSIQNP  
VCWSATAPIHISDGGGGLDTRKRVWTVQKRLEQIHKAMTELPQYHPLALPKVRDDLSDARTFDILNTTFRLLQMSNF  
SLAQDCWLCLKLGPPTPLAIPNPLTYSLADSLANASCIIPPLLVQPMQFSNSSCLSSPFINDTEQIDLGAVTFTNCT  
SVANVSSPLCALNGSVFLCGNNMAYTYLPQNWTRLCVQASLLPDIDINPGDEPVPIPAIDHYIHRPKRAVQFIPLLAG  
LGITAAFTTGATGLGVSVTQYTKLSHQLISDVQVLSGTIQDLQDQVDSLAEVVLQNRRLDLLTAEQGGICLALQEK  
CFYANKSGIVRNKIRTLQEELQKRRESLASNPLWTGLQGFLPYLLPLLGPLLTLTLTIGPCVFNRLVQFVKDRISVV  
**QALVLTQQYHQLKPLEYEP**

#### **BAEV wt**

**MGFTTKIIFLYNLVLVYA**GFDDPRKAIELVQKRYGRPCDCSGGQVSEPPSDRVSQVTCSGKTAAYLMPDQRWKCKSI  
PKDTPSPGPLEQECPCNSYQSSVHSSCYTSYQQCRSGNKTYTATLLKTQTGGTSDVQVLGSTNKLQSPCNGIKG  
QSIWSTTAPIHVSDDGGGLDTRIKSVQRKLEEIHKALYPELQYHPLAIPKVRDNLMVDAQTLNINLNTATYNLLMSN  
TSLVDDCWLCLKLGPPTPLAIPNPLTYSVTRSSDNISCIIPPLLVQPMQFSNSSCLFSPSYNSTEIDLGHVAFSNCT  
SITNVTGPICAVNGSVFLCGNNMAYTYLPTNWTGLCVLATLLPDIDIIIPGDEPVPIPAIDHFIYRPKR  
AIQFIPLLAGLGITAAFTTGATGLGVSVTQYTKLSNQLISDVQILSSTIQDLQDQVDSLAEVVLQNRRLDLLTAEQGGI  
CLALQEKCCFYVKNKSGIVRDKIKTLQEELERRRDLASNPLWTGLQGLPYLLPFLGPLLTLTLTLTIGPCIFNRLTAF  
**INDKLNIIHAMVLTQQYQVLRDDEEAQD**

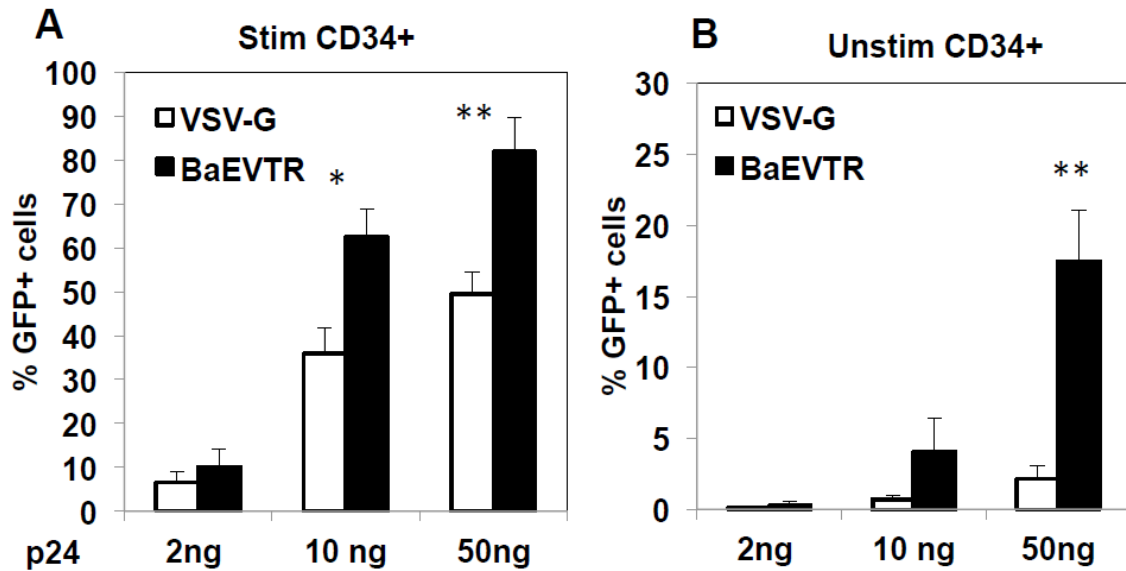
#### **BaEVTR**

**MGFTTKIIFLYNLVLVYA**GFDDPRKAIELVQKRYGRPCDCSGGQVSEPPSDRVSQVTCSGKTAAYLMPDQRWKCKSI  
PKDTPSPGPLEQECPCNSYQSSVHSSCYTSYQQCRSGNKTYTATLLKTQTGGTSDVQVLGSTNKLQSPCNGIKG  
QSIWSTTAPIHVSDDGGGLDTRIKSVQRKLEEIHKALYPELQYHPLAIPKVRDNLMVDAQTLNINLNTATYNLLMSN  
TSLVDDCWLCLKLGPPTPLAIPNPLTYSVTRSSDNISCIIPPLLVQPMQFSNSSCLFSPSYNSTEIDLGHVAFSNCT  
SITNVTGPICAVNGSVFLCGNNMAYTYLPTNWTGLCVLATLLPDIDIIIPGDEPVPIPAIDHFIYRPKR  
AIQFIPLLAGLGITAAFTTGATGLGVSVTQYTKLSNQLISDVQILSSTIQDLQDQVDSLAEVVLQNRRLDLLTAEQGGI  
CLALQEKCCFYVKNKSGIVRDKIKTLQEELERRRDLASNPLWTGLQGLPYLLPFLGPLLTLTLTLTIGPCIFNRLVQ  
**FVKDRISVVQALVLTQQYHQLKPLEYEP**

#### **BaEVrless**

**MGFTTKIIFLYNLVLVYA**GFDDPRKAIELVQKRYGRPCDCSGGQVSEPPSDRVSQVTCSGKTAAYLMPDQRWKCKSI  
PKDTPSPGPLEQECPCNSYQSSVHSSCYTSYQQCRSGNKTYTATLLKTQTGGTSDVQVLGSTNKLQSPCNGIKG  
QSIWSTTAPIHVSDDGGGLDTRIKSVQRKLEEIHKALYPELQYHPLAIPKVRDNLMVDAQTLNINLNTATYNLLMSN  
TSLVDDCWLCLKLGPPTPLAIPNPLTYSVTRSSDNISCIIPPLLVQPMQFSNSSCLFSPSYNSTEIDLGHVAFSNCT  
SITNVTGPICAVNGSVFLCGNNMAYTYLPTNWTGLCVLATLLPDIDIIIPGDEPVPIPAIDHFIYRPKRAIQFIPLLAGLGIT  
AAFTTGATGLGVSVTQYTKLSNQLISDVQILSSTIQDLQDQVDSLAEVVLQNRRLDLLTAEQGGICLALQEKCCFYV  
NKNKSGIVRDKIKTLQEELERRRDLASNPLWTGLQGLPYLLPFLGPLLTLTLTLTIGPCIFNRLTAFINDKLNIIHAM

**Supplementary Figure 1: Amino acid sequences of the MLV, RDTR and BaEV wt and chimeric glycoproteins.** Colors are used to indicate the different domains: **Signal peptides**, **Transmembrane**, **Cytoplasmic tail**, **R peptide**, surface unit (SU) + part of the transmembrane (TM) unit.



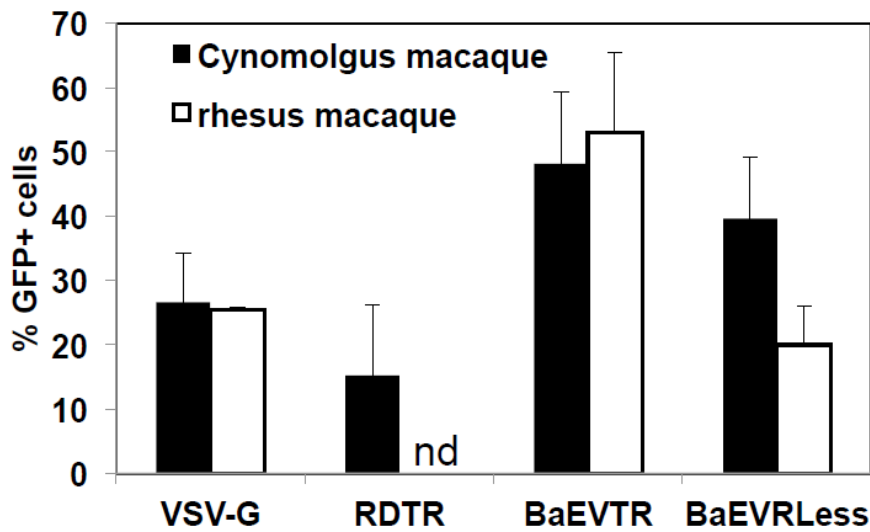
**Supplementary Figure 2: hCD34<sup>+</sup>-cell transduction with different LV pseudotypes at equivalent physical particle doses.**

(A) CD34<sup>+</sup>-cells were prestimulated for 24h (SCF (100ng/ml), TPO, (20ng/ml), Flt3-L, (100ng/ml)) and transduced at the indicated HIV p24 levels with VSV-G-LVs or BaEVTR-LVs. After 24h transduction, cells were washed and cultured in the presence of the same cytokine cocktail for 6 more days. The % of GFP<sup>+</sup> cells was determined by FACS analysis.

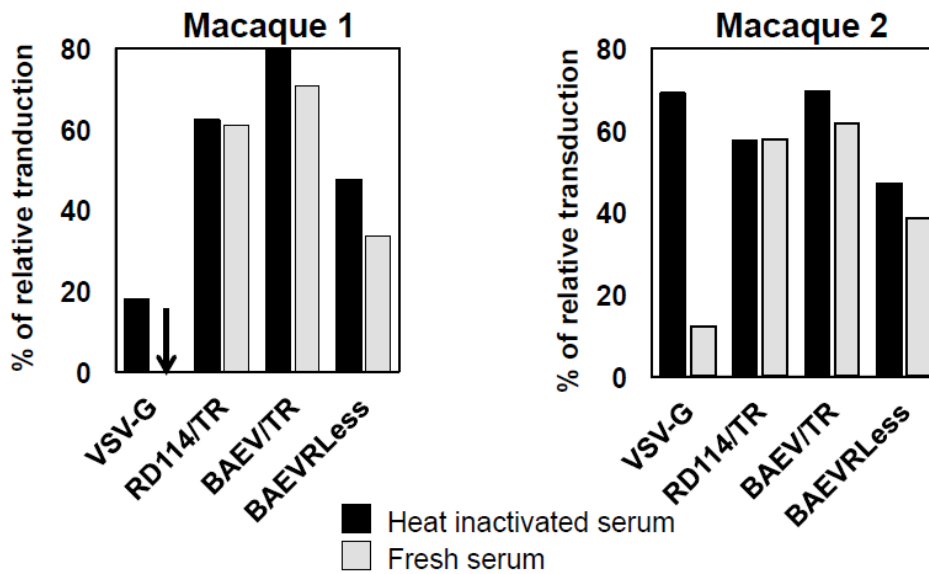
(B) Same transduction protocol as in (A) but using unstimulated hCD34<sup>+</sup>-cells.

\* p<0.05, \*\*p<0.01.

A

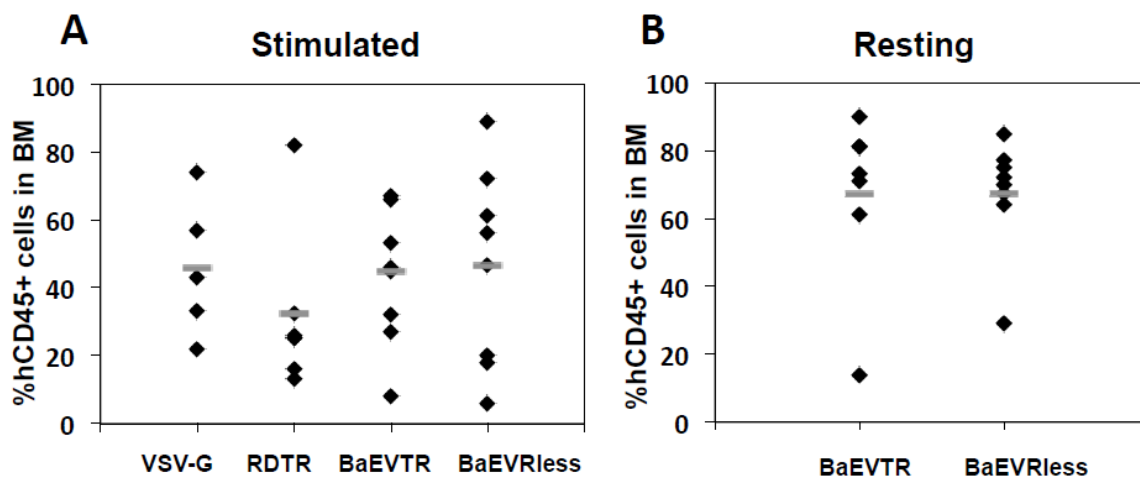


B



**Supplementary Figure 3: SIV vectors pseudotyped with BaEVgps allow efficient transduction of macaque BM CD34<sup>+</sup>-cells**

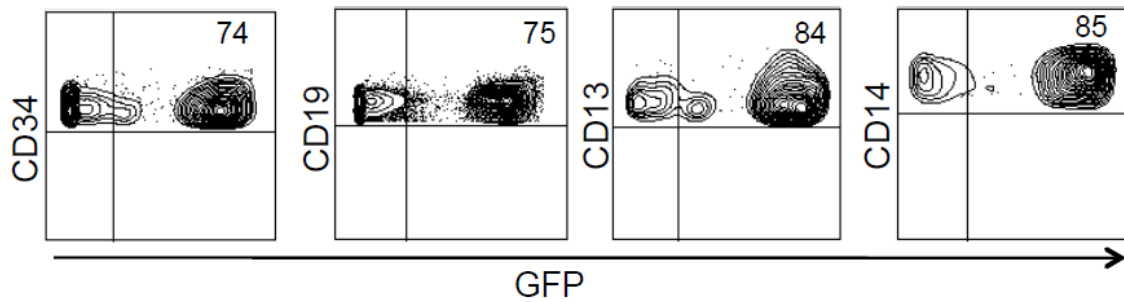
(A) Cynomolgus and rhesus macaque BM CD34<sup>+</sup>-cells were prestimulated with rSCF, rTPO, rFit3-L and IL-3 for 24h and were transduced with SIV-derived lentiviral vectors pseudotyped with RDTR, BaEVTR and BaEVRless gps. All transductions were performed in the presence of retronectin® at MOI of 10 (n=3) except for VSV-G-SIV-LV transductions which were performed at MOI of 100. GFP expression of the cells was analyzed 6 days after transduction by FACS. (B) Percentage of transduction of 293T cells by the indicated LV pseudotypes upon their incubation at 37°C in heat complement inactivated or in fresh macaque sera. Data for 2 different sera are shown.



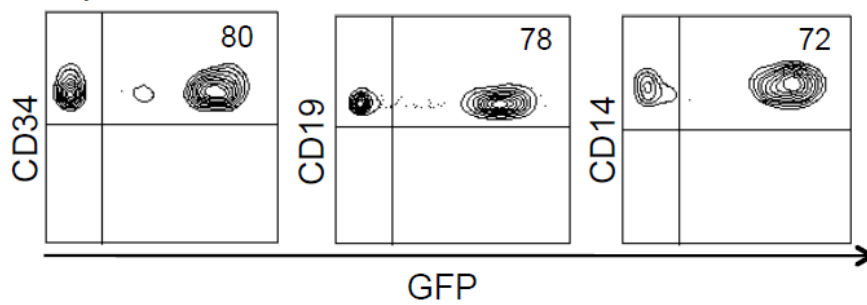
**Supplementary Figure 4: LV-transduced CD34<sup>+</sup>-cell engraftment in NSG mice.**

Prestimulated (A) or resting (B) hCD34<sup>+</sup>-cells were transduced with the indicated LV pseudotypes at MOI=10 and injected intrahepatically into newborn NSG mice. After 12 weeks of reconstitution, the bone marrow (BM) was harvested and assessed for levels of human cell engraftment (hCD45<sup>+</sup>).

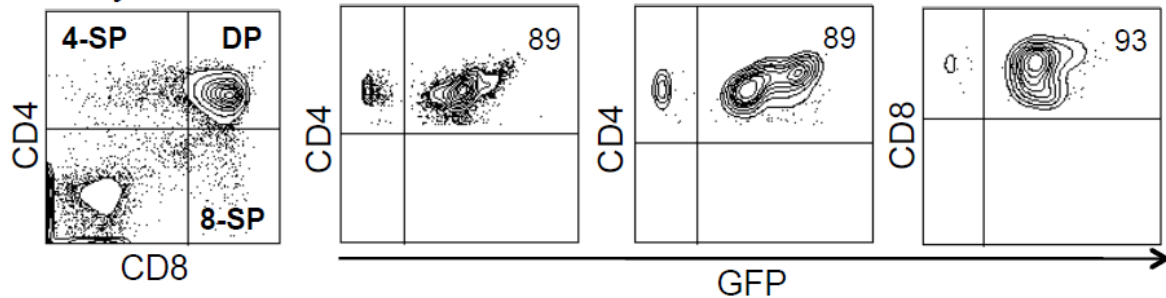
### A. Bone marrow



### B. Spleen

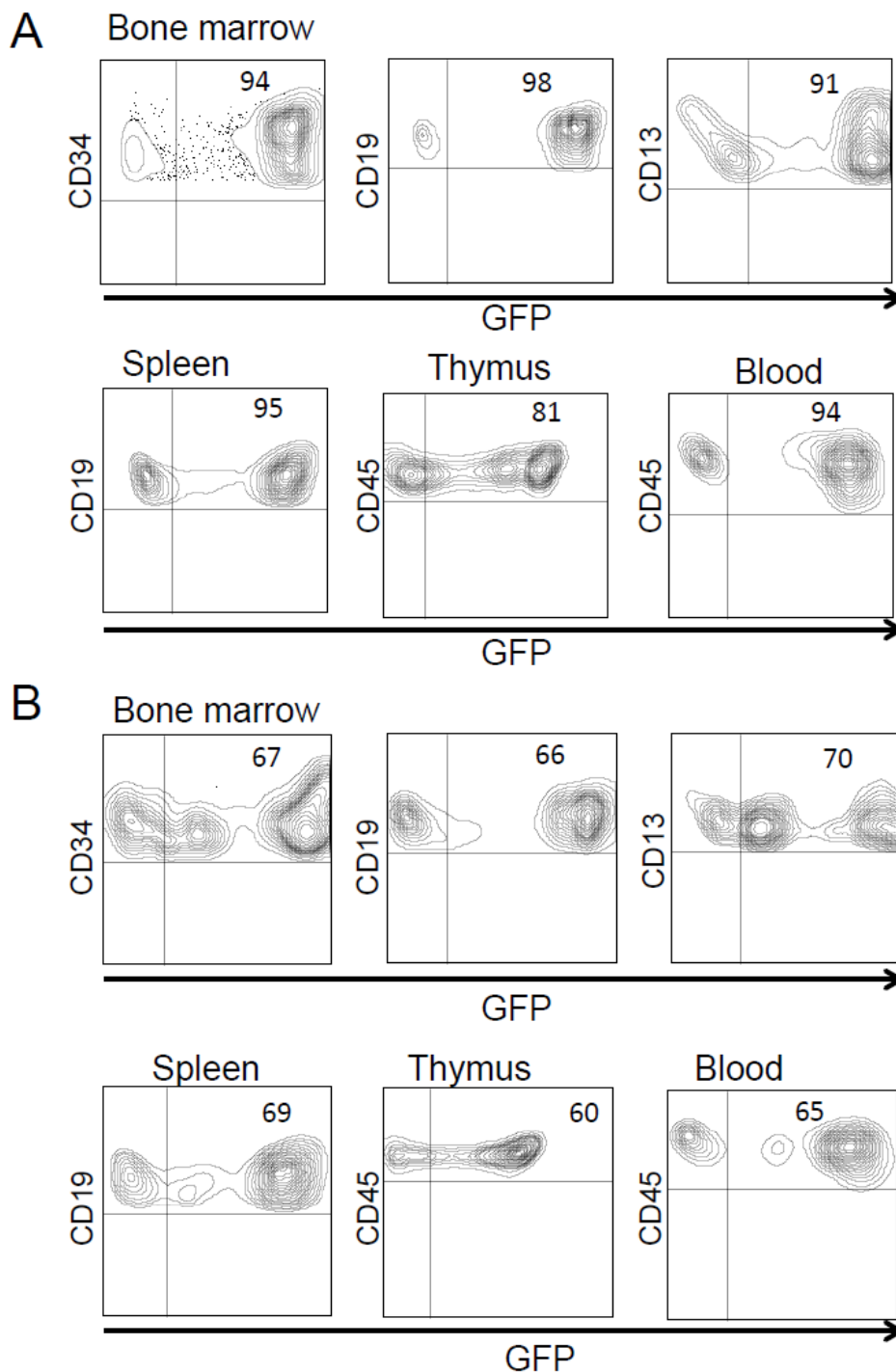


### C. Thymus



### Supplementary Figure 5. BAEV-LVs confer high-level transduction of cytokine-stimulated mobilized hCD34<sup>+</sup>-cells in NSG mice.

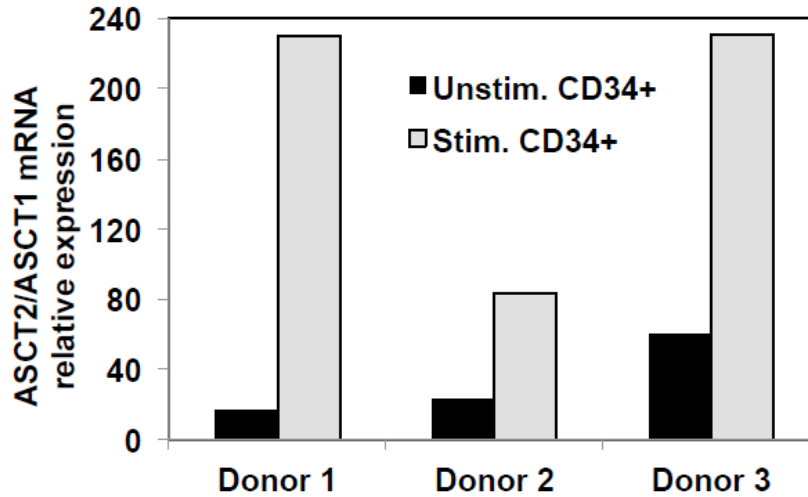
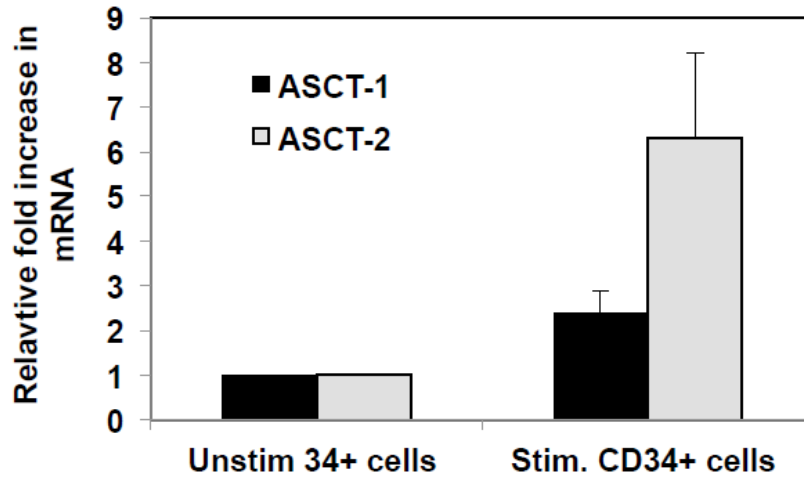
(A) FACS analysis of multilineage engraftment in BM of an NSG mice upon 12 weeks of reconstitution with BaEVRless-LV transduced mobilized hCD34<sup>+</sup>-cells. The upper right quadrants show the GFP<sup>+</sup>-cells within the early progenitors (hCD34<sup>+</sup>), B cells (hCD19<sup>+</sup>), myeloid progenitors (hCD13<sup>+</sup>), and monocytes (hCD14<sup>+</sup>) in the human graft (hCD45<sup>+</sup>-cells). FACS analysis of multi-lineage engraftment in NSG mice of BaEVRless-LV-transduced mobilized hCD34<sup>+</sup>-cells in the spleen is shown in (B), while in (C) the transduced cells in the thymic subpopulations are shown (SP-4 (CD4<sup>+</sup>CD8<sup>-</sup>), SP-8 (CD4<sup>-</sup>CD8<sup>+</sup>) and DP (CD4<sup>+</sup>CD8<sup>+</sup>). The FACS profiles are representative of 2 independent experiments.



**Supplementary Figure 6: BAEVgp-LVs allow efficient transduction of SCID repopulating hCD34<sup>+</sup>-cells**

(A) Analysis of engraftment of transduced human cells (GFP<sup>+</sup>) in the BM, spleen, thymus and blood of secondary transplanted mice reconstituted with hCD34<sup>+</sup>-cells isolated from the primary NSG recipient mice that were reconstituted with pre-stimulated (TPO+SCF+Flk-3L) hCD34<sup>+</sup>-cells transduced with BaEVTR-LVs or BaEVRless-LVs in the presence of retronectin®. (B) Analysis of engraftment of transduced human cells (GFP<sup>+</sup>) in the BM, spleen, thymus and blood of secondary transplanted mice reconstituted with hCD34<sup>+</sup>-cells isolated from the primary NSG recipient mice that were reconstituted with unstimulated hCD34<sup>+</sup>-cells transduced with BaEVTR-LVs or BaEVRless-LVs in the presence of retronectin®.



**A****B**

**Supplementary Figure 7: ASCT-1 and -2 expression levels on unstimulated and cytokine-stimulated CD34<sup>+</sup>-cells.**

(A) Relative mRNA expression levels of ASCT-2 versus ASCT-1 in freshly isolated CD34<sup>+</sup>-cells or CD34<sup>+</sup>-cells stimulated for 24h with TPO(20ng/ml), SCF (100ng/ml) and Flk-3L (100 ng/ml). Data for 3 independent cord blood donors are shown. (B) Relative fold increase in ASCT-1 or ASCT-2 mRNA levels in stimulated CD34<sup>+</sup>-cells (as in (A)) as compared to unstimulated CD34<sup>+</sup>-cells, for which mRNA levels were normalized to 1.

## Supplementary methods

### FACS analysis

In vitro transduction of CD34<sup>+</sup>-cells was detected by anti-CD34-APC antibody.

For the detection of LV transduction of the engrafted human cells in NSG mice, flow cytometry analysis was performed using APC-conjugated anti-hCD45 antibody for the detection of total human cell engraftment in the bone marrow, thymus, peripheral blood and spleen. APC-coupled antibodies are used for the detection of hCD4, hCD56, hCD19, hCD14, and hCD13. Staining of progenitors and early B-cell population in spleen and BM is performed using anti-hCD34-APC, anti-hCD19-PECy7, anti-CD10PE. Staining of thymocytes with anti-hCD3-PE, anti-hCD8-PECy7 and anti-hCD4-APC is performed to screen for thymic subpopulations. Blood samples are stained using anti-CD3APC, anti-hCD19-PECy7, CD14-APC. All antibodies were purchased from BD Biosciences (Le Pont de Claix, France).

Secondary recipient NSG mice for BAEVTR-LV transduction were analyzed for % of GFP<sup>+</sup> human B cells (CD19), human immature progenitor cells (CD34) and myeloid progenitor cells (CD13) by FACS.

### Copy number detection

Estimation of the mean vector copy number per transduced cell was obtained by qPCR performed on human CB CD34<sup>+</sup>-cells ten days after transduction.

Genomic DNA was prepared using NucleoSpin Tissue XS kits (Macherey Nagel, Hoerd, France). Average vector copy number were determined by qPCR with primers amplifying the packaging signal (Primers: Fwd: TGT GTG CCC GTC TGT TGT GT, Rev: GAG TCC TGC GTC GAG AGA GC, Probe: CAG TGG CGC CCG AAC AGG GA) after normalization for endogenous b-actin genes (Primers: Fwd: TCC GTG TGG ATC GGC GGC TCC, Rev: CTG CTT GCT GAT CCA CAT CTG, Probe: CCT GGC CTC GCT GTC CAC CTT CCA). Results were compared with those obtained after serial dilutions of genomic DNA from a cell line containing one copy of the integrated lentiviral vector per haploid genome.

### Production and concentration of lentiviral vectors

Self-inactivating HIV-1-derived vectors were generated by transfection of 2x10<sup>6</sup> 293T cells per 10 cm culture plate in DMEM/10% FCS. Co-transfection by Ca<sub>2</sub>-PO<sub>4</sub> precipitation was performed with the Gag-Pol packaging construct 8.91 (8.6 µg) and the self-inactivating (SIN) HIV-1-derived vector coding for the GFP reporter under the control of a SFFV promoter (SIN-HIVSFFVGFP; 8.6 µg). For display of RDTR, BaEVTR and BaEVRLess gps on LVs, 7 µg of plasmid coding for these gps were co-transfected. For VSV-G-LV production, 3 µg of VSV-G encoding plasmid were transfected. At 16h post-transduction, the supernatant was replaced by Opti-MEM medium supplemented with HEPES (Invitrogen, Carlsbad, Germany). Viral supernatant was harvested 48 h post-transfection and filtrated through a 0.45 µm pore-

sized membrane. Low speed centrifugation of the vectors was performed by overnight centrifugation at 3000xg and 4°C. Concentrated vectors are aliquoted and stored at -80°C. Titration was performed by adding serial dilutions of vector to 293T cells and the MOIs were expressed using 293T titers.

### **Titration of the lentiviral vectors**

#### 1. Titration by FACS analysis

To determine the infectious titers of the HIV vectors, 293T cells were incubated with serial dilutions of the vector supernatants overnight. Medium on the cells is replaced with 2 ml fresh DMEM and cells are incubated for further 72h. The percentage of GFP<sup>+</sup> cells is determined by FACS. To exclude GFP pseudo-transduction and thus verify that expression was only due to integrated vector copies we continued the culture of these 293T cells for 10 more days (4 passages) and determined again the percentage of GFP<sup>+</sup> cells by FACS as also confirmed in parallel the titer by Q-PCR on these same samples (see below).

#### 2. Titration by Q-PCR

Genomic DNA was extracted from the 293T cells (kept in culture for 14 d after transduction) using the genomic DNA purification kit (Nucleospin Tissue XS, Macherey-Nagel, Hoerdt, France). Q-PCR to determine the titer was performed using Taqman gene expression master mix (Life Technologies, St Aubin, France) and following primer pairs and Taqman probes to amplify the lentiviral vector genome:

Lenti-F : 5'-TGT GTG CCC GTC TGT TGT GT-3'

Lenti-R : 5'-GAG TCC TGC GTC GAG AGA GC-3'

Lenti-Probe : 5'-CAG TGG CGC CCG AAC AGG GA-3'

Results were compared with those obtained after serial dilutions of genomic DNA from a 293T cell line containing one copy of the integrated lentiviral vector per haploid genome.

#### 3. Titration for physical particles (HIV p24gag content)

Vectors were titered for physical particles by measuring p24 antigen using an enzyme-linked immunoabsorbant assay (ELISA) following manufacturer's instructions (RetroTek-ZeptoMetrix, Buffalo, NY).

### **ASCT-1 and ASCT-2 mRNA detection by quantitative RT-PCR**

To quantify ASCT1 and ASCT2 gene expression, we used reversed transcription combined with the PCR. Total RNA was isolated from purified unstimulated or SCF/TPO/Flt-3-L stimulated hCB CD34<sup>+</sup>-cells by acid guanidinium thiocyanate-phenol-chloroform extraction. 0.5µg of the total RNA from each sample was reverse transcribed into cDNA using the iScript™ cDNA synthesis kit (BIO-RAD, Marnes-la-Coquette). The amount of ASCT1, ASCT2 and GAPDH mRNA was determined using the Fast Start universal SYBR Green Master MIX (Rox, Roche, France) and the following primers:

- ASCT1 F: 5'- ATT GGT CCT GTT TGC TCT GG - 3'

- ASCT1 R: 5' - TGG GGA GAA TAA ACC TGC TG - 3'

- ASCT2 F: 5'- TAC ATT CTG TGC TGC CTG CT – 3'
- ASCT2 R: 5' – ATG AAA CGG CTG ATG TGC TT – 3'
- GAPDH F: 5' – AAG GTG AAG GTC GGA GTC AA – 3'
- GAPDH R: 5' – AAT GAA GGG GTC ATT GAT GG – 3'

The ASCT1 and ASCT2 mRNA levels were calculated relative to the GAPDH housekeeping gene and normalized to control conditions by using the  $2^{-\Delta\Delta Ct}$  method.

### Complement resistance assay

Stability of vector pseudotypes in human or macaque sera was examined by titrating surviving viral particles after incubation in 1:1 mixtures (vol/vol) of virus preparations with fresh sera for 1 hour at 37°C. Approximately  $5 \times 10^4$  GFP infectious units (IU) of pseudotyped vector particles were used per point. Sera were harvested from healthy human and macaque blood donors. Stability of virions was calculated as the relative percentage of infectivity of macaque or human serum-treated viruses versus viruses treated with foetal calf serum set to 100%.

### Colony-forming-cell (CFC) assays

Three days upon transduction of the CD34<sup>+</sup>-cells, they were washed once and seeded into methyl cellulose containing a cytokine cocktail that supports myeloid differentiation according to manufacturer's recommendation (Stemcell technologies, Grenoble, France). After 14 days of culture in methyl cellulose, GFP<sup>+</sup> colonies were identified by fluorescence microscopy.

**Supplementary table 1: Comparison of titers obtained by Q-PCR and FACS analysis**

	Q-PCR <sup>a</sup> (IU/ml)	FACS <sup>b</sup> (IU/ml)
<b>VSV-G-1</b>	$1.2 \times 10^9$	$1.0 \times 10^9$
<b>VSVG-2</b>	$2.0 \times 10^9$	$2.9 \times 10^9$
<b>BaEVTR-1</b>	$2.5 \times 10^7$	$3.3 \times 10^7$
<b>BaEVTR-2</b>	$2.7 \times 10^7$	$3.0 \times 10^7$
<b>BaEVRLess-1</b>	$4.0 \times 10^7$	$3.5 \times 10^7$
<b>BaEVRLess-2</b>	$1.5 \times 10^7$	$1.1 \times 10^7$

<sup>a-b</sup>: Titer of concentrated vectors obtained by incubation of 293T cells with serial dilution of the vector supernatants. At day 3 post-transduction cells were analyzed for % of GFP positive cells to calculate the vector titer (<sup>b</sup>) and in parallel cells were culture for 14 day, followed by isolation of genomic DNA for determination of titer by Q-PCR(<sup>a</sup>).

**Supplementary table 2.****Titration of the lenti-vector pseudotypes by HIVp24 content**

<b>Pseudotype</b>	<b>p24 (ng/ml) (100x concentrated)</b>
VSV-G	3150±1880
RDTR	3370±1140
BaEVTR	4510±1530
BaEVRLess	3620±1330

**Supplementary table 3. Vector copy number of LV-transduced cytokine prestimulated and unstimulated hCD34<sup>+</sup>-cells.**

<b>Pseudotype</b>	<b>MOI</b>	<b>Vector copy number</b>	
		<b>stimulated CD34</b>	<b>unstimulated CD34</b>
<b>VSV-G</b>	10	3.5 +/- 1.02	ND
<b>VSV-G</b>	100	4.9 +/- 0.89	ND
<b>RDTR</b>	10	2.7 +/- 1.75	ND
<b>BaEVTR</b>	10	4.4 +/- 1.60	3.5 +/- 1.90
<b>BaEVRLess</b>	10	2.8 +/- 1.80	1.3 +/- 1.10

Supplementary Table 4. BaEV-LVs efficiently transduce pre-stimulated hCD34<sup>+</sup>-cells that allow high level NSG engraftment

Pseudotype	BM engraftment	BONE MARROW						SPLEEN						
		CD45+ GFP+ (%)	Early proge-nitor GFP+ (%)	Pre/proB GFP+ (%)	Pro B GFP+ (%)	Mature B GFP+ (%)	Myeloid proge-nitor GFP+ (%)	CD14+ GFP+ (%)	CD45+ GFP+ (%)	Early proge-nitor GFP+ (%)	Pre/proB GFP+ (%)	Pro B GFP+ (%)	Mature B GFP+ (%)	Myeloid proge-nitor GFP+ (%)
<b>VSV-G</b>														
1	22	32	14	15	16	18	20	25	24	17	22	29	22	9
2	33	16	8	30	18	7	11	15	13	7	16	39	16	11
3	43	2	1	1	1	1	0	0	3	10	9	9	9	9
4	74	36	47	31	33	30	49	48	26	ND	ND	ND	ND	ND
<b>RD114/TR</b>														
1	13	37	33	26	33	37	41	46	38	17	27	41	27	27
2	26	69	56	60	74	81	57	45	38	12	17	26	17	11
3	25	12	14	13	12	10	11	5	20	11	6	41	6	11
4	82	34	51	55	31	29	49	41	23	35	61	43	61	27
5	25	52	8	16	51	59	25	17	52	26	27	44	27	12
<b>BaEV/TR</b>														
1	53	29	37	58	33	26	44	41	38	38	49	47	49	49
2	27	92	83	93	89	90	83	73	82	69	84	92	84	80
3	32	69	42	58	55	67	56	71	65	41	60	46	60	62
4	46	75	80	82	78	70	82	69	69	75	71	82	71	65
5	8	88	74	78	81	89	80	67	83	40	67	93	67	65
6	87	56	31	46	62	59	53	47	42	49	39	52	39	44
7	27	74	65	73	73	74	83	75	76	55	80	57	80	50
<b>BaEVR/less</b>														
1	20	40	25	33	32	49	44	41	42	41	27	28	27	25
2	61	52	65	68	52	54	61	60	53	64	54	54	54	61
3	89	87	76	86	87	88	83	84	70	65	66	77	66	73
4	6	95	83	75	86	93	75	80	71	ND	ND	ND	ND	ND
5	56	46	37	39	49	48	57	47	48	53	64	59	64	43
6	72	58	53	65	52	57	57	59	53	46	49	52	49	45
7	18	72	77	69	67	65	69	75	67	60	71	71	71	70

ND : not determined

Supplementary Table 5. BaEV-LVs efficiently transduce quiescent hCD34<sup>+</sup>-cells that allow high-level NSG engraftment

Pseudotype	BM engraftment	BONE MARROW						SPLEEN						
		CD45+ GFP+ (%)	Early proge-nitor GFP+ (%)	Pre/proB GFP+ (%)	Pro B GFP+ (%)	Mature B GFP+ (%)	Myeloid proge-nitor GFP+ (%)	CD14+ GFP+ (%)	CD45+ GFP+ (%)	Early proge-nitor GFP+ (%)	Pre/proB GFP+ (%)	Pro B GFP+ (%)	Mature B GFP+ (%)	Myeloid proge-nitor GFP+ (%)
<b>BaEV/TR</b>														
1	71	8	3	2	10	7	18	19	19	16	20	22	29	
2	73	8	1	1	3	9	6	6	14	3	13	14	25	
3	14	6	5	7	4	34	6	6	8	4	5	5	6	
4	81	16	17	6	17	16	27	12	19	27	26	24	13	
5	90	14	12	10	9	7.3	15	12	16	22	20	18	19	
6	81	36	28	43	29	38	40	42	36	58	44	35	43	
7	61	35	23	29	34	25	27	26	18	20	16	14	27	
<b>BaEVR/less</b>														
1	70	22	12	10	23	24	25	20	16	3	18	16	17	
2	75	22	12	16	19	27	21	14	26	32	29	23	16	
3	29	11	4	2	4	15	11	13	13	15	11	9	11	
4	72	19	12	12	22	17	24	19	20	12	24	22	16	
5	65	11	8	7	14	11	11	10	23	15	28	24	16	
6	77	36	40	55	39	36	39	32	30	43	32	33	40	
7	85	50	49	63	50	47	52	56	52	60	55	50	50	

3) MEASLES VIRUS GLYCOPROTEIN PSEUDOTYPED LVS  
TRANSDUCE CYTOKINE STIMULATED AND RESTING HSCS AT AN  
EFFICIENCY WITHOUT PRECEDENT

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**Title:**

**Measles virus glycoprotein pseudotyped lentiviral vectors transduce cytokine stimulated and resting hematopoietic stem cells at an efficiency without precedent.**

***(Manuscript in preparation)***

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**Running title:** MV-LV high-level transduction of G<sub>0</sub> HSCs

**Keywords:** lentiviral vector, HSCs, pseudotyping, resting cells, hCD34<sup>+</sup>-cells, gene therapy, measles virus

**Personal contribution:**

I designed and performed experiments except for the results presented Figure 3, I analyzed and discussed the data and contribute in writing the manuscriptlj and designing the figures.

## Abstract

Hematopoietic stem cell (HSC) based gene therapy holds promise for the cure of many inherited and acquired diseases. The field is now moving towards the use of lentiviral vectors (LVs) evidenced by several recent clinical trials. In these trials, VSV-G-LVs were applied at high doses to obtain therapeutically relevant transduction levels. Moreover, high gene transfer was only achieved with very strong cytokine-cocktails compromising the 'HSC' character of the cells.

Previously, we have shown that measles virus (MV) glycoprotein displaying LVs (MV-LVs) were able to transduce efficiently stimulated and resting T and B cells, reflecting the natural tropism of MV. Here, we evaluated these MV-LVs for hCD34<sup>+</sup>-cell transduction under mild cytokine prestimulation (TPO, SCF or SCF+TPO) in order to better preserve the 'HSC' characteristics. After a single application at a low vector doses, MV-LVs stably transduced 100% of TPO+SCF stimulated hCD34<sup>+</sup>-cells, where VSV-G-LVs reached 5-10% transduction at the same vector doses. Even more striking was that these MV-LVs allowed at low vector doses efficient transduction of up to 50-70% of quiescent hCD34<sup>+</sup>-cells, an efficacy without precedent. Indeed, VSV-G-LVs only allowed up to 5 % transduction of these quiescent cells even at high vector doses. Importantly, reconstitution of NSG mice with MV-LV transduced pre-stimulated or resting hCD34<sup>+</sup>-cells resulted in transduction levels reaching 100% or 70%, respectively, of all analyzed myeloid and lymphoid engrafted lineages in all hematopoietic tissues. This was also true for secondary mice engraftments. We revealed that the CD46 receptor is required and sufficient for MV-LV entry into hCD34<sup>+</sup>-cells and that a different integration pattern was detected for MV-LVs in resting and stimulated hCD34<sup>+</sup>-cells.

Together, these results strongly suggest that the MV-LVs efficiently transduce true HSCs at very high levels. This paves the way to HSC-based gene therapy of multiple diseases including diseases characterized by low numbers of HSCs such as Fanconi Anemia, for which high level HSC correction is needed to be successful.

## Introduction

Hematopoietic stem cell (HSC) based gene therapy holds promise for the cure of many inherited and acquired diseases. The field is now moving towards the use of lentiviral vectors (LVs) evidenced by recent clinical trials (HSC): successful treatment of patients suffering from adrenoleukodystrophy (ALD)<sup>1</sup>,  $\beta$ -thalassemia<sup>2</sup>, metachromatic leukodystrophy<sup>3</sup> and Wiskott-Aldrich<sup>4</sup> syndrome was achieved by ex vivo correction of autologous HSCs employing LVs. Moreover, new trials for X-SCID and Fanconi anemia, two other severe monogenetic defects in children are being initiated.<sup>5,6</sup>

Genetically modified HSCs should retain similar properties as the unmodified ones. They should be able to both self-renew and differentiate into all the hematopoietic lineages. However, HSCs are poorly permissive for classical vesicular stomatitis virus G (VSV-G)-LV transduction because 75% of them reside in G<sub>0</sub> phase of the cell cycle, thought to be the phase that restricted VSV-G-LV transduction.<sup>7</sup> Indeed, using different lentiviral pseudotypes it was observed that in resting lymphocytes post-entry steps like completion of reverse transcription, nuclear import and chromosomal integration of the transgene do not occur.<sup>7-9</sup> We confirmed in addition a very low expression of the VSV receptor, the low density lipid-receptor (LDL-R;<sup>10</sup>) in unstimulated CD34<sup>+</sup>-cells, perfectly coinciding with their poor VSV-G-LV-mediated transduction.<sup>11</sup> Only 'early-acting-cytokine' stimulation of hCD34<sup>+</sup>-cells, upregulating the LDL-R, permitted high-level VSV-G-LV transduction.<sup>11</sup>

However, intense exposure of HSCs to cytokines might affect their homing, trafficking ability and promote differentiation rather than expansion of the HSC pool.<sup>12,13</sup> High hCD34<sup>+</sup>-cell transduction was only achieved combining high VSV-G-LV doses with strong cytokine-stimulation but the risk for multicopy integration and insertional mutagenesis cannot be neglected under these conditions.<sup>14,15</sup> Therefore, mild cytokine-stimulation allowing efficient HSC transduction is an important objective. Clearly to achieve this objective alternatives to classical VSV-G pseudotypes LVs are urgently needed.

Previously, we engineered LVs pseudotyped with modified measles virus (MV) envelope proteins, hemagglutinin (H), responsible for receptor recognition, and fusion protein (F). Most importantly, they represent the first tool to allow efficient transduction of quiescent human T cells and both healthy and cancerous B cells without inducing entry into the cell cycle or changes in phenotype.<sup>16-18</sup> The MV glycoproteins that are derived from the variant of the MV vaccine strain Edmonston, are truncated in their cytoplasmic tails to allow efficient incorporation into the lentiviral envelope. The respective vector particles (HF-LVs) are able to mediate cell entry via the native MV receptors human CD46 and signaling lymphocyte activation molecule (SLAM).<sup>17,19</sup> CD46 is a complement regulatory molecule expressed on all human nucleated cells<sup>20</sup>, whereas SLAM (CD150) is constitutively expressed at the surface of some T and B subsets and upregulated upon proliferation of T and B lymphocytes and mature dendritic cells.<sup>21,22</sup> Of importance, we found that efficient quiescent lymphocyte transduction only occurs when CD46 and SLAM are correctly engaged by these HF-LVs, which triggers an entry mechanism that strongly resembles macropinocytosis.<sup>23</sup> The recently identified third MV receptor Nectin-4 does not play a role in lymphocyte transduction as it is not expressed on these cell.<sup>24</sup>

Interestingly, the SLAM receptors belong to a new family of cell surface receptors of the immunoglobulin superfamily that regulates several leukocyte functions. These molecules which are cell-cell interaction and signaling receptors, are differentially expressed on distinct type of leukocytes and lymphocytes.<sup>25,26</sup> CD150 receptors are selectively expressed among primitive mouse progenitors in such a way that it is possible to highly purify murine BM and fetal liver HSCs using a simple combination of antibodies (Abs) directed against three of these receptors (CD150+, CD244-, and CD48-).<sup>27,28</sup> Importantly, multipotent progenitors do not express CD150, whereas CD244 and CD48 are expressed. This led to the term “SLAM code,” whose applicability to human CD34<sup>+</sup> HS/PCs is still controversial.<sup>27-31</sup> Indeed, several reports already showed the absence of CD150 on the human CD34<sup>+</sup>CD38<sup>-</sup> hematopoietic early progenitors, a subpopulation highly enriched in stem cells.<sup>30,32</sup>

Here we evaluated for the first time the HF-LVs for transduction of ‘early acting cytokine’ stimulated and unstimulated HSCs. We achieved transduction efficiencies

in prestimulated HSCs close to 100% upon a single step incubation with low doses of HF-LVs. Most remarkably though, was the fact that unstimulated hCD34<sup>+</sup>-cells were transduced up to 70-80%. Even upon transplantation in primary and secondary NSG recipients, this high level of gene modified cell persisted in all hematopoietic organs and in all myeloid and lymphoid lineages, emphasizing the very immature nature of the transduced cells. Thus for the first time, we achieved transduction levels of quiescent HSCs at levels without precedent thereby avoiding loss of 'stem cells' properties.

## **Material and methods**

### **Plasmids**

All Hgps and Fgp are inserted into pCG plasmids under the control of the cytomegalovirus early promoter. Cytoplasmic tails of all Hgps and Fgps were deleted by truncation of 24 and 30 amino acids (aa), respectively. Edm Hgp is the envelope gp form the Edmonston vaccinal strain. The SLAM-restricted vectors (HclinD4/F-LVs), SLAM-blind Hgps (Ha533), H-D4-YG were described previously.<sup>19,33</sup> The pCMV-G plasmid was described previously.<sup>34</sup>

### **Cell lines**

CHO-SLAM (Chinese hamster ovary cells expressing SLAM) cells were grown in RPMI medium (Gibco, Invitrogen, Auckland, New Zealand) supplemented with 10% FCS and 50 µg/ml of penicillin/Streptomycin; 293T (human kidney epithelial cells) were grown in DMEM (Gibco, Invitrogen, Auckland, New Zealand) medium supplemented as for RPMI medium.

### **Lentiviral vector production**

Self-inactivating HIV-1-derived vectors were generated by transient transfection of 293T cells in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) as described previously.<sup>35</sup> Briefly, for codisplay of the different Hgps and Fgps, 3 µg of each envelope plasmid was transfected together with a Gag-Pol packaging plasmid (8.91) and a plasmid encoding a self-inactivating lentiviral vector expressing green fluorescent protein (GFP) under the control of the spleen focus

foamy virus promoter ([SIN]-HIVSFFVGFP). Eighteen hours after transfection, the medium was replaced by Opti-MEM supplemented with HEPES (Invitrogen).

Viral supernatants were harvested 48 h after transfection and filtered. The vectors were concentrated at low speed by overnight centrifugation of the viral supernatants at 3,000g at 4°C. Infectious titers (in transduction units [TU]/ml) were determined by fluorescence-activated cell sorting (FACS) of target cells using serial dilutions of the supernatants added to the appropriate target cell (CHO-SLAM or 293T cells for SLAM- or CD46-tropic vectors, respectively). For detailed titer determination see below.

## **Titration of the lentiviral vectors**

### **1. Titration by FACS analysis**

To determine the infectious titers of the HIV vectors, 293T cells were incubated with serial dilutions of the vector supernatants overnight. Medium on the cells is replaced with 2 ml fresh DMEM and cells are incubated for further 72h. The percentage of GFP<sup>+</sup> cells is determined by FACS. To exclude GFP pseudo-transduction and thus verify that expression was only due to integrated vector copies we continued the culture of these 293T cells for ten more days (4 passages) and determined again the percentage of GFP<sup>+</sup> cells by FACS as also confirmed in parallel the titer by Q-PCR on these same samples (see below).

### **2. Titration by Q-PCR**

Genomic DNA was extracted from the 293T cells (kept in culture for fourteen days after transduction) using the genomic DNA purification kit (Nucleospin Tissue XS, Macherey-Nagel, Hoerd, France). Q-PCR to determine the titer was performed using Taqman gene expression master mix (Life Technologies, St Aubin, France) and following primer pairs and Taqman probes to amplify the lentiviral vector genome:

Lenti-F : 5'-TGT GTG CCC GTC TGT TGT GT-3'

Lenti-R : 5'-GAG TCC TGC GTC GAG AGA GC-3'

Lenti-Probe : 5'-CAG TGG CGC CCG AAC AGG GA-3'

Results were compared with those obtained after serial dilutions of genomic DNA from a 293T cell line containing one copy of the integrated lentiviral vector per haploid genome.

### **3. Titration for physical particles (HIV p24gag content)**

Vectors were titered for physical particles by measuring p24 antigen using an enzyme-linked immunoabsorbant assay (ELISA) following manufacturer's instructions (RetroTek- ZeptoMetrix, Buffalo, NY)

### **Sample collection and isolation of CD34<sup>+</sup>-cells**

Cord blood (CB) samples and mobilized blood were collected in sterile tubes containing the anti-coagulant, citrate-dextrose (ACD, Sigma, France) after informed consent and approval was obtained by the institutional review according to the Helsinki declaration. Human and macaque CD34<sup>+</sup>-cells were isolated as previously described.<sup>36,37</sup>

### **Transduction of human and macaque CD34<sup>+</sup>-cells**

Human and macaque CD34<sup>+</sup>-cells were incubated for 14h or 18-24h (as indicated) in 24-well plates in serum-free medium (CellGro, CellGenix, Germany) supplemented with human recombinant cytokines (as indicated): SCF (100ng/ml), TPO, (20ng/ml), Flt3-L, (100ng/ml), IL-6 (20ng/ml) and IL-3 (20ng/ml) (Preprotech, France). 5x10<sup>4</sup> prestimulated CD34<sup>+</sup>-cells were transduced in 48-well plates with concentrated LVs at indicated MOIs in serum-free medium. Cells were replenished with cytokines every three days. Three, six and fourteen days after transduction, the % of GFP<sup>+</sup>-cells was determined by FACS. Alternatively, CD34<sup>+</sup>-cells were freshly isolated and transduced immediately in absence of cytokines. Where indicated, transductions were performed on Retronectin® coated plates according to manufacturer's instructions (Takara, Japan). For myeloid differentiation of CD34<sup>+</sup>-cells see Supplementary methods.

### **Receptor blocking assay.**

CD34<sup>+</sup>-cells were seeded in 96-well plates and incubated for 2 h with anti-CD46 (clone 13/42; gift from R. Buckland, CIRI, Lyon, France), anti-SLAM (IPO-3; eBioscience, France) or anti-Nectin-4 (R and D System, Mineapolis USA) antibodies prior to infection. Concentrated viral supernatants were added to the cells at an MOI of 10, and three days after infection, transduction efficiency was analyzed by FACS.



## **Conditioning and reconstitution of NSG mice**

NSG mice used were housed in our animal facility (PBES-Lyon, France). Experiments were performed in accordance with the EU guidelines upon approval of the protocols by the local ethical committee (Autorization agreement N° C2EA -15: CECCAPP, Lyon, France).

Two to 3-day-old newborn NSG mice were subjected to 1 Gy irradiation and  $2 \times 10^5$  transduced prestimulated CB CD34<sup>+</sup>-cells were injected intra-hepatically. Unstimulated freshly isolated CD34<sup>+</sup>-cells were transduced for 24h and  $3 \times 10^5$  transduced CD34<sup>+</sup>-cells were injected into NSG mice as above. After 12 weeks of reconstitution, extensive cell phenotyping in the different hematopoietic tissues by flow cytometry (BM, spleen, thymus, blood) of these mice was performed (see Supplementary methods).

For secondary engraftments, the CD34<sup>+</sup>-cells were isolated from the BM of primary engrafted mice as above and  $4-5 \times 10^5$  isolated CD34<sup>+</sup>-cells were injected into newborn NSG mice following the same procedure and phenotypic analysis as above.

## **DNA extraction and Quantitative PCR**

Estimation of the mean vector copy number per transduced cell was obtained by qPCR performed on human CB CD34<sup>+</sup>-cells ten days after transduction.

Genomic DNA was prepared using NucleoSpin Tissue XS kits (Macherey Nagel, Hoerd, France). Average vector copy number were determined by qPCR with primers amplifying the packaging signal (Primers: Fwd: TGT GTG CCC GTC TGT TGT GT, Rev: GAG TCC TGC GTC GAG AGA GC, Probe: CAG TGG CGC CCG AAC AGG GA) after normalization for endogenous  $\beta$ -actin genes (Primers: Fwd: TCC GTG TGG ATC GGC GGC TCC, Rev: CTG CTT GCT GAT CCA CAT CTG, Probe: CCT GGC CTC GCT GTC CAC CTT CCA). Results were compared with those obtained after serial dilutions of genomic DNA from a cell line containing one copy of the integrated lentiviral vector per haploid genome.

## **Cell cycle analysis**

Performed by staining DNA and RNA with 7-amino-actinomycin-D (7AAD) and pyronin-Y as previously described.<sup>16</sup>

## **FACS analysis**

Receptor surface expression was detected with anti-CD46-PE, anti-SLAM-PE (BD Biosciences) and anti-Nectin-4-PE (R and D Systems, Minneapolis, USA ).

In vitro transduction of hCD34<sup>+</sup>-cells was detected by APC-coupled anti-CD34 antibody. Transduced hCD34<sup>+</sup>-cells were also stained with anti-CD34-Pacific blue, anti-CD90-APC and anti CD38PE. Cells dissociated from the CFC colonies were washed once in PBS and stained with anti-GlyA-PE, anti-CD14APC, anti-CD15PECy7, antiCD71PE. All these antibodies were purchased from BD Biosciences.

For the detection of LV transduction of the engrafted human cells in NSG mice flow cytometry analysis was performed using APC-conjugated anti-hCD45 antibody for the detection of total human cell engraftment in the bone marrow, thymus, peripheral blood and spleen. APC-coupled antibodies were used for the detection of hCD4, hCD56, hCD19, hCD14, and hCD13. Staining of progenitors and early B-cell population in spleen and BM was performed using anti-hCD34-APC, anti-hCD19-PECy7, anti-CD10PE. Staining of thymocytes with anti-hCD3-PE, anti-hCD8-PECy7 and anti-hCD4-APC was performed to screen for thymic subpopulations. Blood samples were stained using anti-CD3APC, anti-hCD19-PECy7, CD14-APC. All antibodies were purchased from BD Biosciences.

Secondary recipient NSG mice for BAEVTR-LV transduction were analysed for % of GFP<sup>+</sup> hB cells (hCD19<sup>+</sup>), human immature progenitor cells (CD34<sup>+</sup>) and myeloid progenitor cells (CD13<sup>+</sup>) by FACS.

## **Results**

### **HF-LVs outperform VSV-G-LVs for the transduction of immature hCD34<sup>+</sup>-cells upon mild cytokine-prestimulation and at low vector doses.**

HSCs are important gene therapy targets since upon correction they can reconstitute and correct the entire hematopoietic system.<sup>1-4</sup> Since the lentiviral vectors displaying the measles virus glycoprotein (HF-LVs) developed in our lab were successful in transducing multiple other hematopoietic cells, we tested these vectors for early progenitor hCD34<sup>+</sup>-cell transduction. We pre-stimulated hCD34<sup>+</sup>-cells with multiple cytokines (SCF/TPO/Flt3-L) and transduced them with VSV-G- and HF-LVs at a MOI of 10 (in addition MOI of 100 for VSV-G-LVs was used) in the presence or

absence of the transduction facilitating agent, Retronectin. The HF-LVs outperformed the other pseudotypes for the transduction of hCD34<sup>+</sup>-cells, in the presence of Retronectin (Figure 1A). Importantly, under these conditions up to 100% of the hCD34<sup>+</sup>-cells were transduced by a single exposure to the HF-LVs. Since it is difficult to compare different pseudotypes using their infectious titers, we also transduced stimulated CD34<sup>+</sup>-cells with equivalent amounts of physical particles (HIV p24). Previously, we verified that p24 levels (ng/ml) were equivalent for HF-LVs and VSV-G-LVs.<sup>38</sup> Significantly higher transduction of CD34<sup>+</sup>-cells for HF-LVs as compared to VSV-G-LVs was confirmed (Supplementary Figure 1).

Strong stimulation with a cytokine-cocktail can induce HSC-differentiation and loss of self-renewal and homing capacity. Thus, the less the cells are prestimulated, the more they keep their 'stem cell' characteristics.<sup>12,13,36,37,39</sup> Therefore, we performed LV transductions of hCD34<sup>+</sup>-cells using different cytokine-prestimulation conditions: 1) stimulation with a single cytokine (rSCF or rTPO); 2) rSCF+rTPO; 3) rSCF+rTPO+rFlt3-L (Figure 1B). Remarkably, TPO-stimulation is sufficient to transduce hCD34<sup>+</sup>-cells up to 75% using HF-LVs, while VSVG-LVs transduced on average only 5 % of the hCD34<sup>+</sup>-cells at low vector doses (MOI 10). A single SCF-prestimulation permitted up 90% HF-LV mediated transduction of hCD34<sup>+</sup>-cells, while VSVG-LVs reached maximum transduction levels of 10% at MOI of 10. A combination of TPO+SCF prestimulation and a single hit with HF-LV resulted in up to 98% hCD34<sup>+</sup>-cell transduction, while VSVG-LVs, at same MOI, achieved on average 10% transduction. The triple cytokine-cocktail (TPO+SCF+Flt-3L) resulted still similar transduction levels for the HF-LVs (up to 100 %), while at the same vector doses (MOI =10), VSV-G-LVs did not even transduce 10% of hCD34<sup>+</sup>-cells. Even when VSV-G-LVs were employed at MOI of 100, HF-LVs gene transfer efficiencies were still significantly higher at MOI of 10 and this for all the different stimulation protocols (Figure 1B).

For both rSCF+rTPO and rSCF+rTPO+rFlt3-L stimulations, stable transduction was demonstrated by differentiation of the transduced hCD34<sup>+</sup>-cells into myeloid lineages (Figure 1C and D).

Since a lower vector copy number/cell might reduce the risk of genotoxicity, we tested different vector doses under rSCF+rTPO and rSCF+rTPO+rFlt3-L

stimulation conditions. HF-LVs transduced significantly higher levels of hCD34<sup>+</sup>-cells at low vector doses than the VSV-G-LVs. They reached 90% transduction for both cytokine stimulation conditions at an MOI of 5, while VSV-G-LVs did not even reach 5% transduction (Figure 1E and F). Only at MOI of 100, the VSV-G-LVs allowed efficient hCD34<sup>+</sup>-cell transduction rates (60%), equivalent to the performance of HF-LVs at a MOI of 1 (Figure 1E and F). For as well rSCF+rTPO and rSCF+rTPO+rFlt3-L cytokine-stimulations, HF-LV transduction reached a plateau at 90-100 % for a MOI of 5. As expected, increasing vector doses coincided with increasing vector copy number/cell (Supplementary Figure 2).

Mobilized hCD34<sup>+</sup>-cells are readily accessible valuable target cells for gene therapy in the clinic. Similar to CB hCD34<sup>+</sup>-cells, HF-LVs allowed > 95 % transduction of mobilized hCD34<sup>+</sup>-cells and this transduction level persisted in colony forming cells (CFCs) derived from these cells indicating that short-term progenitors were stably transduced (Supplementary Figure 3).

Since MV can infect non human primates, an important preclinical animal model, we confirmed that BM CD34<sup>+</sup>-cells were efficiently transduced by simian immunodeficiency virus (SIV) derived vectors pseudotyped with H and F MV gps (up to 90% transduction, Supplementary Figure 4).

Concluding, HF-LVs outperformed VSV-G-LVs by far for gene transfer in hCD34<sup>+</sup>-cells, even when using low cytokine-stimulation combined with low vector doses.

### **HF-LV pseudotypes permit high-level transduction of resting hCD34<sup>+</sup>-cells**

Since HF-LVs permitted efficient transduction of hCD34<sup>+</sup>-cells upon stimulation with a single cytokine (e.g TPO or SCF; Figure 1B), we hypothesized that their binding and signaling through the MV receptors might be sufficient to transduce unstimulated hCD34<sup>+</sup>-cells as demonstrated previously for T and B cells.<sup>16,17</sup> Omitting cytokine stimulation in the transduction protocol might avoid differentiation and loss of the HSC subpopulation.<sup>12,13</sup>

Freshly isolated hCD34<sup>+</sup>-cells were therefore transduced with HF- and VSV-G-LVs in the absence of exogenous stimuli. Importantly, the HF-LVs were able to

efficiently transduce resting hCD34<sup>+</sup>-cells at levels not reported before (40-70%) in the absence of any stimulus following a single exposure at low vector doses (MOI=10; Figure 2A). In agreement with our previous reports<sup>11,40,41</sup>, the VSV-G-LVs were unable to transduce efficiently resting hCD34<sup>+</sup>-cells, even when applied at high vector doses (Figure 2A; MOI 100). In contrast to cytokine prestimulated hCD34<sup>+</sup>-cells, their resting counterparts require at least an MOI of 10 for efficient MV-LV gene transfer. Lower vector doses (MOI=5) resulted in a sharp drop of transduction reaching 10% on average (Figure 1E, F versus 2B).

We next verified if HF-LV mediated transduction did not skew the CD34<sup>+</sup>-cell population. Upon HF-LV transduction of resting hCD34<sup>+</sup>-cells we gated on gene modified (GFP<sup>+</sup>) and untransduced cells (GFP<sup>-</sup>). We demonstrated that the most immature population enriched in HSCs, which is characterized by surface markers CD34<sup>+</sup>CD38<sup>-</sup>CD90<sup>+</sup>, was equivalent for both subpopulations indicating that HF-LV transduction did not induce skewing. Importantly, this suggest that this HSC-enriched population was efficiently transduced by the MV-LVs (Figure 2C and data not shown).

As efficient transduction of hCD34<sup>+</sup>-cells with classical VSV-G-LVs requires cytokine-pre-stimulation<sup>11</sup>, which leads to cell cycle entry (Figure 2D) and probably differentiation, we wanted to exclude that HF-LV binding to the MV receptors induced hCD34<sup>+</sup>-cell cycle entry. Using a method that allows simultaneous visualization of DNA and RNA<sup>42</sup>, we evaluated cell cycle entry upon transduction with the different pseudotypes (Figure 2D). As controls, hCD34<sup>+</sup>-cells were incubated without vector in the absence or presence of cytokine stimulation. Although HF-LVs resulted in high-level hCD34<sup>+</sup>-cell transduction (Figure 2A), these cells did not actively enter into the cell cycle but resided into the G0/G1a cell cycle phase. This is essential since the majority of the hCD34<sup>+</sup>-cells with SCID repopulating potential reside in the G0 phase of the cell cycle.<sup>43,44</sup> A low vector copy number/cell was revealed for the HF-LV transduction of CD34<sup>+</sup>-cells (Supplementary Figure 2).

Measles virus infections are characterized by a generalized immune suppression, which involves inadequate activation and maturation of antigen presenting cells such as dendritic cells; also transient lymphopenia is a hallmark of

MV-induced immunosuppression. Reports on the effect of MV on bone marrow cells in vivo and its consequence on hematopoiesis leading to immunosuppression are still contradictory.<sup>30,32</sup> Since Boussaad et al.<sup>32</sup> reported a suppressing effect of MV-LVs on the development of hCD34<sup>+</sup> derived short-term progenitors, we wanted to verify if HF-LVs did not interfere with the differentiation of hCD34<sup>+</sup>-cells into myeloid lineages. HF-LV transduced hCD34<sup>+</sup>-cells were plated into cytokine-enriched methyl cellulose and GFP expression was detected after fourteen days. The HF-LV hCD34<sup>+</sup> gene transfer levels persisted in the myeloid colonies (CFCs) confirming that short-term progenitors were stably transduced (Figure 2E). Equivalent numbers of CFCs for HF-LV transduced and untransduced cells were obtained and FACS analysis of the bulk CFC colonies showed the presence of the different myeloid lineages all transduced to the same extent: erythrocytes, granulocytes, monocytes and macrophages, as also immature proliferating progenitors (Figure 2F). Moreover, the frequencies of BFU-E, CFU-GM, CFU-granulocyte erythroid macrophage megakaryocyte colonies observed for HF-LV transduced cells did not significantly differ from un-transduced counterparts (data not shown).

Thus, HF-LVs allow for the first time very high-level transduction of resting hCD34<sup>+</sup>-cells without skewing this cell population, nor affecting their short term myeloid differentiation capacity.

### **MV-LV gene transfer to high levels of resting and cytokine-activated CD34<sup>+</sup>-cells due to binding and entry through the CD46 receptor**

Since these H/F-LVs are able to transduce early-cytokine stimulated and quiescent hCD34<sup>+</sup>-cells it was important to elucidate the roles of the MV receptors, CD46, SLAM and Nectin-4, in the transduction process of MV-LVs in these primary gene therapy target cells. Previously, we have shown that both SLAM and CD46, are required for MV-LV entry into lymphocytes and dendritic cells.<sup>19,38</sup> Therefore, initially the expression levels of the MV receptors were quantified by FACS analysis on hCD34<sup>+</sup>-cell surface (Fig 3A). CD46 was expressed on the vast majority of cytokine-stimulated and resting hCD34<sup>+</sup>-cells (98% and 99% respectively). Of note, CD46 expression levels were enhanced on cytokine stimulated hCD34<sup>+</sup>-cells, and marked by 2-fold increase in MFI as compared to resting cells (Figure 3A). In contrast, SLAM was almost undetectable at the surface of as well quiescent as stimulated hCD34<sup>+</sup>-

cells in agreement with a previous reports<sup>30,32</sup>, which confirmed that SLAM, a valid surface marker for murine HSCs is not applicable for human HSCs (Figure 3A and <sup>29</sup>). Nectin-4 was also barely present in agreement with its targeted expression on endothelial cells<sup>24</sup>. When CD46 expression was evaluated after different timepoints of early-acting-cytokine stimulation, a gradual upregulation of CD46 density was detected at hCD34<sup>+</sup>-cell surface (Figure 3B). In order to establish a link between CD46 expression and hCD34<sup>+</sup>-cells, as well resting as stimulated hCD34<sup>+</sup>-cells were sorted into two subpopulations, one with high and one with low CD46 surface density and subsequently transduced with HF-LVs. The lowest transduction levels perfectly coincided with the lowest CD46 surface density on these cells (Figure 3C). In order to get a better insight into the roles of the MV receptors in H/F-LV transduction, hCD34<sup>+</sup>-cell were isolated and immediately incubated with specific mAbs against CD46, SLAM or Nectin-4 receptors before transduction with H/F-LVs. The blocking of the CD46 receptor on stimulated and quiescent hCD34<sup>+</sup>-cells completely abrogated H/F-LV-transduction while blocking the SLAM or Nectin 4 receptors had no significant effect (Fig 3D and 3E). Moreover, we already demonstrated before that HF-LVs efficiently bind to as well resting as cytokine-activated CD34<sup>+</sup>-cells.<sup>11</sup>

In order to study the roles of SLAM and CD46 in the transduction of hCD34<sup>+</sup>-cells, we employed a series of LVs pseudotyped with different H envelope glycoproteins (gps) (Figure 3F): 1) Hgp that contains both binding residues to SLAM and CD46 (H;<sup>17,35</sup>); 2) a CD46-tropic H mutant in which one residue (R533A) of Edm-Hgp responsible for binding/fusing through SLAM was ablated (H533)<sup>45</sup>; 3) SLAM-tropic Hgps derived from clinical strains (H-D4)<sup>46,47 48</sup>; 4) a SLAM/CD46 binding mutant was engineered by adding the mutation N481Y (responsible for binding to CD46) to the clinical strain H-D4. The mutation E492G present in Edm strain Hgp was included in addition as it has been shown to stabilize CD46 binding and the mutant was named H-D4-YG<sup>49,50</sup> (Figure 3F).

H533/F-LVs which do not recognize SLAM receptor was capable of transducing cytokine prestimulated hCD34<sup>+</sup>-cells while H-D4/F-LVs which do not recognize CD46 receptor were refractory for transduction of these cells. Introduction of the CD46 binding sites in HD4-YG/F-LVs restored transduction. Results showed

that transduction was impossible when CD46 binding sites in H were absent whereas presence of CD46 binding residues permitted efficient transduction.

These results showed that CD46 was the only MV receptor involved in HF-LVs entry into hCD34<sup>+</sup>-cells and that the level of transduction was highly correlated with CD46 surface density.

### **HF-LVs promote high-level transduction of prestimulated and resting SCID repopulating cells in primary recipient NSG mice.**

Since the NOD/SCID  $\gamma_c^{-/-}$  mice (NSG<sup>51,52</sup>) model allowed high level human cell engraftment is was our model of choice to evaluate the long-term reconstitution capacity of HF-LV-transduced hCD34<sup>+</sup>-cells *in vivo*. To address this question we transplanted prestimulated CB hCD34<sup>+</sup>-cells transduced with HF- and VSVG-LVs into irradiated NSG mice. A comparable high level and variability of engraftment with human cells was detected for both pseudotypes (Figure 4A), indicating that the migration and engraftment of these transduced early progenitors was not impaired.

Overall, VSVG-LVs allowed a much lower transduction of SCID repopulating cells (SRCs) as compared to HF-LVs. HF-LVs transduced hCD34<sup>+</sup>-cells resulted in the highest transduction levels of engrafted human cells in the BM (up to 100 % CD45<sup>+</sup>GFP<sup>+</sup>-cells). These high transduction levels were sustained in the primary recipient mice in all hematopoietic tissues (BM, spleen and thymus; Figure 4B). Detailed flow cytometry analysis of the BM revealed equivalent high level transduction for the different B cell subpopulations (CD19<sup>+</sup> preB/proB, proB and immature/mature B cells), CD13<sup>+</sup> myeloid progenitors and CD14<sup>+</sup> monocytes (Figure 5A). Importantly, for the HF-LVs equivalent high levels of GFP<sup>+</sup> early progenitors (CD34<sup>+</sup>CD19<sup>-</sup>CD10<sup>-</sup>) were detected. In contrast, we observed for VSV-G-LVs a high transduction variability between different tissues and the different cell lineages in the BM (Figure 5A). Consistent with these results, detailed phenotyping of the splenocytes revealed a similar transduction profile (Figure 5B) and also the thymocytes and all thymic subpopulations were transduced to the same extent (Supplementary Figure 5).



In order to evaluate if HF-LVs transduced long-term reconstituting HSCs in the resting hCD34<sup>+</sup>-cell population, we applied a short transduction protocol (18-24h) at low vector doses (MOI=10) without any addition of cytokines before injection into NSG newborn mice. High-level reconstitution with human cells was detected for HF-LV transduced G0 hCD34<sup>+</sup>-cells, indicating that the homing, engraftment and differentiation capacity of these cells was not affected (Figure 4C). Moreover, the human cell engraftment for HF-LV transduced G0 hCD34<sup>+</sup>-cells was as high as for their pre-stimulated counterparts (Figure 4A versus 4C). Interestingly, two groups of mice could be identified: the low transduction group (up to 50%) and the group with high (>80%) transduction efficiencies. For the high transduction groups equivalent high transduction rates in the BM and the spleen were revealed (Figure 4D), while the low transduction group repetitively showed lower thymocyte transduction. As for the prestimulated CD34<sup>+</sup>-cells, detailed flow cytometry analysis of the BM revealed equivalent high level transduction for the different B cell subpopulations (CD19<sup>+</sup> preB/proB, proB and immature/mature B cells), CD13<sup>+</sup> myeloid progenitors and CD14<sup>+</sup> monocytes (Figure 5C). Importantly, for the HF-LVs equivalent high levels of GFP<sup>+</sup> early progenitors (CD34<sup>+</sup>CD19<sup>-</sup>CD10<sup>-</sup>) were detected. Consistent with these results, detailed phenotyping of the splenocytes revealed a similar transduction profile (Figure 5D).

### **H/F-LVs promote high-level transduction of prestimulated and resting HSCs**

To confirm that true HSCs were gene-modified, secondary reconstitutions of NSG mice were performed with hCD34<sup>+</sup>-cells isolated from primary NSG mice previously reconstituted with HF-LV transduced prestimulated hCD34<sup>+</sup>-cells (Figure 6A and B). To improve engraftment into secondary recipients the primary NSG BM hCD34<sup>+</sup>-cells were cultured before injection in SCF and IL-6 to maintain their homing capacity.<sup>53</sup> At 12 weeks post-transplantation, the BM and spleen of the secondary recipient mice were analyzed for the percentage of hCD45<sup>+</sup>GFP<sup>+</sup>-cells (Figure 6A). In the spleen and BM the % of CD45<sup>+</sup>GFP<sup>+</sup>-cells was maintained or even increased in the secondary NSG recipients. Importantly, for HF-LVs high levels of CD34<sup>+</sup>GFP<sup>+</sup> early progenitors, lymphoid (CD19<sup>+</sup>) and myeloid (CD13<sup>+</sup> and CD14<sup>+</sup>) cells were detected in the BM of these secondary recipient mice and equivalent percentages of

GFP<sup>+</sup>-cells were detected in the different lymphoid and myeloid lineages in BM (data not shown).

Secondary reconstitutions of NSG mice were also performed for the HF-LV transduced resting hCD34<sup>+</sup>-cells. The percentage of CD45<sup>+</sup>GFP<sup>+</sup>-cells in the BM was increased in four secondary recipient mice as compared to primary NSG mice (Figure B). Importantly, for HF-LVs high levels of transduced CD34<sup>+</sup> early progenitors, lymphoid (CD19<sup>+</sup>) and myeloid (CD13<sup>+</sup> and CD14<sup>+</sup>) cells were detected in the BM of these secondary recipient mice (Figure 6C). In the spleen of these secondary recipient mice, equivalent percentages of GFP<sup>+</sup>-cells were detected in myeloid cells (CD14<sup>+</sup>) and lymphoid cells (CD19<sup>+</sup>) (Figure 6D).

In summary, these HF-LVs allowed high-level genetic modification of very early progenitor repopulating cells, so-called hHSC in the prestimulated and unstimulated hCD34<sup>+</sup>-cell population, able to home, engraft and differentiate into all the different lineages in primary and secondary NSG mice.

Overall, these data strongly suggest that HF-LVs allowed genetic modification of very early progenitor cells, hHSCs, allowing long-term engraftment and differentiation into all the different cell lineages in vivo.

## **Discussion**

Highly efficient gene delivery without compromising stem cell function is a holy grail in HSC gene therapy. Here we show that measles virus glycoprotein pseudotyped LVs (H/F-LVs) permit to transduce HSCs, not exposed to any exogenous stimulation agent, to an extent not reported before (50-70% transduction). This results were strengthened by the conservation of high transduction levels in lymphoid and myeloid lineages in all hematopoietic tissues of primary recipient NSG mice. Secondary engraftment showed a remarkable enhancement of transduction in the whole hematopoietic lineage in 100% of the NSG mice without any exception. This suggest that, although HF-LVs transduce efficiently very primitive and progenitor hematopoietic cells in the quiescent state, the effect is more pronounced in the primitive HSCs and points toward a preservation of the primitive HSCs. Note that transduction in mildly cytokine stimulated hCD34<sup>+</sup>-cells still increased transduction

(up to 100%). This means that the HF-LVs overcome an important barrier for clinical gene therapy using the classical VSVG-LVs, which demonstrate limited efficiency in particular in resting HSCs<sup>11,40,41</sup>.

It is essential to transduce the HSCs without changing their capacity to self-renewal, homing and differentiation capacity in the whole hematopoietic lineage, which is supported by minimal or no stimulation of these targets. Indeed, culture of HSCs in cytokines leads to loss of engraftment potential in immunodeficient mice while VSV-G-LV transduction remains limited. Thus, there is a trade-off between transduction efficiently and engraftment potential for classical VSV-G-LVs but not for HF-LV mediated resting HSC transduction.

Wang and colleagues<sup>54</sup> showed that rapamycin, an inhibitor of mTOR complexes, strongly facilitated efficient VSV-G-LV transduction in pre-stimulated hCD34<sup>+</sup>-cells also reaching 100% and enhanced marking frequency of longterm engrafting SCID repopulating cells. However, the effect of VSV-G-LV transduction on resting hCD34<sup>+</sup>-cells was not enhanced at all by the addition of this drug<sup>54</sup>. This is according to our recent data due to the absence of the VSV receptor, LDL-R, on resting hCD34<sup>+</sup>-cells whereas we demonstrated a clear upregulation by early acting cytokine stimulation of LDL-R<sup>11</sup>. This was in agreement with an increased binding of VSV-G-LVs to cytokine stimulated hCD34<sup>+</sup>-cells as compared to resting cells. Note that rapamycin treatment does not influence LDL receptor levels at the hCD34<sup>+</sup>-cell surface. These authors suggest that rapamycin enhances post-binding events, leading to increased levels of LV cytoplasmic entry, reverse transcription and genomic integration. Thus, it is tempting to speculate that HF-LV transduction of resting hCD34<sup>+</sup>-cells at low vector doses (MOI 1-3) might still be increased by addition of rapamycin through release of post-entry blocks in resting CD34<sup>+</sup>-cells. Especially, Wang et al. confirmed that the rapamycin transduction enhancing effect was also valid for LV pseudotyped with Lassa virus envelope. However, caution in use of this drug is warranted because of the known block in cell cycle progression that it induces<sup>54</sup>.

Importantly for clinical application, although transient immune suppression is a hallmark of MV infection or vaccination<sup>30,32</sup> we established here that HF-LV

transduction did not affect hCD34<sup>+</sup>-cell short-term differentiation in contrast with wt MV<sup>32</sup>. HF-LVs did also not adversely affect the engraftment ability of hCD34<sup>+</sup>-cells and resulted in normal hematopoiesis *in vivo* upon engraftment in NSG mice. Of importance, we identified the CD46 as the measles virus receptor allowing this spectacular HF-LV transduction efficiency for as well quiescent as cytokine stimulated HSCs.

Several findings pointed toward CD46 as HF-LV entry receptor on hCD34<sup>+</sup> cells: 1) the SLAM receptor co-responsible with CD46 for efficient resting T and B cell transduction<sup>16,17,19</sup> was absent from the surface of as well resting as stimulated hCD34<sup>+</sup>-cells; 2) an antibody interfering with binding of HF-LVs to the cell surface on hCD34<sup>+</sup>-cells abolished transduction completely; 3) HF-LVs blind for CD46 attachment<sup>19,33</sup> were unable to transduce hCD34<sup>+</sup>-cells; 4) cytokine-stimulation increased the CD46 density on the hCD34<sup>+</sup>-cell surface perfectly coinciding with increased transduction efficiency; 5) addition of retronectin, a component of fibronectin approaching vector to the cell surface allowed a significant gain in transduction of quiescent cells probably because this still augmented HF-LV binding to the CD46 complement receptor. Indeed, in the absence of retronectin we obtained equivalent binding of HF-LVs to resting and stimulated hCD34<sup>+</sup>-cells<sup>11</sup>. The mechanism explaining this high-level HF-LV entry and productive transduction needs to be investigated but as for T and B cells CD46 binding might induce an alternative cell entry using macropinocytosis.<sup>19</sup>

During engraftment, transplanted HSCs home to the BM niche, where they initiate both differentiation and self-renewal. There, they reside in a hypoxic niche, where they acquire dormancy and maintain stemness.<sup>52,55</sup> It might be, therefore, crucial to not over-expose the hCD34<sup>+</sup> HSCs to cytokines, currently required for high-level transduction with VSV-G-LVs.<sup>12,13</sup> Indeed, recent clinical trials<sup>1-4</sup> still use strong cytokine-cocktails, although this can affect the cell cycle status and the integrity of the HSC pool.<sup>56-58</sup> Importantly, compared with hCD34<sup>+</sup>-cells in G<sub>0</sub>, cells in G<sub>1</sub> or S/G<sub>2</sub>M phases of the cell cycle demonstrated diminished engraftment.<sup>43,57,59,60</sup> Very recently we demonstrated that a new lentiviral vector pseudotype carrying the Baboon envelopes at their surface (BaEV-LVs) outperformed by far VSV-G-LVs for human HSC transduction<sup>41</sup>. This new LV-pseudotype that conferred high-level

transduction of the HSC subset in the prestimulated hCD34<sup>+</sup>-cell population. Both BaEV- and H/F LVs succeed in transducing efficiently hCD34<sup>+</sup>-cells upon mild cytokine stimulation.

Most interestingly, BaEV- and H/F-LVs manage to transduce up to 30% and 70% respectively of resting HSCs. The latter represents a transduction level never reached before in these resting targets. The clinical interest of transducing resting HSCs are multiple as stated above. H/F-LVs are superior to BaEV-LVs for transducing resting hCD34<sup>+</sup>-cells due to the differential expression of their receptors. Indeed, the expression of both ASCT-1 and ASCT-2 BaEV-LV receptors are upregulated by more than 2- and 6-fold respectively when hCD34<sup>+</sup>-cells are stimulated with early acting cytokines. On the other hand, we report here that resting hCD34<sup>+</sup>-cells already express the human complement receptor CD46 at high density at their cell surface and that this receptor is used for H/F-LV entry. This gain of H/F-LV as compared to BaEV/LVs in transducing unstimulated hCD34<sup>+</sup>-cells, might be important in a clinical setting. Thus, the former vectors still improve HSC-transduction and conserve HSC-potential, a combination needed for successful treatment of diseases in which gene-corrected repopulating cells have no selective advantage (eg hemoglobinopathies). Although, a higher level of HSC correction and conservation is of benefit for all monogenetic diseases, Fanconi Anemia (FA) patients would in particular benefit of resting hCD34<sup>+</sup>-cell transduction. Indeed, the FA genetic defect causes fragility of the hCD34<sup>+</sup>-cells, which are strongly reduced in number in the patients.<sup>61</sup> Moreover, cytokine-stimulation induces FA hCD34<sup>+</sup>-cells into apoptosis. Therefore, minimizing hCD34<sup>+</sup>-cell stimulation combined with efficient gene transfer would be invaluable for FA gene therapy.<sup>6,62,63</sup>

One important question that needs attention is to verify if the genomic accessibility for HF-LV integration differs between resting and stimulated hCD34<sup>+</sup>-cells. Indeed, chromosomal accessibility for LV integration might be altered by cytokine stimulation, inducing mRNA transcription and cell cycle entry. Therefore, the genomic integration profile of HF-LVs such as integrations in or close to oncogenes, in actively transcribed region or in methylated genomic sites in unstimulated resting and cytokine-stimulated hCD34<sup>+</sup>-cells awaits detailed future examination to estimate the long-term safety of these vectors.

Taken together, the efficient transduction of resting HSCs by HF-LVs have major translational implications giving hope for more complex disease such as Fanconi anemia but also for many other inherited or acquired diseases. These HF-LVs have the potential to significantly improve clinical efficacy of gene delivery to HSCs, allowing to use low vector doses, short exposure and omitting cytokine stimulation but nevertheless allowing high enough LV copy numbers per cell. HF-LVs could open the door to for future research in hematopoiesis and clinical applications still out of reach today.

### **Acknowledgement**

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## Figure Legends

### Figure 1: HF-LVs allow close to 100% transduction of prestimulated human CD34<sup>+</sup>-cells.

(A) hCD34<sup>+</sup>-cells were pre-stimulated with a cytokine cocktail (TPO,SCF, Flt3) and transduced with H/F- or VSV-G-LVs at indicated MOIs in the presence or absence of retronectin. Six days post-transduction the cells were analyzed for the % of GFP<sup>+</sup>CD34<sup>+</sup>-cells by FACS (means +/- SD; n=6). (B). hCD34<sup>+</sup>-cells were prestimulated for 18h as followed: 1) SCF or TPO alone, 2) SCF+TPO; 3) SCF+TPO+Flk-3L. Subsequently they were transduced with H/F- or VSV-G-LVs at indicated MOIs in the presence of retronectin. Six days post-infection the cells were evaluated for the % of hCD34<sup>+</sup> GFP<sup>+</sup> cells by FACS. (means +/- SD; n=5). (C) and (D) show the comparison between the transduction level of CD34<sup>+</sup>-cells and the myeloid colonies driven from these cells (GFP<sup>+</sup>CFCs) for CD34<sup>+</sup>-cells prestimulated with SCF+TPO (C) or SCF+TPO+Flk-3L (D). Transductions were performed at MOI 100 for VSV-G-LVs and MOI=10 for HF-LVs. (E, F) hCD34<sup>+</sup>-cells were prestimulated with SCF+TPO (D) or SCF+TPO+Flk-3L (E). Subsequently, they were transduced with H/F- or VSV-G- LVs at decreasing MOIs in the presence of retronectin. Six days post-infection the cells were analyzed for the % of hCD34<sup>+</sup>GFP<sup>+</sup>-cells by FACS.

### Figure 2. HF-LVs allow high-level transduction of unstimulated CD34<sup>+</sup>-cells without altering their immature phenotype

(A) Freshly isolated hCD34<sup>+</sup>-cells were transduced with H/F- or VSV-G-LVs at indicated MOIs in the presence or absence of retronectin. Three days post-infection the cells were evaluated for the % of hCD34<sup>+</sup>GFP<sup>+</sup>-cells by FACS (means +/- SD; n=6). (B) Unstimulated hCD34<sup>+</sup>-cells were transduced with H/F-LVs at increasing MOIs in the presence of retronectin. Three days post-infection the cells were analyzed for the % of hCD34<sup>+</sup>GFP<sup>+</sup>-cells by FACS (means +/- SD; n=6). (C). Surface marking of very immature progenitors (CD34<sup>+</sup>/CD38<sup>-</sup>/CD90<sup>+</sup>) for the transduced (GFP<sup>+</sup>) and untransduced (GFP<sup>-</sup>) cells. The arrows indicate the gated area. (D) Cell cycle progression was monitored by simultaneously visualizing the RNA (Pyronine-Y) and DNA (7-AAD) content of the hCD34<sup>+</sup>-cells three days after transduction. The percentages of cells in the G0/G1a, G1b, S/G2/M phase of the cell cycle are indicated in the dot blots. (E) Comparison between the % GFP<sup>+</sup>CD34<sup>+</sup> quiescent cells

and the % GFP<sup>+</sup> myeloid colonies (CFCs) derived from these transduced CD34<sup>+</sup>-cells. (F) Surface staining of the different transduced myeloid colonies derived from quiescent transduced hCD34<sup>+</sup>-cells (glyA for erythrocytes; CD15 and CD14 for granulocytes, monocytes and macrophages, CD71 for immature proliferating progenitors).

**Figure 3. HF-LVs utilize exclusively the CD46 measles virus receptor for high level transduction of stimulated and quiescent hCD34<sup>+</sup>-cells**

(A) Surface staining for the measles receptors (SLAM, CD46 and Nectin-4) on resting or pre-stimulated hCD34<sup>+</sup>-cells. (B) Quantification of CD46 surface expression (Mean Fluorescence intensity) of hCD34<sup>+</sup>-cells at different timepoints of cytokine stimulation (TPO+SCF+ Flk-3L). (C) Prestimulated and resting CD34<sup>+</sup>-cells were stained for CD46 and each sorted for low and high CD46 expression. Obtained subpopulations were transduced with H/F-LVs in the presence of retronectin and transduction efficiency was analyzed by FACS three days later. (D, E) Prestimulated (D) and resting (E) hCD34<sup>+</sup>-cells were transduced with H/F-LVs (MOI 10) in the absence or presence of anti-CD46, anti-SLAM or anti-nectin-4 blocking antibodies (Ab). Three days post-transduction, GFP expression in these cells was determined by FACS. Transduction levels are presented as GFP expression relative to the H/F-LV transduction in the absence of antibody, set to 100% (means +/- SD; n=3). (F). Schematic representation of the different MV hemagglutinin gp's (Hgps). The MV vaccinal Edmonston strain Hgp contains binding residues for both SLAM and CD46 (H). CD46-tropic mutant Hgp were engineered by mutating one residue of Edm Hgp responsible for binding/fusion through SLAM (R533A; Ha533). SLAM-tropic Hgp was derived from the clinical strain D4 (H-D4). H-D4-YG was obtained by introducing CD46 binding residus (481Y and 492G) into the H-D4 envelope. Prestimulated hCD34<sup>+</sup>-cells were transduced with H/F-, H-D4/F-, H533/F- or H-D4-YG/F-LVs and GFP expression was analyzed by FACS six days later (mean +/- SD, n=4).

**Figure 4: H/F-LVs transduced stimulated and resting hCD34 maintain high-level gene marking upon NSG mice reconstitution.**

hCD34<sup>+</sup>-cells were prestimulated with a cytokine cocktail (TPO+SCF+Flk-3L) for 16 h (A,B) or not (C,D) and transduced with HF-LVs at MOI=10 or VSV-G at MOI=100 in presence of retronectin for 36 h. Subsequently the cells (2E5) were injected into the



liver of irradiated newborn NOD/SCID  $\text{gammac}^{-/-}$  mice (NSG mice). Upon reconstitution for 12 weeks, the bone marrow, spleen and thymus of these primary engrafted mice were analyzed. **(A, C)** Total human cell engraftment analyzed by anti-hCD45 staining using FACS. **(B, D)** The percentage of transduction human cells ( $\text{GFP}^+\text{hCD45}^+$  cells) was analyzed by FACS in the different hematopoietic tissues (bone marrow, spleen and thymus).

**Figure 5. H/F-LV transduced stimulated and resting hCD34 maintain equivalent high gene marking in myeloid and lymphoid lineages in vivo**

$\text{hCD34}^+$ -cells were prestimulated with a cytokine cocktail (TPO+SCF+Flk-3L) for 16 h (A,B) or not (C,D) and transduced with HF-LVs at  $\text{MOI}=10$  or VSV-G at  $\text{MOI}=100$  in presence of retronectin for 36 h. Subsequently the cells ( $2 \times 10^5$ ) were injected into the liver of irradiated newborn NOD/SCID  $\text{gammac}^{-/-}$  mice (NSG mice). Upon reconstitution for 12 weeks, the bone marrow, spleen and thymus of these primary engrafted mice were analyzed. **(A, C)** Transduction levels of the different hematopoietic cell lineages in the BM of NSG reconstituted mice. The percentage of  $\text{GFP}^+$  immature early progenitor cells ( $\text{CD34}^+\text{CD19}^-\text{CD10}^-$ ), pre/pro B cells ( $\text{CD34}^+\text{CD19}^+\text{CD10}^-$ ), pro B cells ( $\text{CD34}^-\text{CD19}^+\text{CD10}^-$ ), immature and mature B cells ( $\text{CD34}^-\text{CD19}^+\text{CD10}^+$ ) and myeloid progenitors ( $\text{CD13}^+$ ) are shown. **(B, D)** Transduction levels of different cell lineages in the spleen of NSG reconstituted mice. The percentage of  $\text{GFP}^+$  pre/pro B cells ( $\text{CD34}^+\text{CD19}^+\text{CD10}^-$ ), pro B cells ( $\text{CD34}^-\text{CD19}^+\text{CD10}^-$ ), immature and mature B cells ( $\text{CD34}^-\text{CD19}^+\text{CD10}^+$ ), monocytes and granulocytes ( $\text{CD14}^+$ ) are shown.

**Figure 6. H/F-LVs transduce long-term reconstituting HSCs at levels approaching 100%.**

Transduced  $\text{hCD34}^+$ -cells were injected into the liver of irradiated newborn NSG mice. Upon reconstitution, the bone marrow and spleen of these primary engrafted mice (1st transplantation) were analysed for transduced human cell engraftment ( $\text{GFP}^+\text{CD45}^+$ ) in the BM and the spleen. Subsequently, the  $\text{hCD34}^+$ -cells, isolated from the BM of these mice, were injected following the same procedure as for the primary NSG engraftments. Eight weeks upon engraftment these secondary engrafted mice were analysed for the % of transduced human cells ( $\text{hCD45}^+\text{GFP}^+$ ) in the BM and spleen. **(A, C)** Analysis of engraftment of transduced human cells ( $\text{hCD45}^+\text{GFP}^+$ ) in the BM and spleen of primary and secondary transplanted mice

reconstituted with hCD34<sup>+</sup>-cells prestimulated **(A)** with a cytokine cocktail (TPO+SCF+Flk-3L) or unstimulated **(C)** and transduced with H/F-LVs. **(B, D)** Detailed phenotypic analysis of transduced human cells in the BM of secondary transplanted mice reconstituted with pre-stimulated (B) or unstimulated (D) H/F-LV transduced hCD34<sup>+</sup>-cells. Analysis of transduced human cells (GFP<sup>+</sup>) in the myeloid progenitors (CD13<sup>+</sup>), monocytes (CD14), progenitors cells (CD34) and B cells (CD19) in secondary recipient mice.

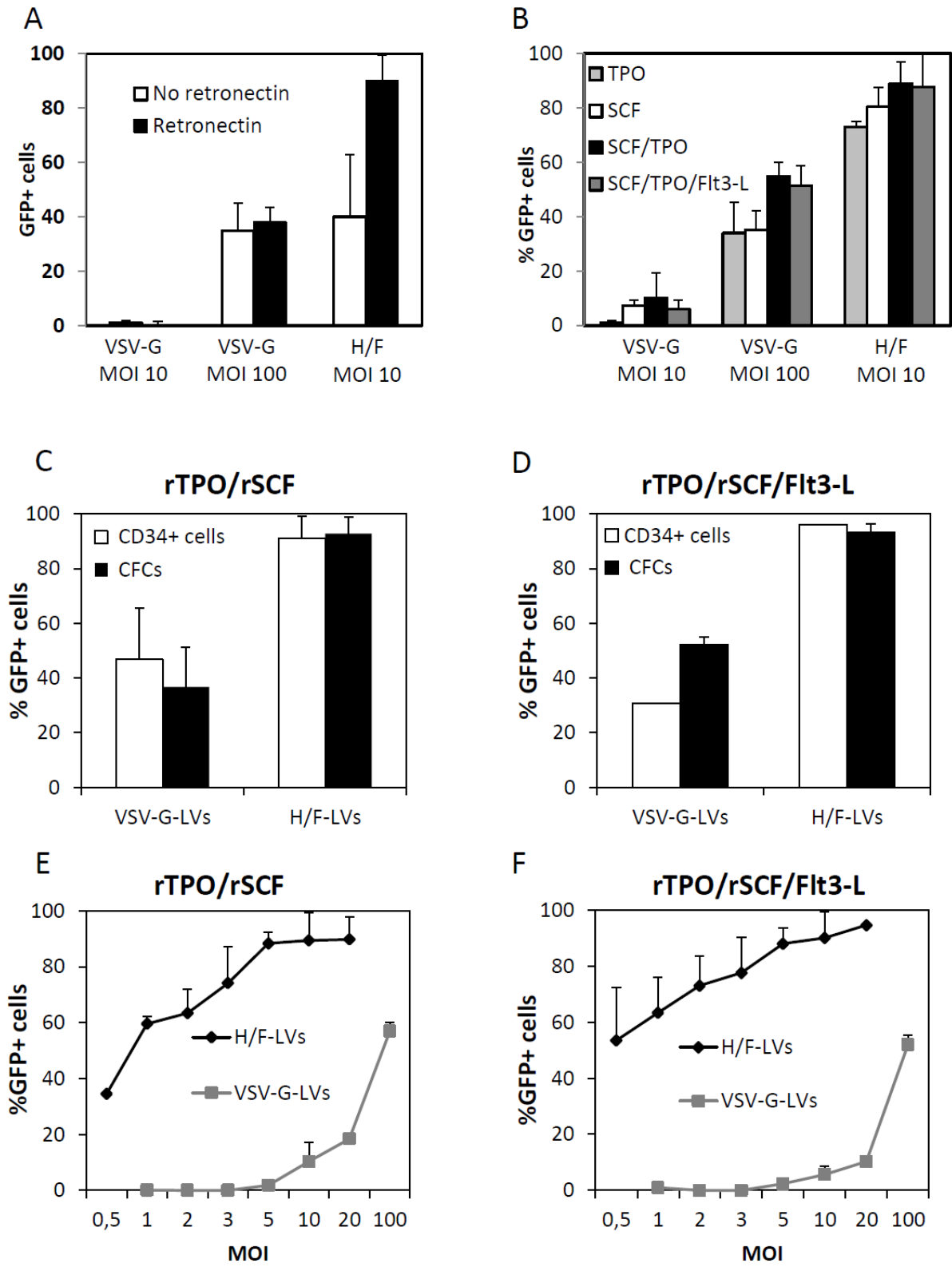
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**Figure 1**

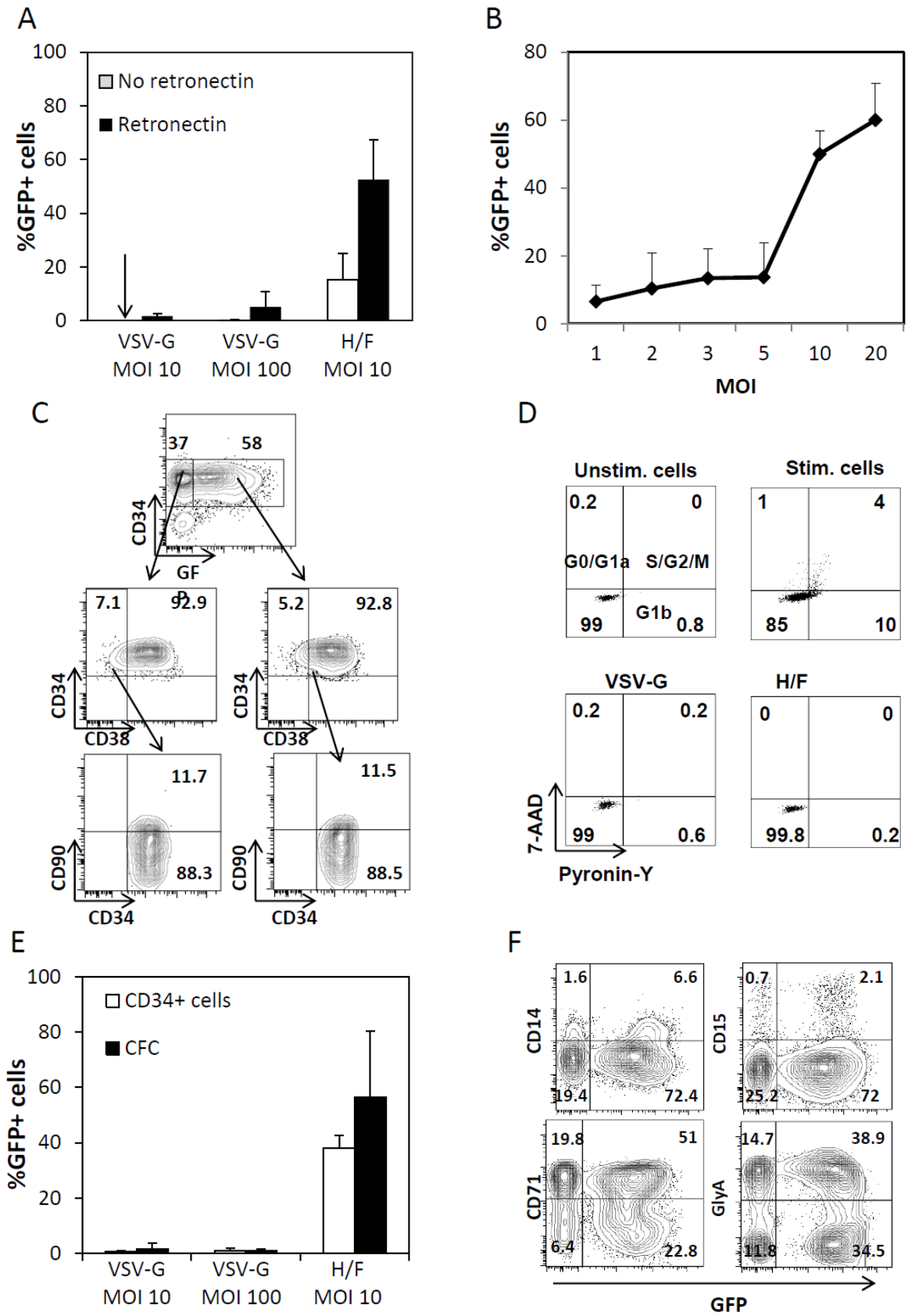


Figure 2.



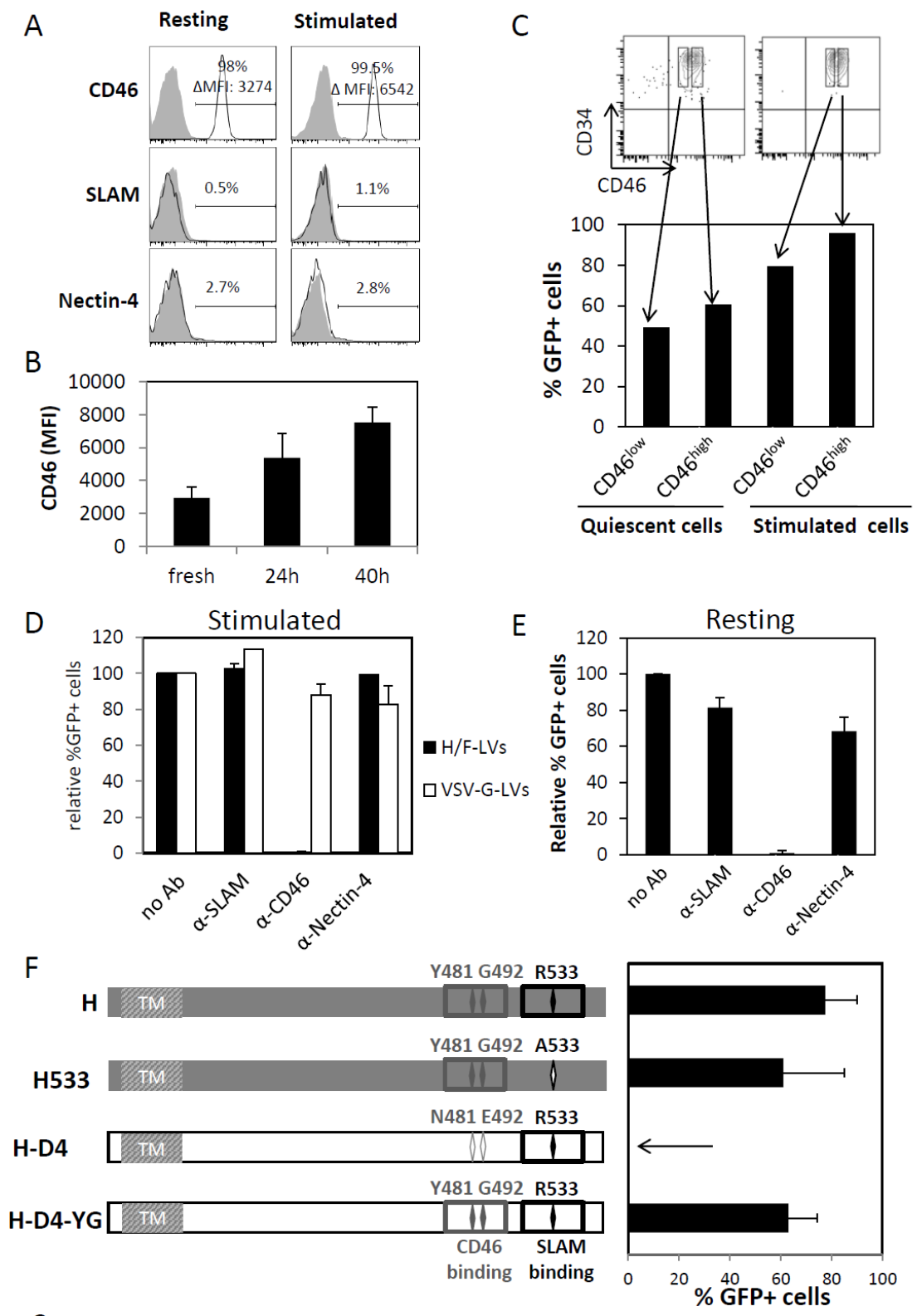
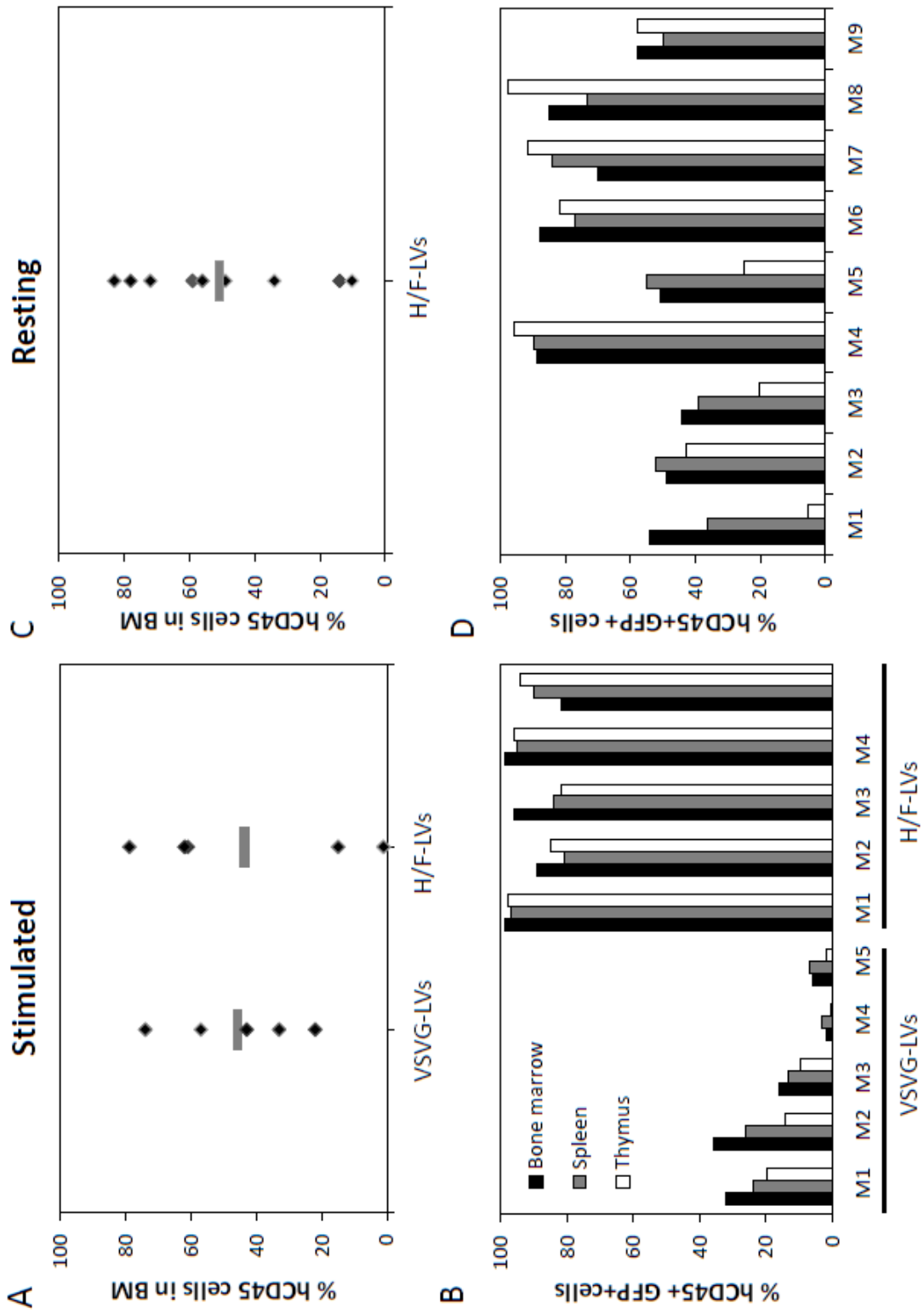
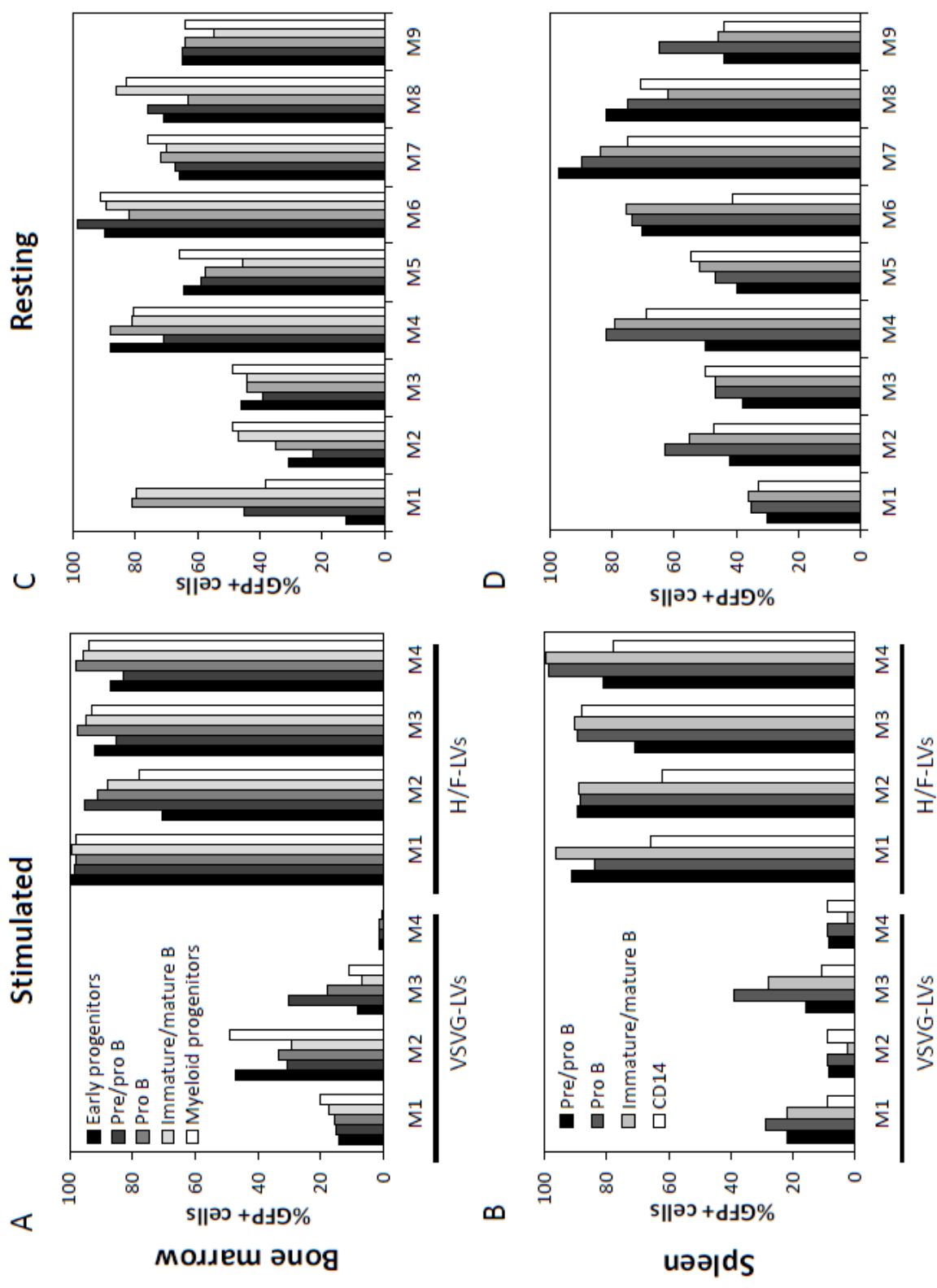


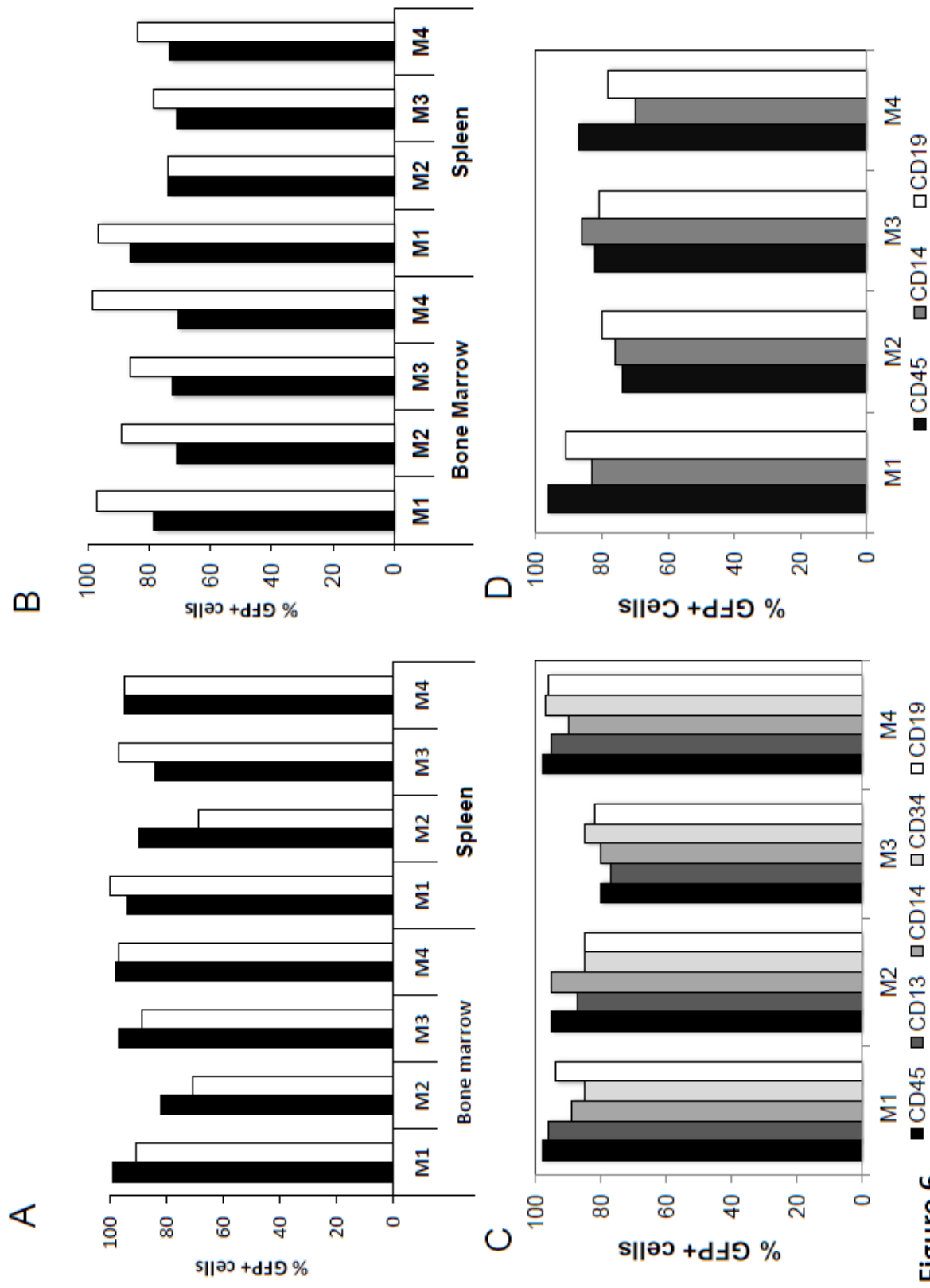
Figure 3.



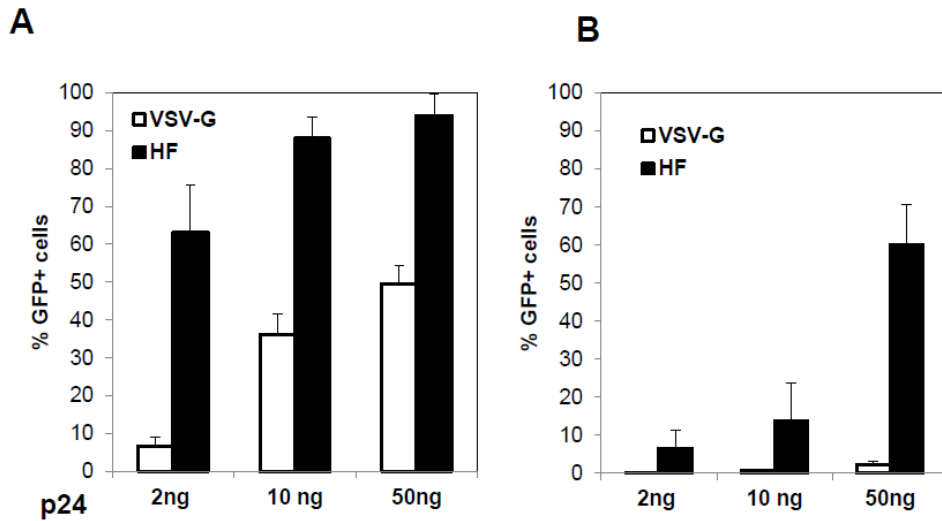
**Figure 4**



**Figure 5**



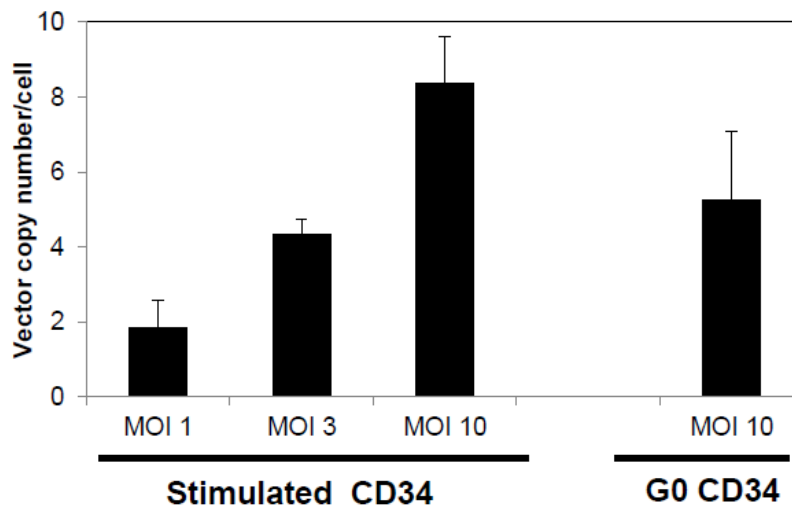
**Figure 6**



**Supplementary Figure 1: hCD34<sup>+</sup>-cell transduction with different LV pseudotypes at equivalent physical particle doses.**

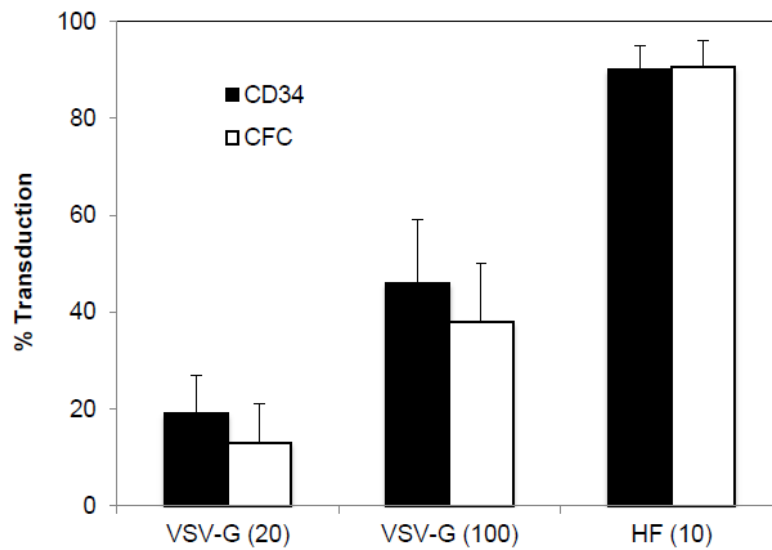
(A) CD34<sup>+</sup>-cells were prestimulated for 24h (SCF (100ng/ml), TPO, (20ng/ml), Flt3-L, (100ng/ml)) and transduced at the indicated HIV p24 levels with VSV-G-LVs or HF-LVs. After 24h transduction, cells were washed and cultured in the presence of the same cytokine cocktail for 6 more days. The % of GFP<sup>+</sup> cells was determined by FACS analysis.

(B) Same transduction protocol as in (A) but using unstimulated hCD34<sup>+</sup>-cells.



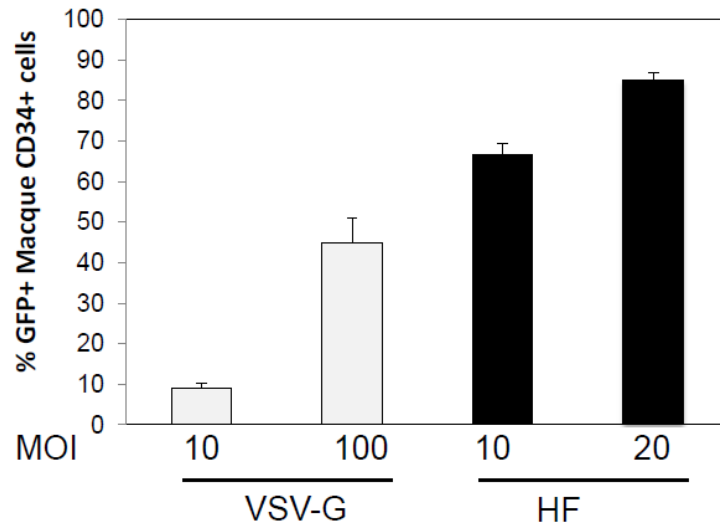
**Supplementary figure 2: Copy number detection in HF-LV transduced CD34<sup>+</sup> cells.**

hCD34<sup>+</sup>-cells were pre-stimulated with a cytokine cocktail (TPO, SCF, Flt3) or left unstimulated (G0 CD34<sup>+</sup>-cells) and transduced with H/F-LVs at indicated MOIs in the presence of retronectin. 10 days post-transduction genomic DNA was isolated from these cells and the copy number of integrated vector / cell was determined by Q-PCR.

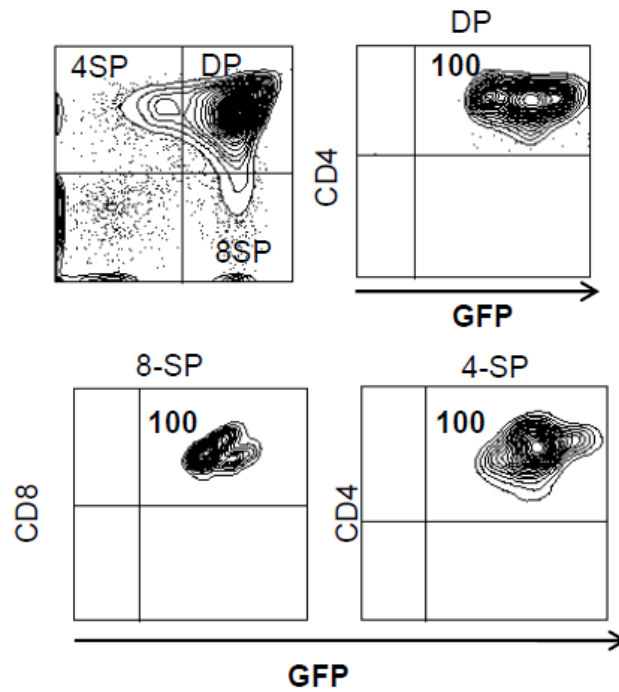


**Supplementary Figure 3: HF-LVs transduce efficiently mobilized hCD34<sup>+</sup>-cells.**

Mobilized hCD34<sup>+</sup>-cells were prestimulated with SCF+TPO+Flt3-L+IL-3 and transduced with the indicated LV pseudotypes in the presence of Retronectin. An MOI of 10 was applied except for VSV-G LVs for which in addition an MOI of 100 was used. Three days after transduction, the cells were seeded in myeloid differentiation medium for 14 days. The % of GFP<sup>+</sup> CD34<sup>+</sup>-cells 3 days post transduction and the GFP<sup>+</sup> colony forming cells (CFCs) at day 14 of differentiation were determined by FACS (means±SD, n=3; p<0.01 when comparing CFC transduction levels of HF-LVs to the VSVG pseudotype).



**Supplementary Figure 4: SIV derived LVs pseudotyped with H and F MV gps, allow efficient transduction of macaque BM CD34<sup>+</sup>-cells**  
 (A) Cynomolgus macaque BM CD34<sup>+</sup>-cells were prestimulated with rSCF, rTPO, rFlt3-L and IL-3 for 24h and were transduced with SIV-derived LVs pseudotyped with HF measles virus envelope glycoproteins or VSV-G-LVs at the indicated vector dosis (MOI). All transduction were performed in the presence of retronectin®. GFP expression of the cells was analyzed 6 days after transduction by FACS.



**Supplementary Figure 5: High-level transduction in human thymocytes after engraftment in recipient NSG mice of transduced cytokine-stimulated hCD34<sup>+</sup>-cells**

hCD34<sup>+</sup>-cells were prestimulated with a cytokine cocktail (TPO+SCF+Flk-3L) for 16 h and transduced with HF-LVs at MOI=10 in presence of retronectin for 36 h. Subsequently the cells (2E5) were injected into the liver of irradiated newborn NSG mice. Upon reconstitution for 12 weeks, thymus of these primary engrafted mice were analyzed. The transduced cells in the thymic subpopulations are shown (SP-4 (CD4<sup>+</sup>CD8<sup>-</sup>), SP-8 (CD4<sup>-</sup>CD8<sup>+</sup>) and DP (CD4<sup>+</sup>CD8<sup>+</sup>). The FACS profiles are representative of 5 independent experiments.





## PART 2 – BAEV PSEUDOTYPED LENTIVIRAL VECTORS ALLOW EFFICIENT TRANSDUCTION OF PROGENITOR T CELLS, THYMOCYTES AND MATURE T CELLS

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### 1) EARLY T CELL PROGENITORS AND NAÏVE T CELLS, INTERESTING TARGETS OF BAEV-LVS FOR T CELL BASED GENE THERAPY AND IMMUNOTHERAPY

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Efficient gene transfer into quiescent T and B lymphocytes for gene therapy or immunotherapy purposes may allow the treatment of several genetic dysfunctions of the hematopoietic system, such as immunodeficiencies, and the development of novel therapeutic strategies for cancers and acquired diseases.

In T cells, completion of reverse transcription, nuclear import and subsequent integration of the VSVG-LV genome do not occur efficiently unless they are activated via the T-cell receptor or by survival-cytokines inducing them to enter into the G<sub>1b</sub> phase of the cell cycle and inducing a skewing in the T cell repertoire generation (Verhoeven et al. 2003), (Korin et al. 1998), (Unutmaz et al. 1999). Moreover, our group have recently reported that VSV-G-LVs cannot enter into resting T cells because they do not express the LDL receptor on their surface (Amirache et al. 2014). Thus, our group have engineered H/F-LVs that efficiently deliver genes into resting and IL-7 stimulated T cells (Frecha et al. 2008). IL-7 stimulation of T cells appears to be more attractive than TCR activation because of its role in supporting T cell survival and homeostatic proliferation (Fry et al. 2001), (Rathmell et al. 2001).

Although H/F-LVs were an interesting advance in the field of T cell gene transfer, they cannot highly transduce the naïve immature UCB T cell population. These naïve immature T cells only express CD46 on their surface without the necessary co-expression of the SLAM receptor. Indeed, our group have suggested that H/F-LVs enter in unstimulated T cells by a sequential engagement of CD46/SLAM that mediates H/F-LVs entry by macropinocytosis (Frecha et al. 2011) which is not possible in this T cell subset. Moreover, H/F-LVs preferentially transduce mature memory cells while naïve T cells are an attractive target because they are

able to induce a response to a novel antigen, have a long-term life span, persist a long-time and are able to generate stable T cell memory populations.

Here, we demonstrate that the new engineered BaEV-LVs are able to transduce IL-7 stimulated immature and naïve T cell subpopulations. The low mRNA expression of both ASCT1 and ASCT2 receptors of BaEV-LVs explain that they cannot transduce resting T cells. Nevertheless, they outperform H/F-LVs allowing a higher transduction of UCB immature naïve T cell, early thymic progenitors and mature naïve T cells. We also propose to develop the co-transplantation of genetically modified HSCs with transduced T cell progenitors from the same donor. This was allowed by the differentiation of CB CD34+ cells in early T progenitors using the promising Notch Ligand DL-4 culture system developed by Reimann and colleagues (Reimann et al. 2012). This is interesting for the development of gene therapy HSCT with an accelerated recovery of the adaptive immune response.

2) NEW BABOON ENVELOPE PSEUDOTYPED LVS EFFICIENTLY  
TRANSDUCE ADULT AND CB T CELLS, THYMOCYTE PROGENITORS  
AND IN VITRO DERIVED T CELL PROGENITORS THAT RECONSTITUTE  
T CELL LINEAGE IN NSG MICE

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## Title

**New Baboon envelope pseudotyped LVs efficiently transduce adult and cord blood T cells, thymocyte progenitors and in vitro derived T cell progenitors that reconstitute T cell lineage in NOD/SCID/ $\gamma$ C<sup>-/-</sup> mice**

***(Manuscript in preparation)***

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**Running title:** Baboon LVs transduce the complete T cell lineages.

**Keywords:** lentiviral vectors, adult T cells, cord blood T cells, thymocytes, T cell progenitors, gene therapy, baboon retrovirus, immunotherapy

**Personal contribution:**

I designed and performed experiments except for the results presented Figure 3 on human thymocytes, I analyzed and discussed the data, designed the figures and contribute in writing the manuscript.

**ABSTRACT**

T cells represent valuable tools for treatment of cancers, infectious diseases and inherited diseases. Remarkable progress has been achieved in cancer treatment by: 1) use of tumor-specific chimeric antigen receptor (CAR) modified T cells with a high success rate; 2) marking of allogenic T cells with a suicide gene conserving their anti-leukemic effect upon transplant but allowing to eradicate them when rejection occurs.

Today, T cell receptor stimulation is still mostly used to allow efficient retroviral vector gene transfer, resulting in skewing of T cells towards a memory phenotype and compromising the longevity of these cells. However, T cell based therapies would strongly benefit from gene transfer into live-long persisting T cells. Several studies indicate that *in vivo* persistence or gene modified T cells correlates with a less differentiated T cell phenotype and suggests that naïve T cells may be optimal in this setting. Therefore, we compared the gene transfer efficiency into more immature naïve T cell with a new lentiviral vector (LV) pseudotyped with the baboon retrovirus envelope (BaEV) to other existing pseudotypes: VSV-G-LVs which is commonly used, RDTR-LVs, carrying the cat retroviral envelope and HF-LVs, displaying the measles virus envelopes.

These BaEV-LVs outperformed the other LV pseudotypes by far for transduction (up to 70%) of naïve adult T cells and cord blood recent thymic emigrants when stimulated by IL-7, which allowed to conserve the naïve phenotype of these target cells. Equivalent transduction levels were revealed for BaEV-LVs in freshly isolated early thymocytes (early thymic progenitors (ETP), progenitors and pre T cells) reaching again 80% transduction whereas the other pseudotypes performed poorly, except for HF-LVs. In agreement, BaEV-LV and HF-LVs transduced ETP and progenitor T cells, differentiated from CD34<sup>+</sup>-cells by culture on DL-4, to the same extent. Of utmost importance, these transduced ETPs and T progenitors allowed efficient reconstitution of NOD/SCID,  $\gamma c^{-/-}$  mice *in vivo*,

maintaining these high transduction levels in all derived T cell lineages and permitted an accelerated T-cell lineage reconstitution as compared to CD34<sup>+</sup> progenitor cell engraftment into this mouse model.

These results indicate that BaEV-LVs are valuable tools for genetic modification of naïve and early T cell lineages, essential targets for gene therapy application where long-lived T cell persistence is important for durable treatment of patients. Additionally, coinjection of LV-corrected autologous T cell progenitors and HSCs might accelerate T cell reconstitution in patients as compared to solely injecting HSCs avoiding a gap in immune reconstitution.



## Introduction

Gene transfer into T lymphocytes is a crucial step in the development of therapeutic strategies for the treatment of several genetic dysfunctions of the hematopoietic system, such as severe combined immunodeficiency (SCID,<sup>1,2</sup>) and the development of novel therapeutic strategies for diseases such as cancers<sup>3,4</sup> and for acquired diseases<sup>5</sup>.

Twenty years ago now, children suffering from the monogenetic disease Adenosine deaminase (ADA)-SCID were treated successfully by T cell gene therapy<sup>6</sup>. Also for HIV infected patients, who are in demand of novel therapies, T cell gene therapy might become an important option in the future since several new combinatorial strategies are proposed<sup>7-9</sup>. More recently, T cells gained field in the treatment of several blood cancers. Firstly, T cell gene therapy has now proven to correct severe side effects of bone marrow transplantation (Allo-BMT). Allogenic T cells as part of the BMT induce a graft versus leukemia effect (GVL), which is unfortunately often accompanied by graft versus host disease (GVHD). To avoid GVHD, retroviral T cell marking with a suicide gene is performed. These gene-marked T cells can then be induced into apoptosis by administration of a specific drug when needed. This strategy has been proven effective in the treatment of GVHD in clinical trials<sup>3,10-12</sup>. Another promising anti-cancer strategy is based on engineering T cells to express an appropriate tumor-specific TCR or a chimeric antigen receptor (CAR)<sup>13-15</sup>. Recent case reports from on-going clinical trials have described durable rejection of previously refractory B-cell malignancies in patients after CD19-directed CAR therapy<sup>4,16-19</sup> with up to an 86% complete response rate in leukemia that is otherwise incurable. Other CARs are directed against :1) CD22 or CD20 for treatment of B cell acute lymphocyte leukemia<sup>20</sup>, 2) CD70 for treatment of glioblastoma<sup>21</sup> 3) GD2 antigen for treatment of neuroblastoma<sup>22</sup>, 4) NY-ESO-1 epitope for melanoma<sup>23</sup>. Concluding, CAR T-cell based therapy offers a valuable alternative for treating cancer patients.

The population of mature adult T cells can be divided into two different subsets, namely, memory and naïve T cells. The clinical efficacy of adoptively transferred T lymphocytes correlates with their ability to persist *in vivo*<sup>24</sup>. Several

studies indicate that *in vivo* persistence correlates with a less differentiated T cell phenotype<sup>13,15,25</sup> and suggests that naïve T cells may be optimal in this setting. Naïve T cells are especially important as gene therapy target cells since they maintain the capacity to respond to novel antigens, are endowed with the highest developmental plasticity and are unique in the sense that they can generate the entire spectrum of immunologic memory. Less differentiated naïve and central memory T cell subsets display superior proliferation, persistence and antitumor responses following infusion when compared to the more differentiated effector memory subset<sup>13,26,27</sup>. Therefore they might be the best T cell population to target for gene therapy when long-live cure is envisaged.

Human umbilical CB T cells, unlike adult-derived PB lymphocytes, are mostly naïve. It is therefore reasonable to speculate whether CB might be an alternative source of T-lymphocytes for genetic engineering. CB T cells differ from their adult counterpart in that the former represent mainly recent thymocyte emigrants<sup>28</sup>. Compared with naïve adult T cells, they are immature with respect to alloantigen recognition, lymphokine production, and T-cell activation profile<sup>29</sup>. Human naïve CD4<sup>+</sup> T cells have recently been shown to contain two subpopulations distinguished by the expression of CD31. The CD31<sup>+</sup> subset is thought to incorporate the population of cells recently emigrated from the thymus, whereas the CD31<sup>-</sup> naïve T cell subset is more mature<sup>30,31</sup>. Interestingly, IL-7 may play a preferential role in the maintenance of CD31<sup>+</sup> naïve CD4<sup>+</sup> T cells during adult life<sup>32</sup>. IL-7 seems to exert a preferential effect on umbilical cord blood (CB) naïve T cells that proliferate significantly more than adult peripheral blood naïve T cells in response to IL-7<sup>33,34</sup>. These characteristics led to the first CAR CB T strategies for treatment of cancer and infectious diseases<sup>35-38</sup>

The thymus is the primary site of T cell development and thymocytes are important for treatment of auto-immune disorders since they play a key role in the induction of self tolerance. Targeting the thymus with an antigen of interest can result in the induction of tolerance. This has been achieved by the direct injection of soluble antigen or viral vectors harboring an antigen of interest<sup>39-41</sup>. Moreover, *in situ* correction of a genetic immunodeficiency (ZAP-70) by intrathymic injection of a lentiviral vector expressing the correcting gene was demonstrated<sup>42</sup>. Early T cell

development depends on the interaction of thymocytes with its microenvironment through Notch-Ligand signaling pathways. DL-4 was identified as the essential Notch 1 activator in the T-cell engagement of hematopoietic stem cells<sup>43-45</sup>. By extending this approach, Reimann et al.<sup>46</sup> confirmed that immobilized DL-4 supported the in vitro generation of T-cell progenitor cells from CB CD34<sup>+</sup> cells in a feeder-cell-free culture system. These DL-4 generated T-cell progenitors displayed the phenotypic and molecular signatures of very immature thymic precursors. Upon transfer in NOD/SCID/gc<sup>-/-</sup> (NSG) mice, these progenitors were capable of migrating to the thymus and differentiating into mature polyclonal T cells and of accelerating T-cell reconstitution in vivo as compared to HSCs<sup>46</sup>. Therefore, transfer of in vitro-generated T-cell progenitors has emerged as a promising strategy for accelerating T-cell reconstitution when coinjected with autologous HSCs.

It is now generally accepted that resting T cells cannot be readily transduced by classical VSV-G pseudotyped lentiviral vectors (LVs)<sup>47-49</sup>. In addition, we revealed that this is due to the absence of the low density lipid VSV receptor (LDL-R) on resting T cells<sup>50</sup>. It is though also of the utmost importance that the responses of T cells to antigens are not dramatically altered by the gene transfer protocol. We and others have reported that inducing cell cycle entry into G<sub>1b</sub> via stimulation through the TCR allows efficient transduction of adult naïve T cells by VSV-G-LVs<sup>48,49,51</sup> (Verhoeven 2003, Korin and Zack, Maurice). Note that TCR-stimulation remarkably up-regulated the LDL-R surface expression coinciding with high level VSV-G-LV transduction<sup>50</sup>. However, TCR-stimulation of T cells alters their half-life and immune-competence, results in an inversion of the CD4/CD8 ratio, and is associated with loss of naïve T cell subsets and a skewed TCR repertoire<sup>52,53</sup>. In this regard, it is interesting to note that exposure of adult T cells to cytokines such as interleukin-2 (IL-2), IL-15, and IL-7 renders them permissive to VSV-G LV transduction<sup>47,49,54-56</sup>. Clearly, IL-7 stimulation allowing lentiviral T-cell transduction could preserve a functional T-cell repertoire and maintain an appropriate proportion of naïve and memory CD4<sup>+</sup> and CD8<sup>+</sup> T cells<sup>49,54</sup>. Naïve and memory T cells respond differently to rIL-7 stimulation. In agreement with this, naïve adult T cells need longer IL-7 stimulation than memory cells to allow efficient LV transduction<sup>34</sup>

In conclusion, LV transduction of IL-2, IL-15 or IL-7 stimulated T cells

overcomes the limitation of TCR-mediated LV gene transfer since they avoid skewing of the T cell populations.

As mentioned, efficient transduction of early naïve T cells would mean a step forward in the field since this might guarantee long-term engraftment. However, classical VSV-G-LVs do not allow high-level transduction of these valuable naïve T cell targets under stimulation conditions (e.g IL-7) that conserve their naïve phenotype<sup>47,49,55</sup>. Clearly, to reach this objective alternatives to VSV-G-LV pseudotypes are urgently needed. We previously engineered LVs carrying the measles virus (MV) envelope glycoproteins, H and F, from a vaccinal strain at their surface (H/F-LVs), which conserved the original MV tropism through the complement receptor, CD46, and SLAM (signaling lymphocytes activating molecule) receptor. These were the first LVs to allow efficient transduction of quiescent human memory and naïve T and B cells without inducing entry into the cell cycle while conserving T and B cell phenotype<sup>57-59</sup>. Some time ago, we engineered LVs displaying the feline retrovirus envelope glycoprotein, RD114/TR, which confer efficient transduction of human T cells, however, only upon TCR stimulation<sup>60</sup>. RD114 belongs to the betaretroviruses, also including the baboon endogenous retrovirus (BaEV). They both use the neutral amino acid (aa) transporter 2 (ASCT-2), present on T cells<sup>61,62</sup>. Interestingly, BaEV also recognizes another aa transporter, ASCT-1, not recognised by RD114.<sup>63</sup> Since BaEV uses in addition to ASCT-2 also ASCT-1, it demonstrated a broader tropism than RD114.<sup>64</sup> Very recently we demonstrated that BaEV-LVs outperformed by far VSV-G-, RD114/TR-, H/F-LVs for human HSC transduction<sup>17</sup>. Therefore, we hypothesized that BaEV-LVs might be an excellent candidate for naïve T cell and T-cell progenitor transduction. Thus, we carefully compared these new pseudotypes here for the transduction of naïve adult and cord blood RTE cells, thymocytes and early T cells progenitor generated in vitro with the classically used VSV-G-LVs and the HF- and RD114/TR-LVs to identify the best candidate for transduction of the long-lived naïve and progenitor T cell, both invaluable targets for gene or immunotherapy applications.

## Materials and Methods

### Plasmids

RDTR, BAEVTR and BAEVRless envelope glycoproteins were previously described<sup>17,60</sup>. Briefly, the cytoplasmic tail of BaEVwt and RD114wt was replaced by the one of MLV-A resulting in BAEVTR and RDTR, respectively. The BAEVRless-gp was constructed by deletion of the R-peptide sequence from BaEVwt-gp. H $\Delta$ 24 and F $\Delta$ 30 code for cytoplasmic-tail deletion mutants of the Edmonston measles virus glycoproteins, H and F described earlier<sup>58</sup> (Supplementary Figure 1). All chimeric envelope gps were expressed in the pCMV-G expression plasmid<sup>51</sup>.

### Production of lentiviral vectors

Self-inactivating HIV-1-derived vectors encoding green fluorescent protein (GFP) or mcherry under the control of a spleen focus foamy virus promoter were generated by transfection of 293T cells described in detail elsewhere<sup>17</sup>. Briefly, for display of RDTR, BAEVTR or BAEVRless-gps on LVs, 7  $\mu$ g of the respective envelope gp encoding plasmids were transfected with gagpol and the SIN transfer vector; 3  $\mu$ g of VSV-Ggp encoding plasmid was used for VSV-G-LV production, for co-display of H and F measles virus mutant gps on LVs, 2.75  $\mu$ g of each envelope encoding plasmid was co-transfected.

### Titration of lentiviral vectors

To determine the infectious titers of the HIV vectors, 293T cells were incubated with serial dilutions of the vector supernatants overnight. Medium on the cells is replaced with 2 ml fresh DMEM and cells are incubated for further 72h. The percentage of GFP<sup>+</sup> cells is determined by FACS. To exclude GFP pseudo-transduction and thus verify that expression was only due to integrated vector copies we continued the culture of these 293T cells for ten more days (4 passages) and determined again the percentage of GFP<sup>+</sup> cells by FACS as also confirmed in parallel the titer by Q-PCR on these same samples.

For titration by Q-PCR genomic DNA was extracted from the 293T cells (kept in culture for fourteen days after transduction) using the genomic DNA purification kit (Nucleospin Tissue XS, Macherey-Nagel, Hoerd, France). Q-PCR to determine the titer was performed using Taqman gene expression master mix (Life Technologies, St Aubin, France) and following primer pairs and Taqman probes to amplify the lentiviral vector genome:

Lenti-F : 5'-TGT GTG CCC GTC TGT TGT GT-3'

Lenti-R : 5'-GAG TCC TGC GTC GAG AGA GC-3'

Lenti-Probe : 5'-CAG TGG CGC CCG AAC AGG GA-3'

Results were compared with those obtained after serial dilutions of genomic DNA from a 293T cell line containing one copy of the integrated lentiviral vector per haploid genome.

### **Isolation of human primary T cells, thymocytes and CD34<sup>+</sup>-cells**

Cord blood (CB) from full-term pregnancies and adult blood samples were collected in sterile tubes containing the anti-coagulant, citrate-dextrose (ACD, Sigma, France). Blood samples and thymus resections were obtained after informed consent according to the Helsinki declaration.

Adult peripheral blood (PB) CD3<sup>+</sup> T lymphocytes and CB CD4<sup>+</sup> T lymphocytes were isolated by negative selection using the Rosette Sep T-cell and CD4<sup>+</sup> T-cell isolation kits (Stem Cell Technologies, Grenoble, France) to avoid cell activation. The purity of T cell isolation was monitored by FACS using anti-CD3 and anti-CD4 antibodies.

Thymocytes were isolated by crushing the thymic lob in RPMI medium and centrifugation over a ficoll gradient to isolate the white mononucleated cells. Subsequently, they were put into cell culture for transduction or CD34<sup>+</sup>-cells enrichment was performed by means of positive selection using magnetic cell separation beads and columns according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Since CD34<sup>+</sup> enrichment was envisaged only one passage through a magnetized column was performed. Human CD34<sup>+</sup>-cells were isolated from CB as previously described.<sup>17</sup> PBMC were isolated from human peripheral blood by a ficol gradient.

Freshly isolated lymphocytes and thymocytes were immediately seeded in RPMI 1640 medium (Gibco, Invitrogen) supplemented with 10% fetal calf serum and penicillin/streptomycin (Gibco, Invitrogen).

### **Transduction of primary cells**

PB T lymphocytes were prestimulated for 24h with anti-CD3 and anti-CD28 antibodies (1 µg/mL, BD Pharmingen) in presence of hIL-2 (1 ng/mL) and CB and PB T cells were pre-stimulated for two and three days, respectively with rIL-7 (10 ng/mL; BD Biosciences)<sup>49</sup>.

5E4 CB or PB T cells were seeded in 48-well plates upon TCR- or cytokine stimulation and transduced with concentrated LVs at indicated MOIs. Cells were replenished with cytokines every three days. Three and six days after transduction, the percentage of GFP<sup>+</sup>-cells was determined by FACS. Where indicated, transductions were performed on Retronectin® coated plates according to manufacturer's instructions (Takara, Japan).

5E5 freshly isolated thymocytes or CD34<sup>+</sup> enriched thymocytes were seeded in Retronectin® coated 48-well plates in RPMI/10%FCS in the presence of rIL-7 (20 ng/mL) with concentrated LVs at indicated MOIs. Cells were replenished with cytokines after three days. Four days after transduction, the percentage of GFP<sup>+</sup>-cells was determined in the thymocyte subpopulations by FACS.

### **Transduction of early T cell lineages during CB hCD34<sup>+</sup>-cell exposure to Delta-4**

CB CD34<sup>+</sup>-cells were cultured in 48-well plates previously coated with DL-4 Fc fusion protein (5 µg/ml, 4°C overnight; <sup>46</sup>) Cultures were initiated at concentration of 1E4 CD34<sup>+</sup>-cells per well in X-vivo 20 medium (BioWhittaker, Walkersville, MD), supplemented with 20% defined fetal calf serum (Hyclone, Thermo Fisher Scientific, Illkirch, France) and human cytokines IL-7, Flt3-L, SCF and TPO (all at 100 ng/ml; Myltenyi). After 24 h of culture, the LVs were added to the cells at the indicated MOIs. Before LV addition to the cells, the vectors were pre-incubated with the transduction facilitating agent Vectofusin (12 µg/ml) for 10 min. After seven days of culture on DL-4, cells were transferred to freshly DL-4 coated 24-wells at a concentration of 1E5 cells/well for further differentiation and transduction evaluation. The % of GFP<sup>+</sup>-cells was determined in the thymocyte subpopulation by FACS at day three, day seven and day fourteen of differentiation.

## **FACS analysis**

For the detection of LV transduction of different adult and cord blood T cell subpopulations flow cytometry analysis was performed using anti-CD3-APC, CD62L-PECy7, CCR7-PE, CD4- or CD8-Vioblue, CD45RA-APC, CD45RO-PE and CD31-Pacific Blue antibodies. Staining of total thymocytes with anti-hCD3-PE, anti-hCD8-PECy7 and anti-hCD4-APC is performed to evaluate transduction of thymic subpopulations. Staining of ELP, ETP, Pro T and Pre T thymocyte populations as well as CD34<sup>+</sup>-enriched cells during hCD34<sup>+</sup>-cell differentiation toward T cell lineage was evaluated by FACS using anti-CD34-APC, -CD7-PECy7, -CD5-PE and -CD1a-Vioblue.

For FACS analysis of the NSG reconstituted mice with progenitor T cells we used APC-conjugated anti-hCD45 antibody for the detection of total human cell engraftment in the bone marrow, thymus, peripheral blood and spleen. Thymocytes and peripheral T cell subpopulations are evaluated by anti-hCD3-PE, anti-hCD8-PECy7 and anti-hCD4-APC. Staining of ELP, ETP, Pro T and Pre T early thymocyte population was evaluated by FACS using anti-CD34-APC, -CD7-PECy7, -CD5-PE and -CD1a-Vioblue. Maturation of T cells was detected by CD3-APC, TCR $\alpha/\beta$ -PE or TCR $\gamma\delta$ -PE in the thymus, blood and spleen. For co-injection of T cell progenitors and CD34<sup>+</sup>-cells, anti-CD3-PE, anti-CD19-PECy7, anti-CD14-APC, anti-CD13-APC antibodies are used for phenotyping of the BM. Staining of progenitors and early B-cell population in spleen and BM is performed using anti-CD34-APC, anti-CD19-PECy7, anti-CD10-PE. Staining of thymocytes with anti-hCD3-PE, anti-hCD8-PECy7 and anti-hCD4-APC is performed to screen for thymic subpopulations. Blood samples are stained using anti-CD3-APC, anti-CD19-PECy7, CD14-APC.

## **ASCT-1 and ASCT-2 mRNA detection by quantitative RT-PCR**

To quantify ASCT1 and ASCT2 gene expression, we used reversed transcription combined with the PCR. Total RNA was isolated from purified unstimulated, IL-7 and TCR stimulated adult peripheral blood T cells by acid guanidinium thiocyanate-phenol-chloroform extraction.

0.5  $\mu$ g of the total RNA from each sample was reverse transcribed into cDNA using the



iScript™ cDNA synthesis kit (BIO-RAD, Marnes-la-Coquette). The amount of ASCT1,

ASCT2 and GAPDH mRNA was determined using the Fast Start universal SYBR Green

Master MIX (Rox, Roche, France) and the following primers:

- ASCT1 F: 5'- ATT GGT CCT GTT TGC TCT GG – 3'

- ASCT1 R: 5' – TGG GGA GAA TAA ACC TGC TG - 3'

- ASCT2 F: 5'- TAC ATT CTG TGC TGC CTG CT – 3'

- ASCT2 R: 5' – ATG AAA CGG CTG ATG TGC TT – 3'

- GAPDH F: 5' – AAG GTG AAG GTC GGA GTC AA – 3'

- GAPDH R: 5' – AAT GAA GGG GTC ATT GAT GG – 3'

The ASCT1 and ASCT2 mRNA levels were calculated relative to the GAPDH housekeeping

gene and normalized to control conditions by using the 2-delta-Ct method.

### **Conditioning and reconstitution of NSG mice**

NSG mice were housed in our animal facility (PBES-Lyon, France). Experiments were performed in accordance with the EU guidelines upon approval of the protocols by the local ethical committee. Two to 3-day-old newborn NSG mice were subjected to 1 Gy irradiation and  $5 \times 10^5$  transduced progenitor T cells derived from hCD34<sup>+</sup>-cells were injected intra-hepatically. Pre-stimulated freshly isolated CD34<sup>+</sup>-cells from the same donor were transduced with an mcherry coding vector for 24h and  $2 \times 10^5$  transduced CD34<sup>+</sup>-cells were co-injected with  $5 \times 10^5$  autologous human progenitor T cells transduced with a GFP encoding vector into NSG mice as above.

After four, six and eight to twelve weeks of reconstitution, extensive cell phenotyping in the different hematopoietic tissues (BM, spleen, thymus, blood) of these mice was performed (see FACS analysis).

### **Statistical analysis**

Statistical analysis was performed using student *t* test.

## Results

The new baboon gp LV pseudotypes engineered recently by us (BaEVTR- and BaEVRless-LVs<sup>17</sup>) were compared to other reported LV pseudotypes that allow T cell transduction: 1) Measles virus gp (HF-LVs<sup>58</sup>), 2) RD114 cat retrovirus gp (RDTR-LVs<sup>60</sup>) and 3) the generally used VSV-G gp (VSV-G-LVs<sup>49</sup>). The different gps are depicted in supplementary figure 1.

Up to date the majority of the clinical trials including those using CAR modified T cells<sup>4</sup> employ T cell receptor (TCR) stimulation to allow efficient gene transfer into the target T cells. The BaEVTR- and BaEVRless-LVs (BaEV-LVs) reached 80-90 % T cell gene transfer after TCR (anti-CD3+antiCD28) stimulation at low vector doses (MOI = 10) and thus reached a transduction levels slightly higher that obtained with VSV-G- and RDTR-LVs (Figure 1A). Importantly, these BaEV pseudotypes are dependent on the facilitating agent retronectin for high T cell transduction (Figure 1A). HF-LVs reached similar transduction levels as the BaEV-LVs in the presence of retronectin, which allowed still an increase in HF-LV T cell transduction as compared to no retronectin addition (Figure 1A and <sup>58</sup>). As mentioned above this strong signalling through the TCR changes T cell phenotype and thus a milder stimulation protocol might be warranted allowing conservation of naïve T cells which are the long-living cells that, when corrected, might persist over years. Since IL-7 stimulation does not skew the T cell population we tested the performance of the BaEV-LVs as compared to the other LV pseudotypes on IL-7 stimulated adult T cells.

Both BaEVTR- and BaEVRless-LVs allow efficient transduction of rIL-7 pre-stimulated total human T cells and were superior over VSV-G-LVs at the same (MOI=10) or at 10-fold higher (MOI=100) vector doses in the presence of retronectin (% transduction, BaEVTR-LV=29-43%, BaEVRless = 35-60%, VSV-G-LV= 2-12% (MOI 10); Figure 1B). These BaEVTR- and BaEVRless-LVs allowed an efficient high transduction level of both naïve and memory IL-7 prestimulated T cells (Figure 1B). HF-LVs resulted in over 80% transduction of IL-7 stimulated T cell in presence of retronectin. In this case the HF-LVs target primarily memory T cells for transduction confirming our previous results (Figure 1B and <sup>58</sup>).

Strict gating on CD45RA, CD62L and CCR7 expressing naïve T cells confirmed high-level BaEV-LV transduction in these CD4<sup>+</sup> and CD8<sup>+</sup> subpopulations (Figure 1C). Surprisingly, the RDTR-LVs allowed much lower transduction efficiency of rIL-7 prestimulated naïve and memory T cells as compared to BaEV-LVs (RDTR-LV= 5-15%). As expected, BaEVTR- and BaEVRless-LVs allowed high-level transduction of IL-7 stimulated T cells without compromising their naïve phenotype or skewing CD4/CD8 T cell population (data not shown).

Previously, we have shown that HF-LVs allow gene transfer into resting T cells<sup>58,65</sup> but we confirmed here that as well RDTR- as BaEVTR- and BaEVRless-LVs perform poorly on these quiescent target cells (Supplementary Figure 2). This might be explained by a distinct receptor usage for these pseudotypes. RDTR-LVs only bind to the hASCT-2 receptor for cell entry while the BaEV-LVs additionally bind to the amino acid transporter, hASCT-1. Importantly, both ASCT-1 and ASCT-2 mRNA levels are upregulated upon IL-7 cytokine-stimulation and this upregulation is even more pronounced upon TCR activation of T cells. Upregulation might strongly increase the affinity of BaEV-LVs to T cells and explain the significantly higher transduction levels in these stimulated cells as compared to their resting counterparts (Figure 1D and 1A and B).

Overall, the novel BaEV/TR- and BaEVRless-LVs are promising gene transfer tools for adult T cell gene therapy. Indeed, if one considers the need for a life time correction, we need to transduce the long-lived naïve T cells which are shown here to be highly susceptible to BaEVTR- and BaEVRless-LV mediated gene transfer.

### **BAEVgp-LVs allow high-level transduction of recent thymocyte emigrants**

It might be of high interest for T cell gene therapy or immunotherapy to genetically modify T cells at an as early stage as possible, at a stage where the cells will persist for the entire life-span of an individual. Therefore, we focused on cord blood (CB) T cells since they contain for the majority highly immature naïve T cells. The majority of CB CD4<sup>+</sup> T cells (70-80%) are identified by CD31, CD62L and CD45RA positive staining which have the features of recent thymocyte emigrants

(RTEs). Genetic modification of these cells in the context of allogeneic transplantation to increase their graft-versus-leukemia effect without increasing GVHD would be beneficial. Therefore, we tested several lentiviral vector pseudotypes for their transduction efficiency of CB RTEs.

To conserve the T cell naïve phenotype we prestimulated freshly isolated total CB T cells with the T cell survival cytokine, IL-7, for 24 h and subsequently transduced them with the different vectors: VSV-G-, RDTR-, BAEVTR-, BAEVRless- and HF-LVs coding for the GFP reporter at an MOI of 10 or 20, except for VSV-G-LVs, for which a higher vector dose (MOI of 50) was applied. BAEVTR-, and BAEVRless-LVs permitted 40-55% transduction at an MOI =10, outperforming VSV-G- (< 20%) or RDTR-LVs (<10%; Figure 2A). HF-LVs barely achieved 20% transduction in the presence of retronectin, signifying that RTEs and mature naïve CB T cells are poorly permissive for HF-LVs confirming our previous results (Frecha et al. et al. 2008). Importantly, BAEVTR- and BAEVRless-LVs reached easily 60 to 70 % transduction when the vector dose increased 2-fold (MOI = 20; Figure 2B). In all cases, the immature naïve recent thymocyte emigrants (CD69L<sup>+</sup>CD45RA<sup>+</sup>CD31<sup>+</sup>) were transduced to the same extent as the more mature naïve CB CD4<sup>+</sup> T cells (CD69L<sup>+</sup>CD45RA<sup>+</sup>CD31<sup>-</sup>; figure 2B). Transductions had no effect on the distribution of these two naïve CB T cell populations as compared to untransduced control cells (Figure 2C).

### **BAEV-LVs promote high-level transduction of thymocytes in early stages of differentiation.**

It would be of major interest to correct T cells or genetically change T cell functions as early in T cell differentiation as possible to assure a long-term benefit to patients and allow long-term in vivo expansion of these cells.

Thymocyte differentiation is a highly regulated process that occurs via the interaction with the thymic stroma cells and cytokine stimulation (Figure 3 A). Hematopoietic stem cells (CD34<sup>+</sup> CD1a<sup>-</sup>) are migrating from the BM to the thymus where they lack the expression of CD8 and CD4 markers (CD8<sup>-</sup>CD4<sup>-</sup> double negative (DN) stage; 2-5% of total thymocytes). The DN cells subsequently gain expression of

the pre-T cell receptor (pre-T) and develop into immature CD4 single positive cells (ISP) (Figure 3A). The appearance of the CD8 surface marker together with CD4 and maturation of the TCR is called the double positive (DP) stage of thymocyte development (80-90%). Upon selection in the thymus, DP thymocytes develop into CD8<sup>+</sup> CD4<sup>-</sup> (SP8) and CD8<sup>-</sup> CD4<sup>+</sup> (SP4) thymocytes.

Because of the potential value of thymocyte gene transfer for immune modulation and gene therapy, we evaluated the performance of BAEGp LVs for the transduction of all these different thymocyte subpopulations. Upon isolation of mononuclear cells from human thymus, we transduced them in the presence of the survival cytokine IL-7 with RDTR-, BAEVTR-, BAEVRless- and HF-LVs encoding the GFP reporter at an MOI of 10. VSV-G-LVs were used at higher vector doses (MOI 50). For all transduction experiments retronectin coated plates were used. Three days later we determined the transduction levels by FACS in the different thymic subpopulations using CD4/CD8/CD3 multiple staining. VSV-G-LVs do not allow efficient transduction of all these different subpopulations reaching between 10 and 20 % of transduced cells (Figure 3B). RD114/TR-LV, BAEVTR-LVs and BAEVRless-LVs though allow preferential transduction of the thymocytes in the early stages of development (DN and ISP; Figure 3A). They transduce the more mature thymocytes but to a much lower extent (Figure 3B). As already detected for adult human T cells, RDTR-LVs transduction of all different thymocyte subpopulations was significantly lower as compared to BaEV-gp-LVs ( $p < 0.05$ ). Interestingly, HF-LVs did not show a preference for the most immature thymocytes but assured an efficient transduction of all the different thymocyte populations (Figure 3B). Of importance, the transduction with the different LV pseudotypes had no effect on the distribution of different thymocyte subpopulations (Supplementary Figure 3).

The DN population can still be subdivided in early lymphoid progenitors (ELP), followed the early thymocyte (ETP) progenitor stage, which then differentiate into pro T and pre T cells. These distinct DN thymocyte stages can be identified by CD34, CD7, CD4 and CD1a surface markers as indicated in Figure 3A.

Since the DN stage is only a very small fraction of the total thymocytes, we enriched the thymocytes for the CD34<sup>+</sup> marker to obtain enough ELP, ETP and Pro T

and Pre T cells for transduction experiments. The gating strategy of these different thymic fractions is depicted in Figure 3C (Upper blot). Again the BaEVgp-LVs target transduction to the most immature DN thymocytes, with BaEVRless-LVs reaching 100 and 90% of transduced ETP and Pro T cells, respectively (Figure 3D). A representative experiment is shown in Figure 3C for BaEVTR-LVs. The VSV-G and RDTR pseudotypes result in transduction levels way below that. HF-LVs, on the contrary mediate intermediate transduction levels, equivalent in ETP, proT, Pre T and ISP cells (Figure 3D).

This indicates that BAEV-LVs are excellent tools for transduction of thymocytes and that they are highly superior over VSV-G- and RDTR-LVs for the transduction of ETP and pro T and ISP thymocytes. Moreover, they allowed high-level transduction upon mild IL-7 stimulation avoiding skewing of these populations.

### **BAEV-LVs mediates efficient transduction in human T lymphoid progenitors generated *in vitro***

Here we will make use of a new culture system based on immobilized Notch Ligand Delta-like-4 (DL-4) that we described earlier<sup>46</sup>. Culture of human CD34<sup>+</sup> CB progenitors in this system enabled *in vitro* generation of large amounts of T cell progenitors, displaying the same characteristics as ETPs. Since the co-injection of LV-corrected autologous T cell progenitors and HSCs might accelerate T cell reconstitution in the patients as compared to solely injecting HSCs, we evaluated the BAEV-LVs for their capacity to confer gene transfer into *in vitro* generated ETP and Pro T cells.

Purified CB CD34<sup>+</sup>-cells were cultured on DL-4 coated plates in the presence of a cytokine cocktail (IL-7, TPO, SCF and Flt3-L) inducing T cell differentiation. After 24h incubation, the different vector pseudotypes were added to these cell cultures at an MOI of 3. At day three of differentiation, the CD34<sup>+</sup>-cells began to express CD7 allowing to distinguish the ELP and ETP thymocyte-like populations (Figure 4A). In perfect agreement with the results obtained for freshly isolated human ETP cells, BAEV-LVs transduced at high level ELPs (65-75%) and ETPs (70-80%) (Figure 4C vs Figure 3D). Accordingly, VSV-G- and RD114TR-LVs permitted only poor

transduction of ELPs (15-20 % on average) and ETPs (8-10% on average), whereas HF-LVs achieved intermediate transduction levels of 30-50% (Figure 4A and C).

In accordance with our previous results<sup>46</sup>, the CD34<sup>+</sup>CD7<sup>+</sup> ETP population further increased and a new population of CD7<sup>+</sup>CD34<sup>-</sup> pro T cells appeared by day seven of differentiation (Figure 4B). We obtained the same picture as for day three of differentiation: BaEV-LVs conferred high-level gene transfer into as well ELP and ETP and now also into proT cells while VSV-G and RDTR-LVs were much less efficient for transduction of these cell populations (Figure 4D). In contrast, the HF-LV transduction levels at day seven of T cell differentiation were similar to the BaEV-LVs (Figure 4D). Representative results for day seven are shown in Figure 4B. Importantly, differentiation into ETP and pro T cells was not perturbed by any of the LV pseudotypes as compared to untransduced cells (Supplementary Figure 4). By day 14, a subset of CD34<sup>-</sup>CD7<sup>++</sup> starts to express CD5, which allows to distinguish ProT1 (CD7<sup>++</sup>/CD5<sup>-</sup>) and ProT2 (CD7<sup>++</sup>/CD5<sup>+</sup>). When the latter population gains expression of CD1a we can identify newly developed pre T cells (Figure 4E). As expected, the high-level transductions obtained for BaEV-LVs at day three and seven persisted at day fourteen for the ELP, ETP and pro T1 and T2 thymic populations but was also detected in the newly differentiated pre T cell stage (Figure 4E). Since the original CD34<sup>+</sup>-cells have undergone 50-fold expansion after 14d of T cell differentiation, stable transduction in the different populations was confirmed.

### **H/F-LV transduced progenitor T cells seed the thymus and allowed accelerated thymopoiesis in vivo in NSG mice**

To evaluate the T cell differentiation potential of LV transduced progenitor T cells we used the immunodeficient NSG mice model since it supports efficiently T cell lineage differentiation. We choose to engraft the cells into newborns NSG mice since it was reported to support better human cell engraftment<sup>46</sup>.

CD34<sup>+</sup>-cells isolated from one single CB donor were divided into two. Half of these cells were plated on DL-4 coated culture wells in presence of a T cell differentiation cytokine cocktail (IL7, TPO, Flt3-L) for 24h. They were subsequently transduced with HF-LVs at a MOI of 10 in the presence of Vectofusin. The other half

of the CD34<sup>+</sup> cells was defrosted eight days later and these cells were stimulated by a cocktail of early acting cytokines (SCF, TPO, Flt3-L) for 24h and they were subsequently transduced with HF-LVs at a MOI of 10 in the presence of retronectin. HF-LV transduced T cell progenitors and HF-LV transduced CD34<sup>+</sup> cells were separately injected into sub-lethally irradiated newborn NSG mice after 10 days of differentiation and 24 hours of cytokine stimulation, respectively. For both conditions untransduced control cells were injected in parallel. Human thymocyte reconstitution was assessed six weeks after injection into NSG mice by flow cytometry. We found efficient thymic engraftment in for both T cell progenitors and CD34<sup>+</sup> cells independent of transduction or not. Intrathymic T cell development was accelerated in DL-4 treated compared to hCD34<sup>+</sup> cell recipient mice as suggested by higher percentage of mature CD3<sup>+</sup>/TCR $\alpha/\beta$  cells (Figure 5A; 10-fold increase) and CD4 and CD8 single positive cells coinciding with decreased DP thymocyte numbers (Figure 5B). Of high importance, this was true for as well untransduced as HF-LVs transduced progenitor T cells underlining that transduction did not affect T cell potential in vivo. To evaluate the proportion of the DN thymic subpopulations CD34/CD7/CD5/CD1a labeling was performed of human thymocytes. We distinguish a much higher proportion of ProT2 (CD7<sup>++</sup>/CD5<sup>+</sup>) than ProT1 (CD7<sup>++</sup>/CD5<sup>-</sup>) cell for injected T cell progenitors as compared to hCD34<sup>+</sup> cells (Figure 5 C and D). The pre T cells expressing, CD1a at the surface next to CD7 and CD5 was highly enriched in the mice injected with T cells progenitors (Figure 5 C and D). Of importance, high level HF-LV transduction detected ex vivo in the T cell progenitors persisted in all the thymic subpopulations in vivo.

In conclusion, high level gene marked T cell progenitors generated in vitro, efficiently repopulate the thymus of NSG mice, allow efficient thymopoiesis and speed up T cell reconstitution.

*REMARK: For the moment only the first HF-LV transduction data are available since these experiments in newborn NSG mice are timeconsuming and difficult to plan. New NSG reconstitution experiments are ongoing for HF-LV and BaEV-LV progenitor T cells. Finally, we will co-inject HF-LV and BaEV-LV transduced progenitor T cells gene-marked by the GFP reporter and hCD34<sup>+</sup> cells marked by mcherry to evaluate*



*carefully the reconstitution of the T cell lineages in a single experiment mimicking a preclinical setting for gene therapy.*

## **Discussion**

Here we highlight the exceptional capacity of the engineered Baboon envelope pseudotyped LVs (BaEV-LVs) to efficiently transduce naïve adult and cord blood T cells and early T cell thymic subpopulations. Of note, we recently demonstrated that these new BaEV-LVs outperformed VSV-G- and RD114/TR-LVs by far for gene delivery into HSCs.<sup>17</sup> Here, we report that these BaEV-LVs succeeded in transducing up to 70% of IL-7 stimulated naïve adult T cells and CB recent thymic emigrants. Remarkably, they transduced up to 80% of early thymocytes such as ETP, proT and preT cell lineages differentiated from CD34<sup>+</sup> cells using a feeder-cell free DL-4 culture system<sup>46</sup>. In contrast, as well for cord blood RTEs as early thymic populations, transduction levels using VSV-G-LVs or RDTR-LVs were very poor. Of note, although we reported that H/F-LVs were the first vectors to allow efficient gene delivery in resting adult T cells<sup>58,59</sup>, they were unable to high-level transduction of naïve immature CB T cells and conferred preferential transduction of memory over naïve T cells<sup>58,59</sup>. In comparison with H/F-LVs, BaEV-LVs appeared a promising tool for the transduction of naïve and early progenitor T cells while H/F-LVs is complementary in its capacity to efficiently transduce memory T cells and late stage thymocytes: CD4/CD8 double positive and CD4 or CD8 single positive cells. Surprisingly, BaEV-LVs do not, in contrast to H/F-LVs, permit efficient transduction of resting T cells probably due to a low mRNA expression profile of ASCT-1 and ASCT2, the two receptors used for BaEV-LV cell entry<sup>63,66</sup>. In agreement, we confirmed an important upregulation of both receptors upon IL-7 and TCR stimulation of T cells coinciding with increased BaEV-LV mediated gene transfer.

A T-cell immunotherapy that currently deserves attention consists of engineering autologous or allogenic T cells to express chimeric antigen receptors (CAR) specific for: 1) viruses to induce an antiviral response in vivo or 2) tumor antigens to allow successful treatment of cancers such as melanoma, B-cell lymphoma or B-cell leukemia<sup>20-23</sup>. Although these CAR T cell strategies were using initially autologous adult T cell modification, recently this has been translated to

immature naïve cells from cord blood<sup>35,36,38</sup>. Micklethwate et al.<sup>14</sup> transduced CB T cells with a CAR against CD19, which also co-expressed TCRs specific for three different viruses that can affect patients after a cord blood transplant (CMV, EBV and adenovirus).

Naïve CB T cells as compared to adult T cell show a greater tolerance to HLA disparity<sup>67,68</sup> and thus induce less graft-versus-host-disease. Therefore, they could be considered as a convenient alternative source of allogenic lymphocytes for adoptive cellular therapy against viral infection or/and cancer. In particular, for example, when cancer patients do not have enough autologous T cells. Although the advantages of LVs as compared to retroviral vectors are clearly recognized, the absence of adapted tools for transducing naïve CB T cells incited people to continue using retroviral vector gene delivery in TCR stimulated CB T cells<sup>18</sup>. The development of CAR CB T cell strategies based on the genetic modification of IL-7 mildly stimulated CB T cells by the use of BaEV-LVs could answer to numerous current issues. Indeed, since BaEV-LVs provide next to 70% of gene delivery in IL-7 stimulated CB T cells, they could allow to compensate the low number of modified effector T cells derived from CB.<sup>69</sup> This might represent a huge advantage for CAR therapy since naïve CB T cell subsets show high capacity to expand, and could provided longer persisting anti-tumor responses in vivo than adult T cells, allowing to protect against relapse after transplant<sup>32,34</sup>

Upon the infusion of allogenic cord blood or bone marrow HSC engraftment, very often serious infections are detected in patients as a side-effect that can become fatal. This is undoubtedly related to delayed T cell immune reconstitution and impaired de novo thymopoiesis<sup>70,71</sup>. The same is detected upon engraftment of autologous gene therapy corrected HSCs, where T cell reconstitution can take months. Moreover, complete restoration of the T cell compartment with the polyclonal T cell repertoire takes even longer, which fragilizes the patients for more than a year<sup>70,72</sup>.

A promising approach for accelerating de novo T cell development consists in adoptive transfer of high numbers of in vitro generated T cell progenitors. In the case of gene therapy, autologous gene-corrected T cell progenitor administration might be

an option. We showed here that we can genetically modify these T cell progenitors derived from CD34<sup>+</sup>-cells at high levels (80%) using the BaEV-LVs or HF-LVs. Combining transduction with DL-4 culture of hCD34<sup>+</sup> cells, resulted in expansion of highly gene marked ETPs and ProT1 cells, the most primitive T cell lineages. Moreover, these cells retained in vivo intra-thymic T cell differentiation and displayed a conventional polyclonal CD4, CD8 and TCR $\alpha/\beta$  repertoire and T cell reconstitution was accelerated as compared to hCD34<sup>+</sup> cell engraftment in NSG mice<sup>46</sup>.

This might allow treatment of severe combined immunodeficiencies e.g. ZAP70<sup>-/-</sup> or also viral infections like HIV where the thymic environment is not ideal for HSC differentiation into T cell lineage. Indeed, infusion of autologous corrected DL-4 induced T cell progenitors together with corrected HSCs could accelerate immune reconstitution and offer a safe way to reduce the long phase of immune system recovery, which might reduce post-transplant complications such as infections. In this context, we have recently shown that BAEV-LVs not only allow high-level transduction of ETPs and proT cells but also of prestimulated and even unstimulated human HSCs<sup>17</sup>. Indeed, we demonstrated that NSG mice T cell lineage differentiation of injected genetically modified progenitor T cells was accelerated as compared to injection of transduced early immature progenitor hCD34<sup>+</sup>-cells.

In conclusion, BaEV-LVs and HF-LVs represent very useful new tools for T cell gene therapy and immunotherapy applications because of their unique quality to complementarily transduce efficiently naïve adult and cord blood T cells under conditions that avoid skewing of T cell phenotype.

Moreover, a strategy could be proposed based on the co-injection of BaEV-LV corrected autologous HSCs and corrected T progenitors to allow short term T cell immune response recovery combined with longterm correction in the patients.

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## Figure Legends

### **Figure 1 BAEV-LVs allow high level transduction of as well naïve as memory adult human T cells**

(A) Fresh peripheral blood T cells isolated by negative selection were pre-activated through the TCR (anti-CD3+ anti-CD28 antibodies + IL-2) for 24 h and transduced in the absence and presence of retronectin with the different LV pseudotypes at indicated MOIs. Three days after transduction the % of GFP<sup>+</sup> T cells was analyzed by FACS (means +/- SD; n=4).

(B) and (C) Freshly isolated T cells were pre-activated for three days with IL-7 and transduced in the presence of retronectin with the different LV pseudotypes at an MOI 10 or 100 as indicated. Three days after transduction the % of GFP<sup>+</sup> T-cells was analyzed by FACS for total T cells (anti-CD3) and the naïve (CD45RA<sup>+</sup>) or memory (anti-CD45RO<sup>+</sup>) T cells subsets in (B). More detailed phenotyping of these cells (C) shows the % GFP<sup>+</sup> naïve (CD45RA<sup>+</sup>CD62L<sup>+</sup>CCR7<sup>+</sup>) CD4 T cells and CD8 T cells (means +/- SD; n=3).

(D) ASCT-1 and -2 expression levels in unstimulated and TCR- or IL-7-activated total T cells (as in A and B, respectively). Relative fold increase in ASCT-1 or ASCT-2 mRNA levels in stimulated T cells as compared to unstimulated T cells, for which mRNA levels were normalized to 1 (means +/- SD; n=3).

### **Figure 2: BAEVgp-LVs allow efficient transduction of immature naïve cord blood T cells.**

Freshly isolated total CB CD4<sup>+</sup> T cells were pre-stimulated with IL-7 for 48 h and subsequently transduced in the presence of retronectin with the LV pseudotypes, VSV-G-, RDTR-, BAEVTR-, BAEVRless- and HF-LVs, encoding the GFP reporter at an MOI of 10 (A) or 20 (B), except for VSV-G-LVs for which a higher vector dose was applied as indicated. Three days after transduction cells were stained for human surface markers: CD45RA, CD62L, CD31 and the % of GFP<sup>+</sup> cells in the recent thymocyte emigrants (CD45RA<sup>+</sup>/CD62L<sup>+</sup>/CD31<sup>+</sup>) and more mature naïve CB CD4<sup>+</sup> T

cells (CD45RA<sup>+</sup>/CD62L<sup>+</sup>/CD31<sup>-</sup>) were determined by FACS analysis (means +/- SD; n=3).

(C) Distribution of the recent thymocyte emigrants (CD45RA<sup>+</sup>/CD62L<sup>+</sup>/CD31<sup>+</sup>) and more mature naïve CB (CD45RA<sup>+</sup>/CD62L<sup>+</sup>/CD31<sup>-</sup>) CD4<sup>+</sup> T cell subpopulations upon incubation of CB T cells with the different vector pseudotypes as compared to untransduced T cells (means +/- SD; n=4).

### **Figure 3. BAEVgp-LVs confer high-level transduction of human immature thymocyte populations**

(A) Schematic representation of human stem cell migration to the thymus and thymocyte development. For each differentiation stage phenotypic surface markers are indicated. HSPC= human stem progenitor cells; ELP = early lymphocyte progenitor, ETP= early thymocyte progenitor, ISP= immature single positive T cell, DP = double positive, SP = mature single positive T cell.

(B) Freshly isolated human thymocytes were transduced in the presence of IL-7 on retronectin coated culture plates with the indicated LV pseudotypes at an MOI of 10 except for VSV-G-LVs, for which an MOI 50 was applied. Four days post-transduction the thymocytes were stained for human surface markers (CD3, CD4 and CD8) and the % of GFP<sup>+</sup>-cells in the different thymocyte subpopulations analyzed by FACS (means +/- SD; n=5; CD4<sup>-</sup>CD8<sup>-</sup> =DN; CD4<sup>+</sup>CD3<sup>+</sup>CD8<sup>-</sup> = SP-CD4; CD4<sup>-</sup>CD3<sup>+</sup>CD8<sup>+</sup> = SP-CD8; CD4<sup>+</sup>CD8<sup>+</sup> =DP; CD4<sup>+</sup>CD3<sup>-</sup>CD8<sup>-</sup>=ISP).

(C,D) Freshly isolated human thymocytes were enriched for CD34<sup>+</sup>-cells to increase the DN thymocyte population and they were transduced as in (B). (D) Four days post-transduction the thymocytes were stained for human surface markers (CD34, CD7, CD1a and CD5) and the % of GFP<sup>+</sup> cells in the different thymocyte subpopulations were determined (means +/- SD, n=4). Gating strategies for FACS analysis are shown in (C) for a representative result of BAEVRless-LV transduction of CD34<sup>+</sup> enriched thymocytes

### **Figure 4. BAEVgp-LVs confer efficient gene transfer into in vitro generated early T cell progenitors**

CB CD34<sup>+</sup>-cells were plated on DL-4 coated culture wells in presence of a cytokine cocktail (IL7, TPO, Flt3-L) for 24h and they were subsequently transduced with VSV-G-, RDTR-, BAEVTR-, BAEVRless- and HF-LVs encoding the GFP reporter at an

MOI of 10, except for VSV-G-LVs for which a higher vector dose (MOI of 50) was applied. At day three (A,C) and seven (B,D) of culture, the cells were analysed by FACS for surface expression of CD34, CD7, and CD5. The gating strategy to determine GFP expression in the subpopulations ELP, ETP and Pro T and are shown in (A) and (B) for a representative experiment. The transduction levels for day three and seven of differentiation towards T cell lineage in these subpopulations are summarized in the histograms in (C) and (D) respectively. (E) At day fourteen of culture, the cells were analysed by FACS for surface expression of CD34, CD7, CD5 and CD1a; GFP expression in the subpopulations ELP, ETP, Pro T1, T2 and pre T cells at day fourteen of differentiation towards T cell lineage is shown for the different pseudotypes.

**Figure 5. H/F-LV transduced progenitor T cells allowed accelerated thymopoiesis in vivo in NSG mice as compared to HF-LV transduced CD34+ cells**

CB CD34<sup>+</sup>-cells from the same donor were subdivided in two parts. One part of these cells was plated on DL-4 coated culture wells in presence of a cytokine cocktail (IL7, TPO, Flt3-L) for 24h and they were subsequently transduced with HF-LVs in the presence of Vectofusin encoding the GFP reporter at a MOI of 10. At day six of differentiation, the other part of the CD34<sup>+</sup> cells was defrosted and these cells were stimulated by a cytokine cocktail (SCF, TPO, Flt3-L) for 24h and they were subsequently transduced with HF-LVs encoding the GFP reporter at a MOI of 10 in the presence of retronectin. HF-LV transduced T cell progenitors and CD34<sup>+</sup> cells were injected intrahepatically into separate sub-lethally irradiated newborn NSG mice after ten days of differentiation and 24h transduction, respectively. For both conditions untransduced control cells were injected in parallel. Human thymocyte reconstitution was assessed six weeks after injection into NSG mice by flow cytometry. CD3/TCR $\alpha/\beta$  a/b (A), CD4/CD8 (B), CD34/CD7/CD5/CD1a (C, D) labeling was performed for humanized thymus and compared between untransduced and H/F-LV transduction conditions.

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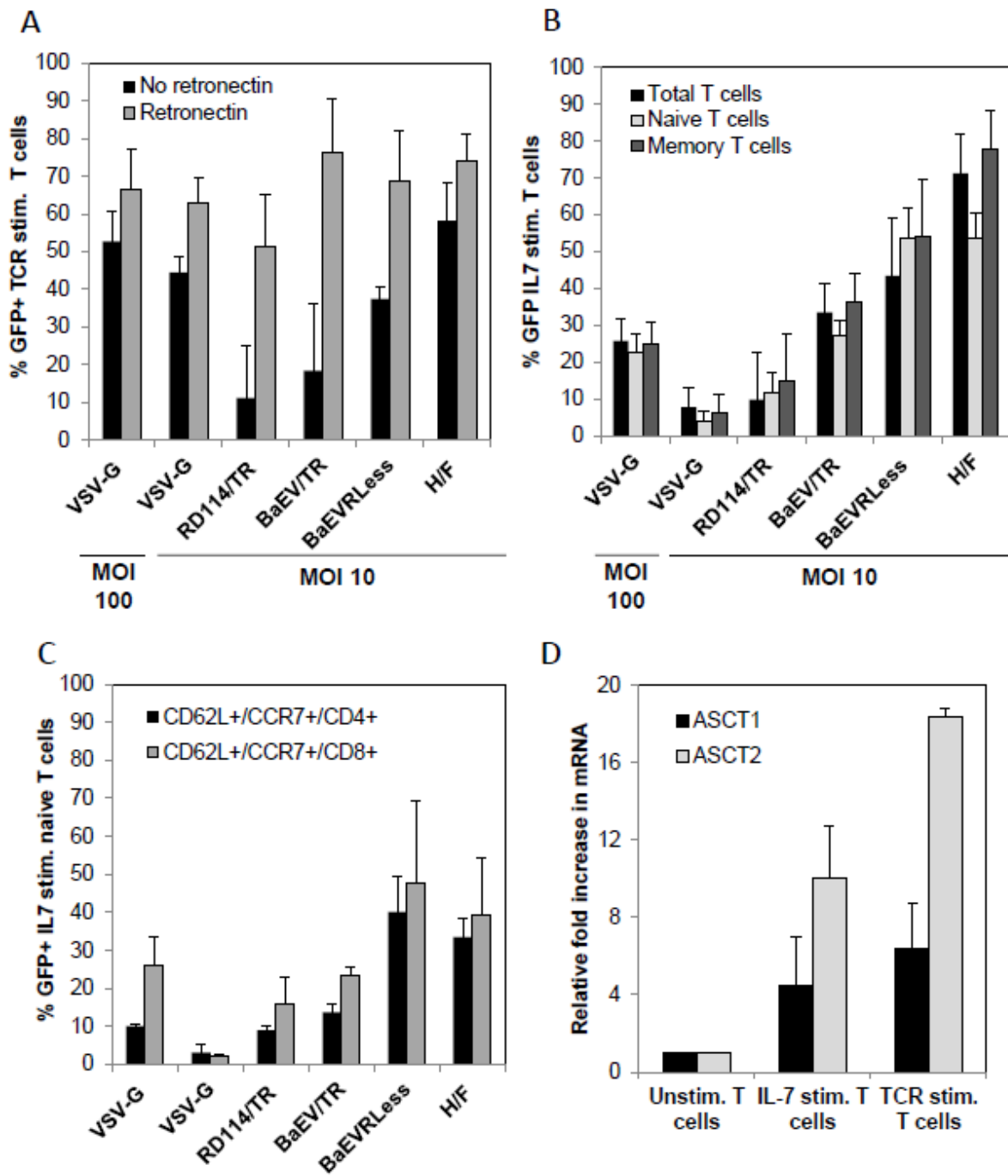


Figure 1

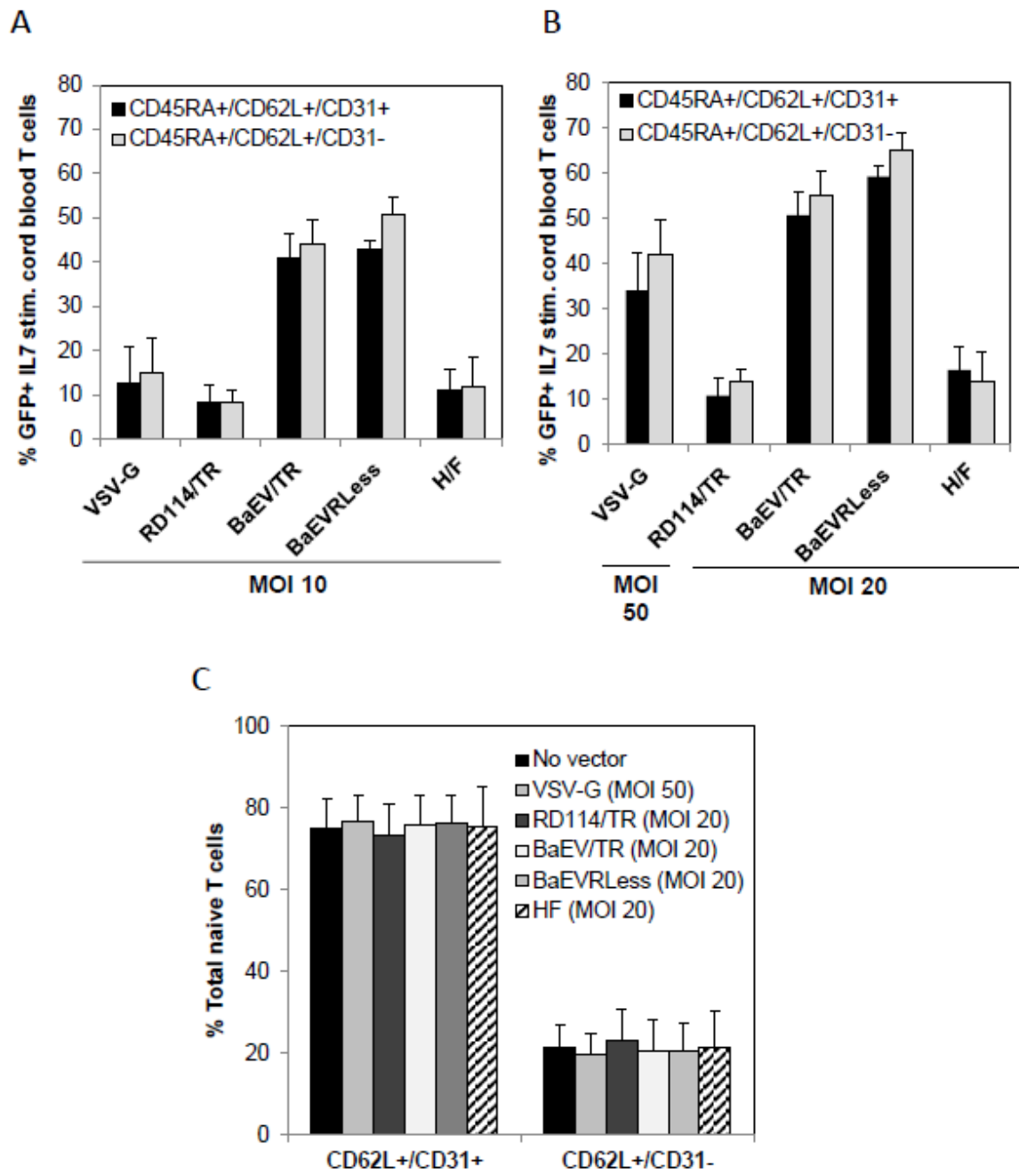


Figure 2

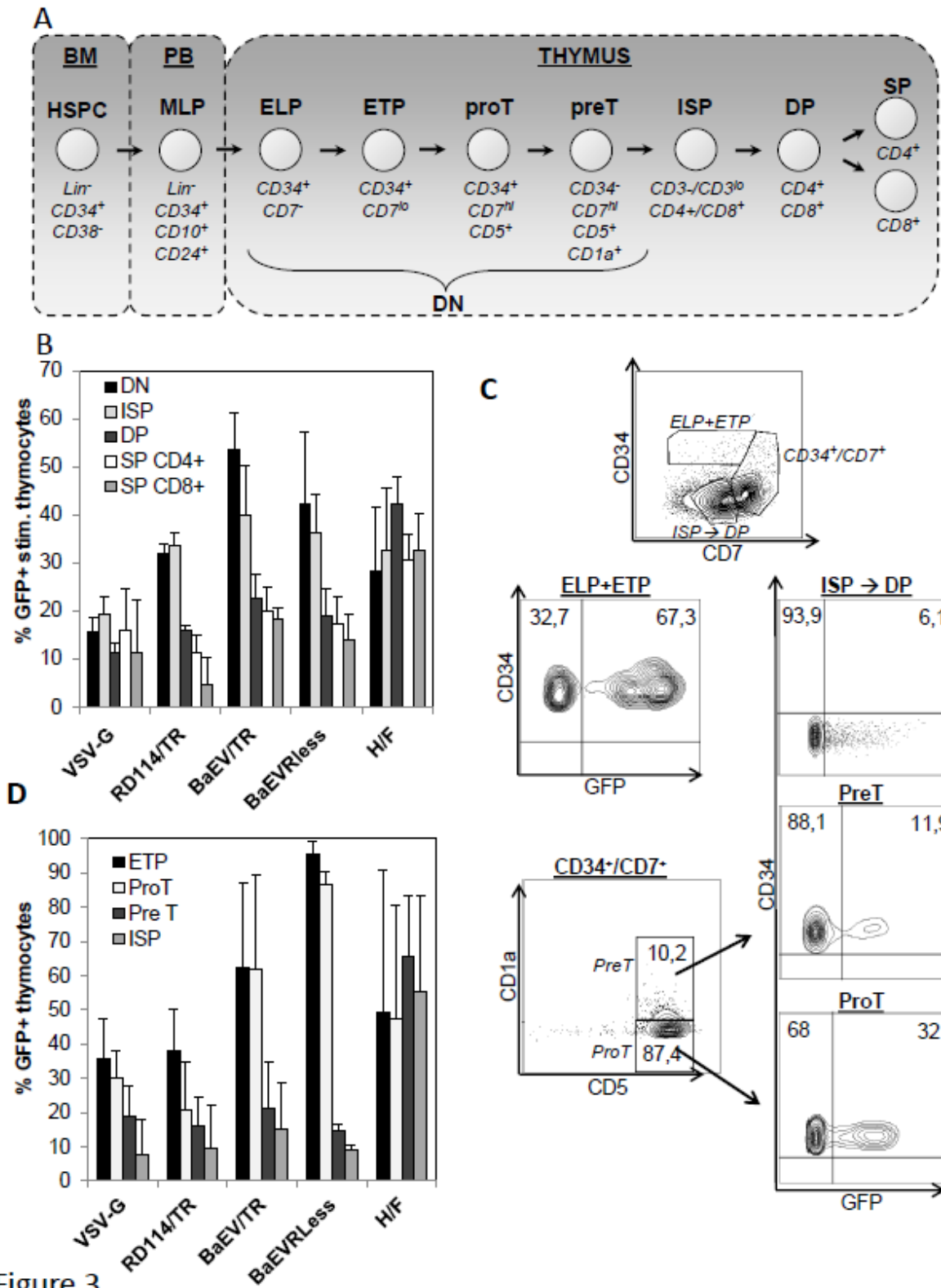


Figure 3

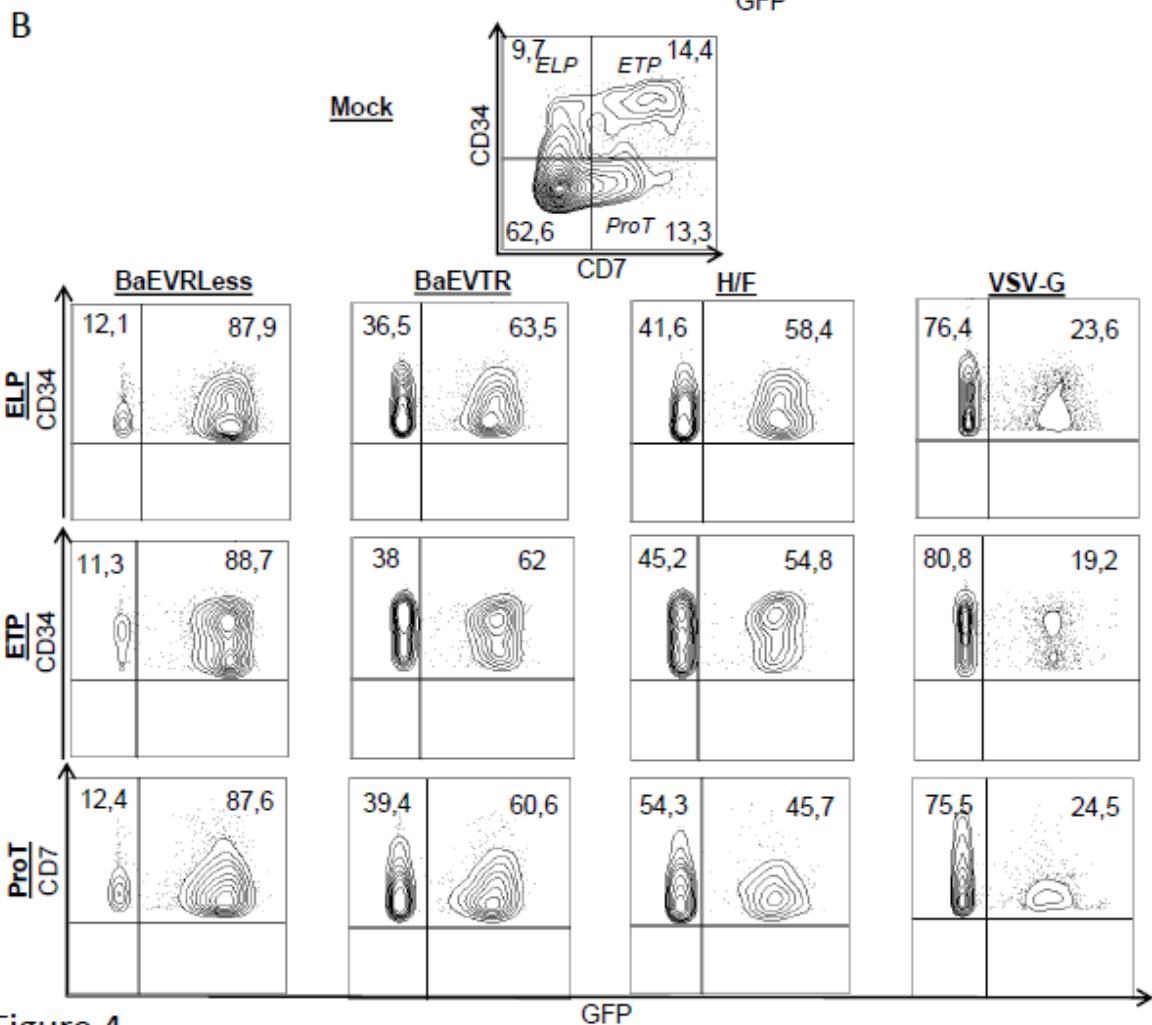
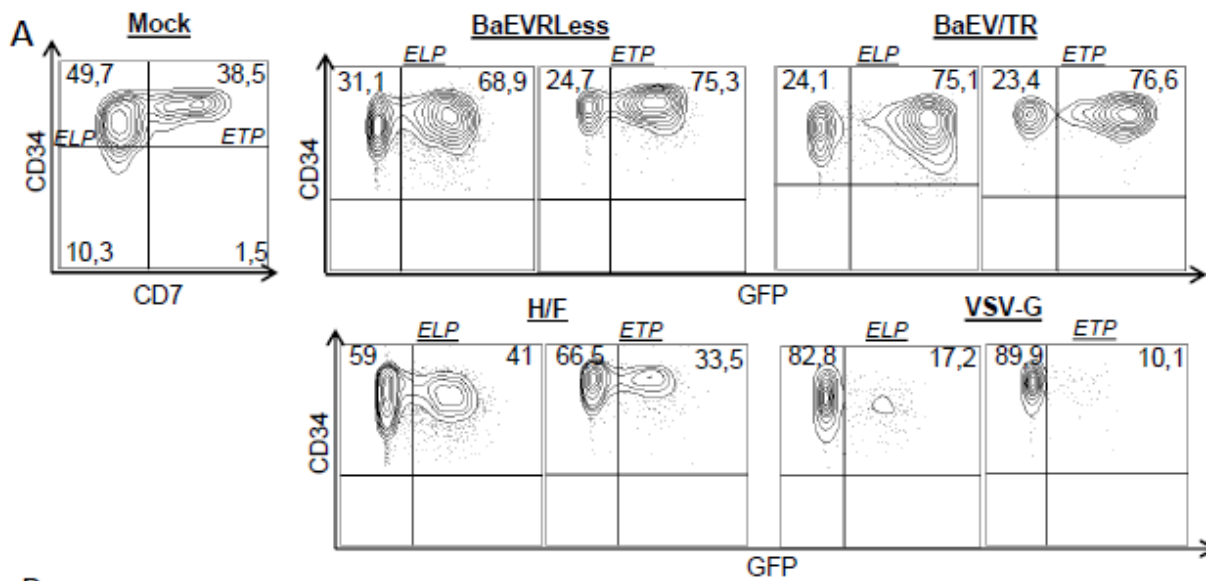


Figure 4

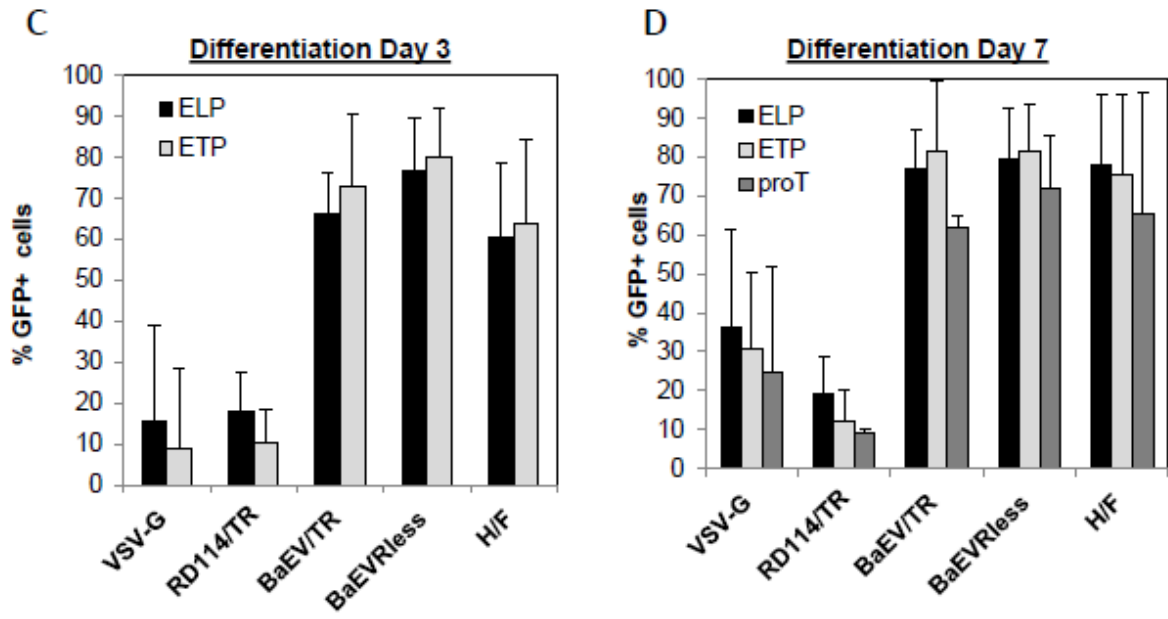


Figure 4



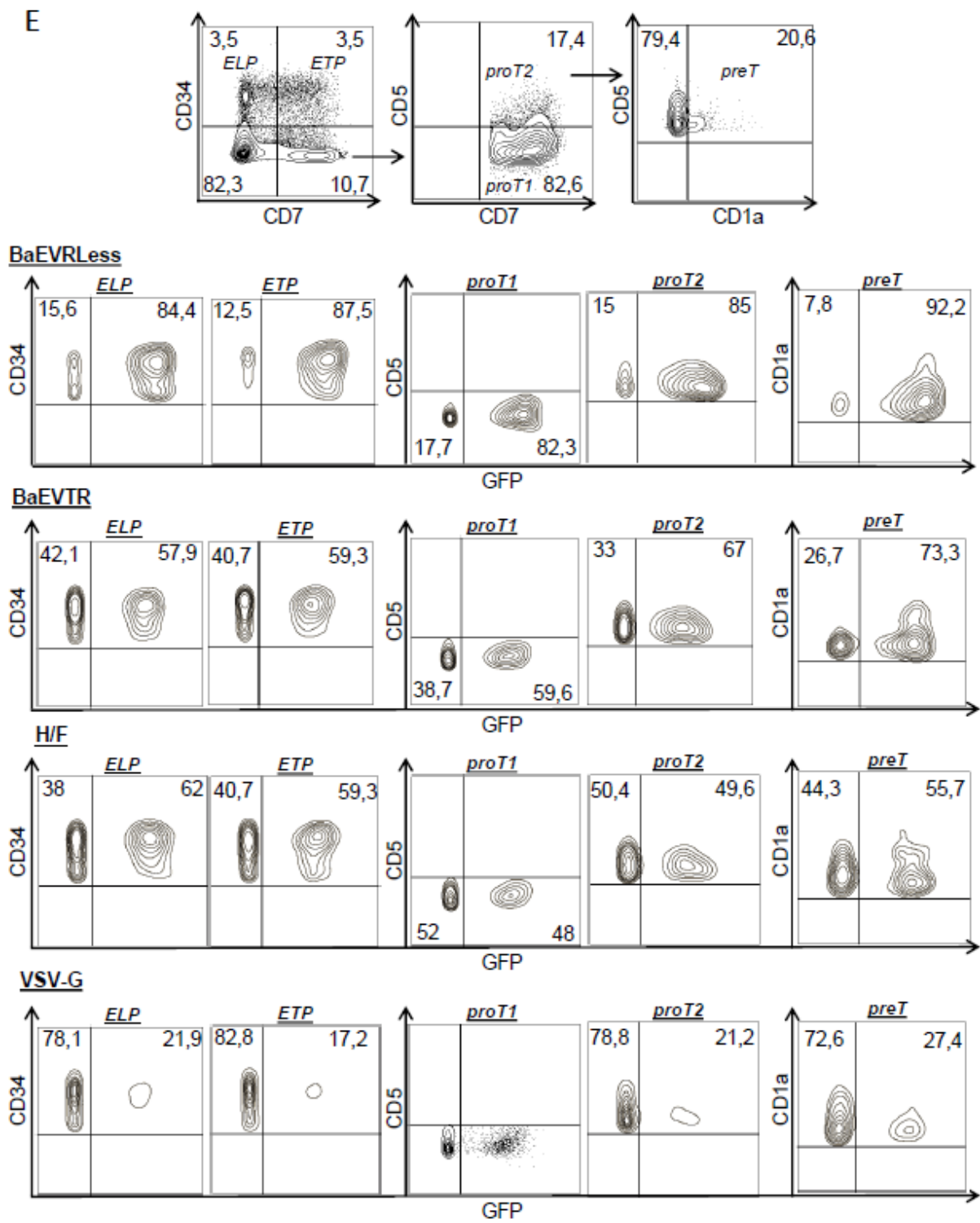


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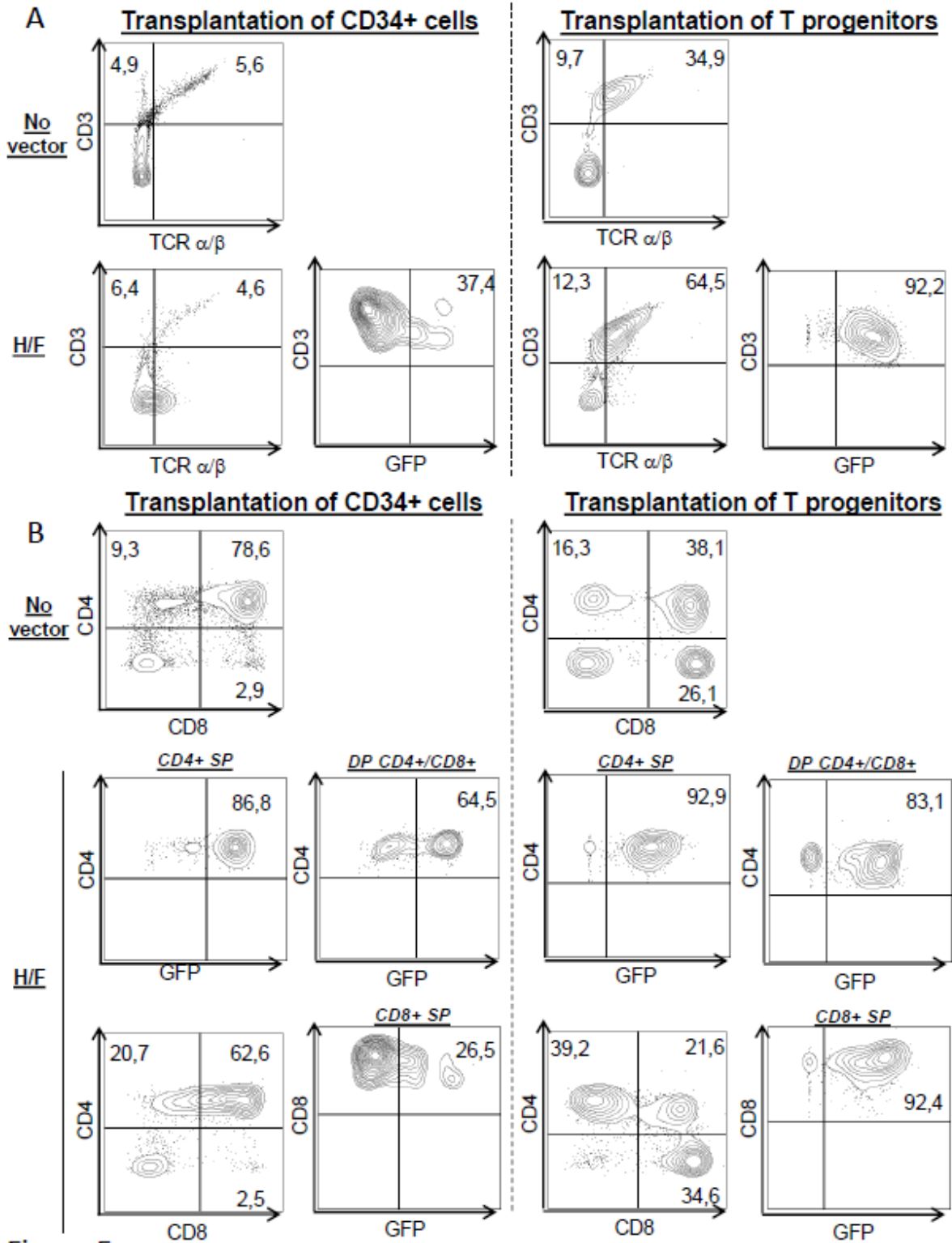
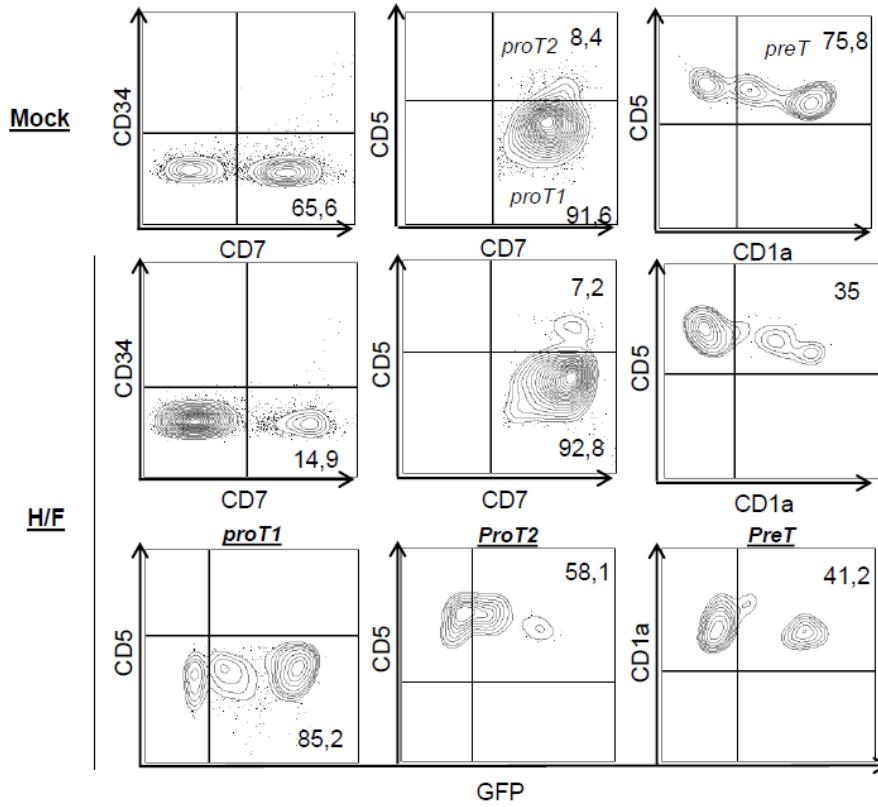


Figure 5

C

**Transplantation of CD34+ cells**



**Transplantation of T progenitors**

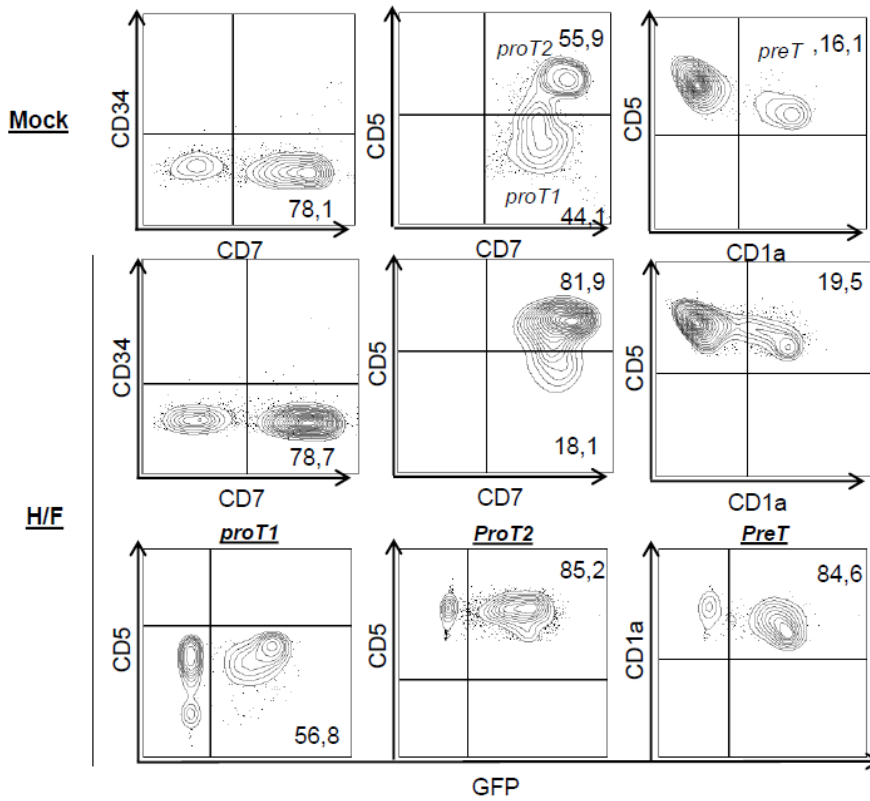
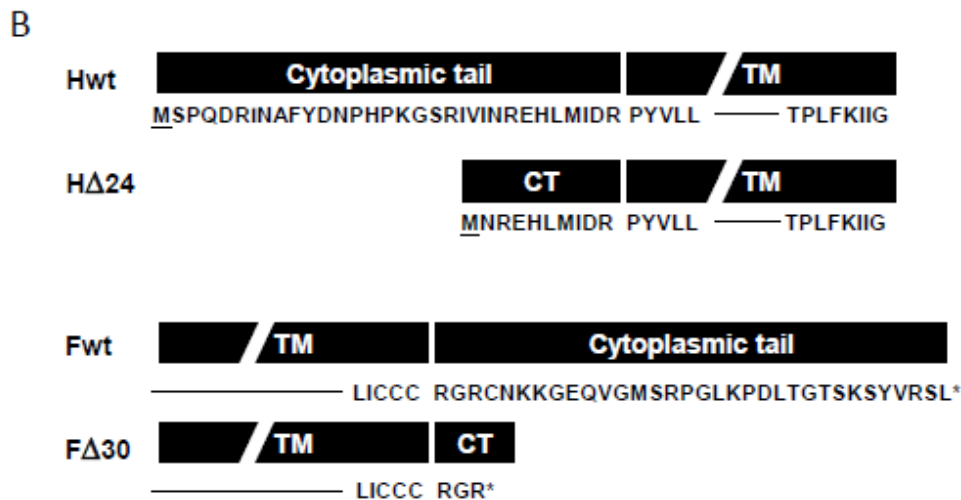
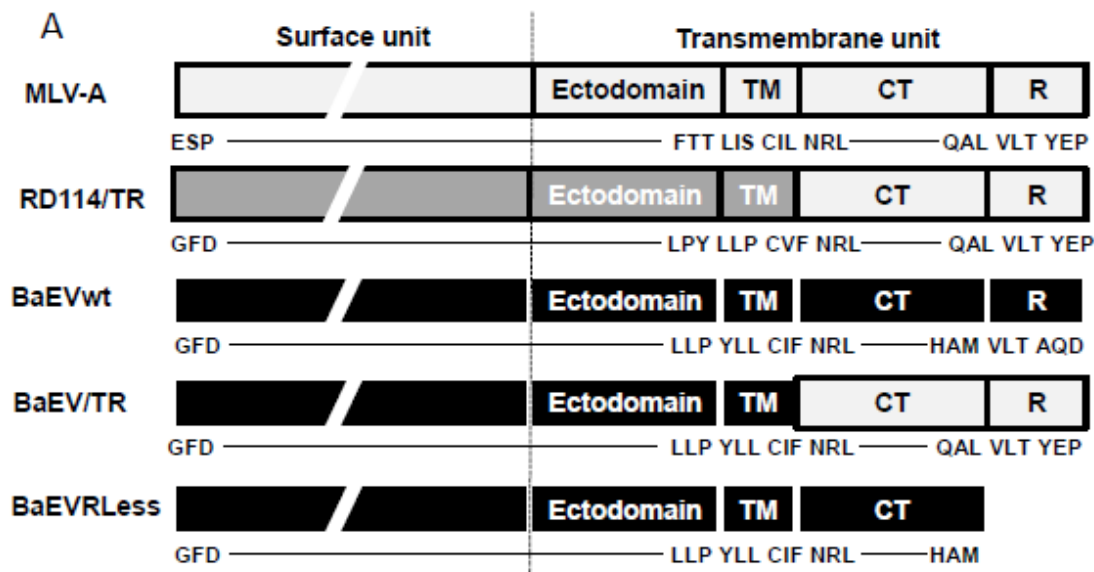
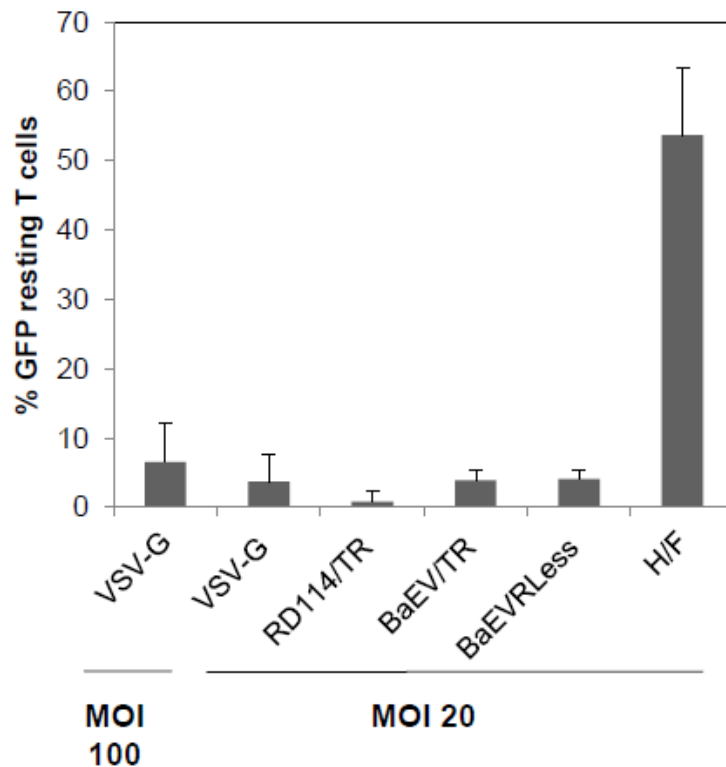


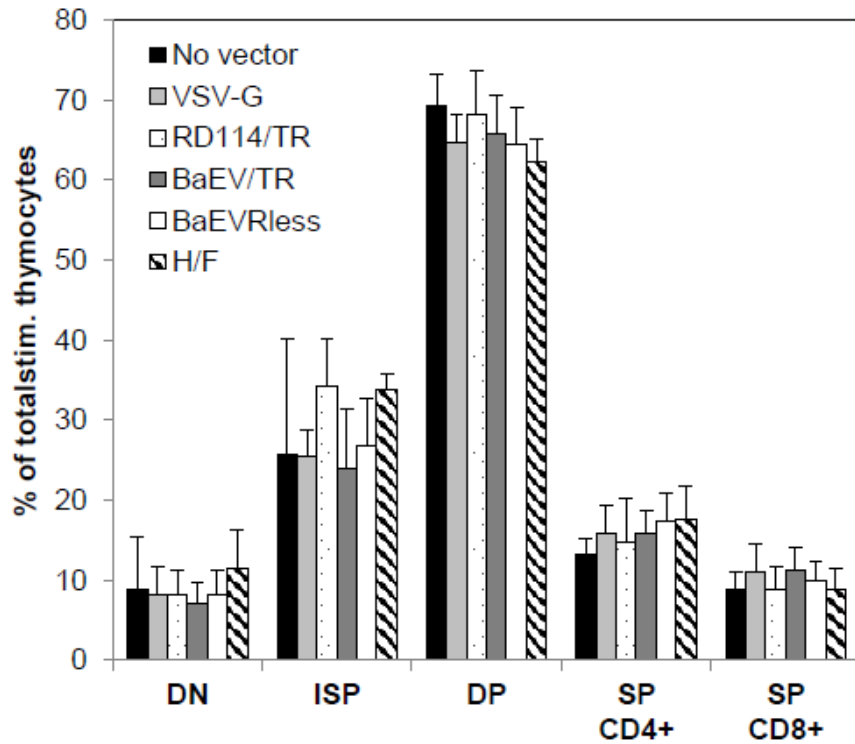
Figure 5



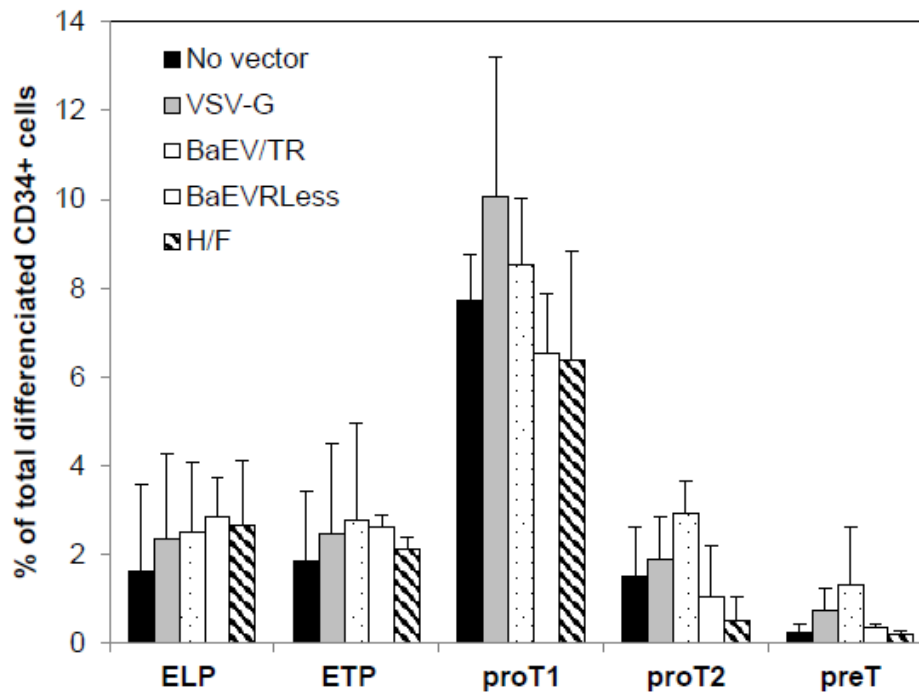
**Supplementary Figure 1. Schematic representation of the envelope glycoprotein constructs.** CT: cytoplasmic tail, TM: transmembrane. (A) Schematic representation of the wt (BaEVwt) and mutant BaEV (BaEV/TR, BaEVRless), RD114/TR- and MLV-A-glycoproteins (gps). The 17 amino acid long cytoplasmic domain of the cat and baboon retroviral gps, RD114 and BaEVwt, respectively, was exchanged for the one of the MLV-A glycoprotein resulting in the chimeric RD114/TR- and BaEV/TR-gps, respectively. The R-peptide of the cytoplasmic tail of BaEVwt was deleted resulting in the BaEVRless mutant gp. (B) Schematic presentation of wild-type or mutant F and H gps from the Edmonston MV strain. HΔ24 is a H mutant with deletion of the cytoplasmic tail. FΔ30 contains only 3 residual amino acids of the F wt cytoplasmic tail.



**Supplementary Figure 2. BAEV-LVs poorly transduce resting T cells as compared to H/F-LVs.** Highly purified resting adult T cells were isolated by negative antibody-mediated selection to avoid cell activation and then transduced with RD114/TR-, BaEV/TR-, BaEVRless-, H/F- or VSV-G-pseudotyped GFP encoding lentiviral vectors at an MOI of 20 or 100 in the absence of exogenous stimuli. After 3 days, the transduced cell cultures were washed and continued in the presence of human rIL-7 for 3 more days to verify stable GFP expression and the % of GFP+ T cells was analyzed by FACS for total T cells (means  $\pm$  SD; n = 4).



**Supplementary Figure 3. Distribution of the thymocyte subsets upon incubation with LVs as compared to untransduced thymocytes.** Freshly isolated human thymocytes were transduced in the presence of IL-7 on retronectin coated culture plates with the indicated LV pseudotypes at an MOI of 10 except for VSV-G-LVs, for which an MOI 50 was applied. Four days post-transduction the thymocytes were stained for human surface markers (CD3, CD4 and CD8) (means +/- SD; n=5; CD4<sup>-</sup>CD8<sup>-</sup> = DN; CD4<sup>+</sup>CD3<sup>-</sup>CD8<sup>-</sup> = SP-CD4; CD4<sup>-</sup>CD3<sup>+</sup>CD8<sup>+</sup> = SP-CD8; CD4<sup>+</sup>CD8<sup>+</sup> = DP; CD4<sup>+</sup>CD3<sup>-</sup>CD8<sup>-</sup> = ISP).



**Supplementary Figure 4. Distribution of the CB CD34 differentiated subsets upon incubation with LVs as compared to untransduced cells.** CB CD34<sup>+</sup>-cells were plated on DL-4 coated culture wells in presence of a cytokine cocktail (IL7, TPO, Flt3-L) for 24h and they were subsequently transduced with VSV-G-, RDTR-, BAEVTR-, BAEVRLess- and HF-LVs encoding the GFP reporter at an MOI of 10, except for VSV-G-LVs for which a higher vector dose (MOI of 50) was applied. At day 14 of culture, the cells were analyzed by FACS for surface expression of CD34, CD7, CD5 and CD1a.

## PART3 – SUPPLEMENTARY RESULTS: BAEV PSEUDOTYPED LENTIVIRAL VECTORS ALLOW EFFICIENT TRANSDUCTION OF ADULT B CELLS

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### 1) INTRODUCING LV MEDIATED GENE DELIVERY INTO B CELLS

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As compared T cells LV, which are highly transduced with LVs upon a proliferating stimulus, transduction of B cells is another matter since even under conditions inducing B cell proliferation, only very low transduction with classical VSV-G-LVs is detected (Frecha 2009), (Serafini 2004), (Levy 2010). Indeed, different B-cell activation/stimulation protocols have been set up in order to achieve stable gene transfer into human B-cells. One method used addition of antiCD40-crosslinking antibodies and IL-4 that induces proliferation and entry into S/G<sub>2</sub>/M phase of the cell cycle. However, even under these conditions VSVG-LVs allow only very poor transduction of B-cells (Serafini et al. 2004).

VSVG-LVs allow efficient transduction of human B cells only upon cytokine stimulation and proliferation in a complex co-culture system using murine thymoma cells in the presence of a cocktail of cytokines (Bovia et al. 2003). However, this system did not only led to proliferation but subsequent plasmoid differentiation of all naïve and memory human B cell subsets (Bovia et al. 2003).

More elaborated approaches have been developed using LVs that display an anti-CD20 scFv to target gene transfer to B cells (Ziegler et al. 2008) but binding of anti-CD20-targeting vectors to B cells can result in a proliferative stimulus by BCR activation (Buchholz et al. 2009). It is essential that the initial characteristics of the target B-cell remain as intact as possible upon transduction and that the vector does not induce any activation and/or differentiation as a secondary effect for further clinical applications.

Our group recently showed that H/F-LVs allow high and stable gene transfer of primary quiescent B-cells and cancer B-cells, which now makes it possible to study with ease gene functions in these cells (Frecha et al. 2009), (Lévy et al. 2010).



Nevertheless, H/F-LVs are difficult to produce at very high titers and these LVs are neutralized by circulating MV antibodies due to vaccination. As a consequence it is currently not possible to use these vector for direct *in vivo* gene delivery in B cells. Therefore, we decided to evaluate the novel BaEV LV pseudotypes for the transduction of B cells

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## 2) ADDITIONAL MATERIAL AND METHODS

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### 2.1) B CELLS ISOLATION

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Adult peripheral blood samples, obtained from healthy adult donors after informed consent, were collected in acid citrate dextrose (ACD) containing tubes. CD19+ B cells were purified by negative selection to avoid B cell activation using the Rosette tetrameric complex system (StemSep Technologies, Vancouver, Canada). Purity of isolated B cells was monitored using anti-hCD19APC antibody, and was analyzed by fluorescence-activated cell sorting (FACS CANTOII; BD Biosciences). B lymphocytes were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% FCS and penicillin/streptomycin.

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### 2.2) TRANSDUCTION OF B CELLS

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Freshly isolated B cells were immediately seeded for transduction or pre-stimulated through the B-cell receptor (BCR) during 24h with 0.001% of Staphylococcus Aureus Cowan (SAC) (Calbiochem) and 1ng/ml rhIL-2 (SIGMA). Briefly,  $1 \times 10^5$  cells were seeded in 48-well-plates and concentrated vector was added at indicated doses. At two days after the transduction, B-cells were washed twice and transferred to an MS5 cell monolayer in RPMI supplemented with 10ng/ml rhIL-15 and 5ng/ml rhIL-2 (SIGMA). The percentage of GFP<sup>+</sup> cells was determined by flow cytometry two days and six days post-transduction.

### 3) BAEV-LVS EFFICIENTLY TRANSDUCE STIMULATED AND RESTING B CELLS

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#### 3.1) BAEV PSEUDOTYPED VECTORS MANAGE TO TRANSDUCE QUIESCENT B CELLS

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We evaluated the ability of BaEV-LVs to transduce unstimulated B cells. Two days after the transduction, unstimulated B cells were cultured on a monolayer of MS5 cells in order to allow their survival for six days. We analyzed the GFP expression six days after the transduction. In absence of retronectin, BaEV/TR LVs do not transduce B cells and BaEVRLess-LVs present a low rate of transduction. Because of its positive effect on the transduction of CD34+ cells, we tempted to transduce B cells in presence of retronectin (Figure 24 A). All vectors were applied at a MOI of 10 except for VSV-G-LVs (MOI of 10 and 100).

BaEV-LVs transduced up to 20% of unstimulated B cells and the transduction by BaEVRLess-LVs appeared less donor dependent than observed for H/F-LVs ( $p < 0.03$ ). As expected, we shown that under these culture conditions, VSV-G-LVs did not transduce unstimulated B cells at all.

As it has been shown above for unstimulated CD34+ cells, it was important to check if BaEV-LVs can transduce truly quiescent B cells and that they did not involve cell cycle entry. Unstimulated B cells were incubated for two days with VSV-G-, H/F-, BaEV/TR-, BaEVRLess-LVs (MOI=100 and 10 for VSV-G-LVs and MOI=10 for other LV pseudotypes). As a positive control of cell-cycle progression, cells were incubated with no vector in absence or in presence of a SACI/IL-2 stimulation (BCR stimulation). Using 7-AAD(DNA)/PY staining(RNA), we observed that unstimulated B cells were in G0/G1a phase before transduction and that transduction with both BaEV-LVs did not induce cell cycle entry into the G1b phase (Figure 24 B).

The conservation after transduction of the initial phenotype of the cell is essential for therapeutic applications. Therefore, two days after transduction, we evaluated the expression of two activation markers of B cells, hCD69 and hCD71. In view of the importance of CD86 (B-7 molecule) for interaction with T cells we also

checked the rate of expression of this costimulatory molecule (Figure 24 D). Both BaEV-LVs did not induce up-regulation of activation markers of B cells but induce a slight increase of CD86 expression. No major phenotypic change was observed after transduction by both BaEV/LVs although we will need to confirm that the increase in CD86 expression could not have negative effect in T to B cell interaction.

Remarkably, both BaEV-LVs transduced up to 20% of quiescent B cells without inducing major phenotypic changes and cell cycle entry. The ability of BaEVRLess-LVs to transduce quiescent B cells was donor independent in contrast with H/F-LVs and BaEV/TR transduction of quiescent B cells was not statistically different than the one of H/F-LVs.

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### 3.2) BOTH BAEV LV PSEUDOTYPES ALLOW EFFICIENT TRANSDUCTION OF BCR STIMULATED AND LEUKEMIC HUMAN B CELLS

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Since quiescent B cells were efficiently transduced by BaEV-LVs, we evaluated subsequently their ability to transduce BCR stimulated cells to check if their transduction rate could be increased. Adult B cells were prestimulated by SACI/IL-2 for 24 hours and two days after transduction, B cells were cultured on a monolayer of MS5 cells in order to allow their survival for six days. We analyzed the GFP expression six days after the transduction. All vectors were applied at a MOI of 10 except for VSV-G-LVs (MOI=100 and 10) and transduction was performed in the presence of retronectin (Figure 25 A). Under these culture conditions, VSV-G-LVs did not manage to transduce BCR-stimulated B cells whereas BaEV/TR- and BaEVRLess-LVs transduced these cells up to 30% and 40% respectively. Importantly, there were no significant difference between BaEV-LVs and H/F-LV transduction levels using this B cell stimulation protocol.

Gene transfer into cancer B cells need new efficient tools, in particular for basic research on cancer physiopathology. Firstly, we evaluated BaEV-LVs in primary marginal zone lymphoma (MZL) B cells (Figure 25 B). MZL is an indolent B-cell lymphoma and can be subdivided in three types based on its site impact in spleen, lymph nodes and extranodal mucosa-associated lymphoid tissue. Few trials are available for the treatment of this lymphoma lacking standard guidelines and also

a precise description of its physiopathology (Joshi et al. 2012). Transduction was realized in the presence of retronectin at an MOI of 10 for each LV pseudotype except for VSV-G-LVs (MOI=100 and 10). H/F-LVs transduced 50% of MZL B cells and BaEVRLess-LVs 60%, thus, both vectors did not present significant difference in transducing these cells. However, BaEV/TR LV pseudotype appeared to be superior for MZL B cells transduction with 70% of transduced cells. Secondly, we tested BaEV-LVs on primary chronic lymphocyte leukemia B cells (B-CLL) (Figure 25 C). In this cancer, B cells are blocked in G0/early G1 phase of the cell cycle. They do not well respond to proliferating stimuli such as BCR stimulation. We used the same transduction conditions as employed for MZL B cells. The transduction of these B cells by H/F-LVs reaches 25% and is clearly dependent on the expression of the signaling-activating-molecule (SLAM) (Lévy et al. 2010) and consequently transduction varies between donors. BaEV-LVs transduced less than 10% of these cells and were thus less suited for B-CLL transduction than H/F-LVs.

BaEV-LVs appeared to be a good alternative to H/F-LVs for BCR stimulated B cells and MZL B cells. In B-CLL cells, they could be interesting in case of donor presenting a very low expression of SLAM and thus that cannot be transduced by H/F-LVs.

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### 3.3) BAEV/TR LV PSEUDOTYPE HIGHLY TRANSDUCE MEMORY B CELLS

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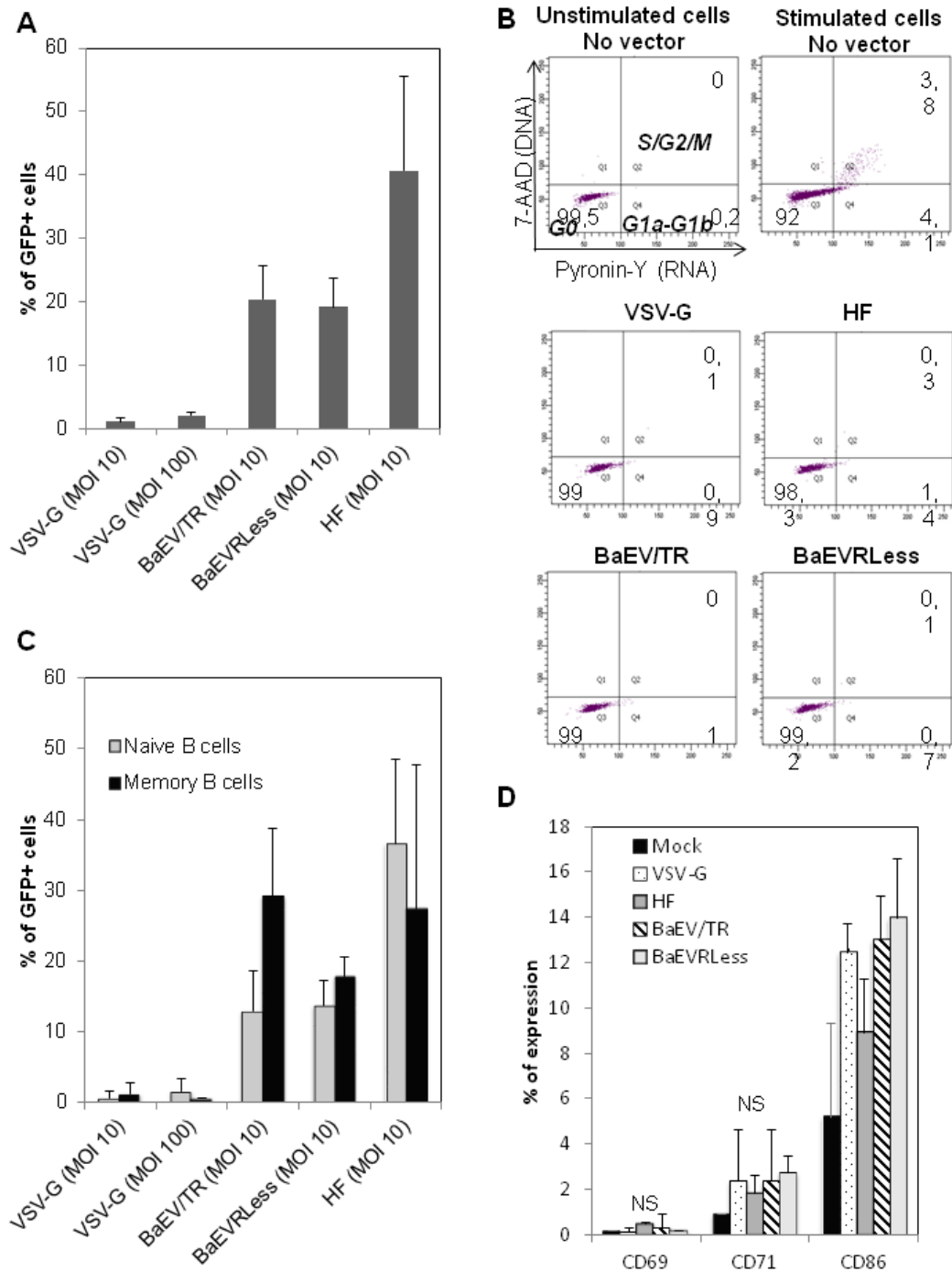
For therapeutic purposes, it could be important that BaEV-LVs transduce as well naïve and memory cells in order to use them for various applications. We applied the same transduction protocol as related before and we analyzed six days after the transduction the levels of GFP+ memory (CD27+) and naïve (CD27-) B cells. For resting (Figure 24 C) and stimulated B cells (Figure 25 A), we showed that BaEVRLess-LVs transduce as well naïve as memory B cells similarly to H/F-LVs.

#### 4) TRANSDUCTION OF B CELLS BY BAEV-LVS, ENCOURAGING PRELIMINARY RESULTS

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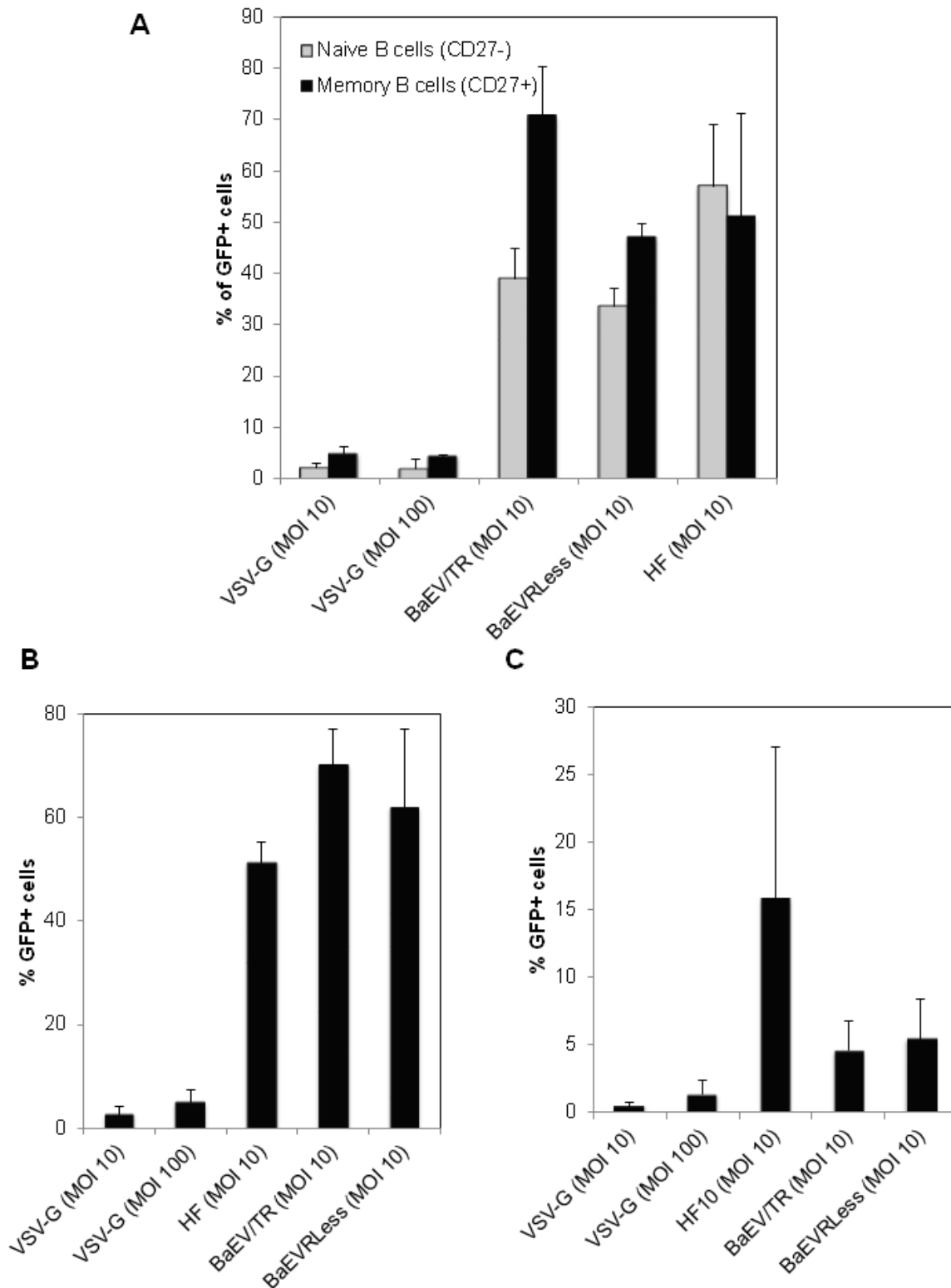
BaEVTR- and BaEVRless-LVs allow high-level stable gene transfer of primary quiescent B cells that now makes it possible to study with ease gene function and therapeutic gene transfer in these cells. These vectors transduce B cells without changing their phenotype except for CD86 expression which increased slightly after transduction with BaEV-LVs. It will be important to evaluate the true effect in T to B cell interactions and to take this into account for further applications. It was also interesting to study the mechanisms of entry of BaEV-LVs in B cells as proposed for CD34+ cells. Regarding their ability to transduce BCR-stimulated B cells and leukemic B cells such as MZL B cells and B-CLL cells and to transduce both naïve and memory B cells, these novel vectors hold promise for multiple clinical research applications. They may allow expression of co-stimulatory molecules such as CD40L or B7 molecules in non-dividing tumor B-cells such as B-CLLs in order to elicit immune responses against gene modified and unmodified cells.

Although we need to evaluate extensively the impact of the transduction of B cells by BaEV-LVs and by adoptive transfer in mouse, these first results suggest that BaEV-LVs might open the way to improved genetic vaccination strategies against cancer, infectious or autoimmune diseases as well as gene therapy of genetic diseases. For instance, in case of the recent B cell based anti-HIV strategy proposed by Luo and colleagues (Luo et al. 2009), LVs were used to transduce HSC which were subsequently differentiated into antibody-secreting autologous B cells. Alternatively, direct lentiviral transduction of memory B cells and reinfusion of autologous B-cells would allow a quick and continuous supply of HIV neutralizing antibodies *in vivo*. We could also envisage the use of BaEV LV pseudotypes as shRNAs in fundamental research in order to understand the cellular mechanisms of B or cancerous B cells such as MZL B cells.



**Figure 24. BaEV lentiviral vector pseudotypes transduce quiescent B cells conserving their phenotype.**

(A) Adult B cells were transduced in presence of retronectin by different LV pseudotypes at a MOI of 10 (and 100 for VSV-G LV pseudotype) ( $n > 4$ ). At day 2 after transduction, part of the B cells was continued on a monolayer of MS5 cells. Analysis for GFP expressing total B cells (A), CD27- naïve B cells and CD27+ memory B cells (C) were performed 6 days after transduction. (B) Cell cycle progression was monitored by simultaneous visualization of RNA (Pyronin-Y) and DNA content (7-AAD) at 2 days after transduction of unstimulated B cells by different LV pseudotypes. As a control, B cells were stimulated by overnight incubation with SAC/IL-2. (Representative data of 2 experiments) (D) Resting B cells were transduced with H/F-LVs, BaEV/TR-LVs, BaEVRLess-LVs at an MOI of 10 and VSVG-LVs at an MOI of 100 respectively, or incubated without vector. Surface staining of activation markers (CD69, CD71) and of CD86 2 days after transduction is shown ( $n = 3$ ).



**Figure 25. Efficient transduction of human stimulated and leukemic B cells by BaEV/TR and BaEVRIless pseudotyped LVs.**

B cells were transduced in presence of retronectin with VSV-G-, HF-, BaEV/TR- and BaEVRIless-pseudotyped LVs at MOI of 10 and 100 for VSV-G-LV pseudotypes. At day 2 after transduction, part of the B cells was continued on a monolayer of MS5 cells. Analysis for GFP expressing cells was performed 6 days after transduction. (A) Freshly isolated B cells prestimulated 24h with SACI/IL-2 were transduced as indicated ( $n > 5$ ) and naive and memory B cells were phenotyped (B) MZL B cells from 3 different donors were transduced as indicated ( $n > 5$ ). (C) B-CLL cells from 3 different donors were transduced as indicated ( $n > 4$ ).

## DISCUSSION

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## 1) BAEV- AND H/F-LVS, COMPARISONS AND PROSPECTS

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Few efficient tools are available for the transduction of hematopoietic cells, this thesis describes two major lentiviral vector pseudotypes and their capacity to transfer genes into hematopoietic cells such as HSCs, T cell lineages and B cells.

### 1.1) BAEV- AND H/F-LVS EFFICIENTLY TRANSDUCE HSCS

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Although classical VSV-G-LVs are reported to be able to transduce non-dividing cells, fully quiescent  $G_0$  cells are not efficiently transduced (Frecha et al. 2008), (Korin et al. 1998), (Sutton et al. 1999), (Verhoeyen et al. 2003). Therefore, HSCs are poorly permissive for classical VSV-G-LV transduction because 75% of them reside in  $G_0$  (Sutton et al. 1999). Moreover, our group confirmed a very low expression of the VSV receptor, the low density lipoprotein receptor (LDL-R) (Finkelshtein et al. 2013) (Amirache et al. 2014) in unstimulated  $CD34^+$ -cells, perfectly coinciding with their poor VSV-G-LV-mediated transduction (Amirache et al. 2014). Only 'early-acting-cytokine' stimulation of  $hCD34^+$ -cells, upregulating the LDL-R, permitted high-level VSV-G-LV transduction (Amirache et al. 2014). However, intense exposure of HSCs to cytokines might affect their homing, extravasation ability and promote differentiation rather than expansion of the HSC pool (Ahmed et al. 2004), (Peled et al. 1999). Moreover, when combining high vector doses with strong cytokine-stimulation, the risk for multicopy integration and insertional mutagenesis cannot be neglected (Hacein-Bey-Abina et al. 2008), (Hacein-Bey-Abina et al. 2003).

In this thesis, we present two new LV-pseudotypes that confer high-level transduction on the HSC subset in the  $CD34^+$  cell population. Both BaEV- and H/F LVs succeed in transducing up to 90% of  $CD34^+$  upon mild cytokine stimulation. We decided to stimulate here  $CD34^+$  cells only with SCF, TPO and Flt-3-L, since IL-3 commonly added in the  $CD34^+$  cytokine cocktails used in the clinic was thought to increase  $CD34^+$  cell differentiation (Millington et al. 2009).

Most interestingly, BaEV- and H/F-LVs transduce up to 30% and 70% respectively of resting HSCs, a transduction level never reached before in these targets. The clinical interest of transducing resting HSCs are multiple and extensively discussed below.

Considering HSC transduction levels, BaEV- and H/F-LVs are equally efficient for CD34+ cells stimulated with a mild cytokine cocktail. H/F-LVs are superior to BaEV-LVs for transducing resting CD34+ cells due to the differential expression of their receptors. Indeed, the expression of both ASCT-1 and ASCT-2 receptors for BaEV-LV are upregulated by more than 2- and 6-fold respectively when CD34+ cells are stimulated with early acting cytokines. In conclusion, both ASCT-1 and ASCT-2 receptors are needed in sufficient levels at the hCD34+ cell surface to allow vector transduction. On the other hand, we reported that resting CD34+ already express CD46 at high density at their cell surface and that this receptor is used for H/F-LV entry as also for the vaccinal strain of measles virus; This superiority of H/F-LV in transducing unstimulated CD34+ cells gives advantages as compared to BaEV/LVs.

In order to confirm that both BaEV-LVs and H/F-LVs transduce the stem cell subset in the CD34+ cell population, we performed highly specific secondary engraftments in NSG mice. Indeed, for each primary transplanted mice, we isolated bone marrow human CD34+ cells and we transplant them into a secondary mouse without mixing CD34+ isolated from several primary mice. This protocol allow us to show that even for quiescent cells BaEV and H/F-LVs transduce HSCs. This was evidenced by the important increase of the GFP+ proportion in the human CD45+ engrafted cells in secondary recipient mice as compared to the first engraftment. For instance, we showed that although BaEV-LVs transduce less than 30% of resting CD34+ cells, we obtain up to 65% of CD45+ GFP+ cells in secondary transplanted mice suggesting that BaEV-LVs preferentially transduce the HSC sub-population in the CD34+ cell subset.

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## 1.2) BAEV- AND H/F-LVS ARE COMPLEMENTARY PARTNERS FOR THE TRANSDUCTION OF THE T CELL LINEAGE

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Our group has developed H/F-LVs for the transduction of resting and IL-7 stimulated T cells with an advantageous transduction of adult memory T cells. Moreover, it appeared that H/F-LVs were not able to efficiently transduce the naïve immature UCB T cell population although they express CD46 on their surface (Frecha et al. 2008). This reduced transduction is due to the lack of SLAM receptor, another measles virus receptor on these naïve UCB T cells (Frecha et al. 2008). Thus, we decided to evaluate the capacity of the new BaEV-LV pseudotypes to

transduce the more naïve and immature T cell subpopulations following several objectives.

As developed above, the commonly used TCR stimulation of T cells in order to allow VSV-G-LV transduction induce an important skewing of T cell repertoire protocols (Verhoeyen et al. 2003), (Markt et al. 2003). Therefore, we focused on IL-7 mild stimulation notably because of its role in supporting T cell survival and homeostatic proliferation (Fry et al. 2001), (Rathmell et al. 2001) without inducing alteration of T cell populations.

BaEV-LVs cannot transduce unstimulated T cells probably due to a very low mRNA expression of both ASCT1 and ASCT2 receptor of BaEV-LV in resting T cells. Of interest, both ASCT1 and ASCT2 are strongly upregulated on adult T cells upon TCR and IL-7 stimulation, perfectly in agreement with increasing transduction levels obtained with the BaEV-LVs.

H/F-LVs maintain their advantage for the resting T cell population reaching more than 50% of transduction in presence of retronectin. Our group suggested that H/F-LVs enter into resting T cells by a concerted or sequential SLAM/CD46 engagement that may induce macropinocytosis and LV entry.

Of interest for T cell based gene therapy and immunotherapy, BaEV-LVs transduce as well naïve and memory T cells as compared to H/F-LVs which better transduce memory T cells. Our group previously explained the preference of H/F-LVs for memory cells because naïve cells express less SLAM than memory cells (Frecha et al. 2011). Since BaEV-LVs transduce as well naïve and memory IL7 stimulated T cells, we could hypothesize that IL-7 stimulation induce ASCT1 and ASCT2 upregulation in both naïve and memory T cells.

As previously detailed, naïve T cells are especially important as gene therapy target cells since they are able to respond to novel antigens and are essential in immunologic memory generation. Moreover, BaEV-LVs are highly superior to H/F-LVs in transducing UCB immature naïve recent thymic emigrants and more mature naïve CB CD4+ T cells reaching more than 50% of transduction. This is very promising for gene therapy and immunotherapy application. Indeed, the early immature naïve T cell subsets are characterized by their high capacity to proliferate,

to persist during the individual lifespan and to differentiate in long lived memory T cells (Szabolcs et al. 2003).

Because of the interest of correcting T cells at the earliest stages of differentiation for immune modulation and gene therapy, we also evaluate and compare BaEV- and H/F-LVs in mediating gene transfer in thymocytes. BaEV/TR- and BaEVRless-LVs resulted in a preferential transduction of early thymic progenitors and Pro T subsets while H/F-LVs transduce at the same level of above 60% all thymic subsets. Nevertheless, the transduction level of thymocytes by H/F-LVs depends on the donor, probably in connection with the cell activation state and expression of its CD46 and SLAM receptors.

Using the Notch Ligand DL-4 culture system (Reimann et al. 2012), we showed that BaEV- and H/F-LVs transduced HSCs during differentiation into lymphoid progenitors without altering the T cell differentiation process, allowing us to have a 10-fold expansion of the cells.

As developed below, it could be of particular interest to develop a co-transplantation protocol using HSC and T progenitors to accelerate the recovery of the adaptative immune response after gene therapy HSCT.

To conclude, BaEV-LV seems to be an interesting tool for the transduction of naïve and early progenitor T cells while H/F-LVs present a complementary capacity by efficiently transducing memory cells and the latest stages of thymic differentiation. Of importance, H/F-LV remains the only LV pseudotype allowing efficient transduction of resting T cells.

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### 1.3) CAN THE PRODUCTION OF BAEV- AND H/F-LVS, GO BOTH TOWARDS THE CLINIC?

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Although we demonstrated that BaEV- and H/F-LVs are powerful tools for the transduction of HSCs and T cells, their potential development for clinical purposes differs according to their own characteristics.

### 1.3.1) ARE THEY EASY TO PRODUCE AT HIGH INFECTIOUS TITERS?

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The need of high infectious titer of lentiviral vector is essential in order to reduce the difficulty and the cost of production for laboratory and clinical applications. Despite its numerous disadvantages, the currently used VSV-G LV pseudotype can be produced on average at the high concentrated titer of  $1.10^9$  IU/ml and we reach titers of  $6.10^7$  IU/ml for RD114/T-LVs . Modifications in the cytoplasmic tail of the BaEV glycoprotein allows to obtain relatively high concentrated titers of  $5.10^7$  IU/ml and  $2.10^8$  IU/ml for the BaEV/TR and BaEVRless constructs respectively. BaEV/TR-LVs present equivalent titers as obtained for RD114/TR LVs. For H/F-LVs, the H $\Delta$ 24/F $\Delta$ 30 truncated envelopes improved their incorporation on LV and allowed to produce an average of  $1.3.10^7$  IU/ml (Frecha et al. 2011 et al. 2008).

As a consequence, BaEV-LVs and especially BaEVRless-LVs can be produced at significantly higher titers as compared to H/F-LVs. They approach the titers of the currently used VSV-G-LVs. In addition they allow high level of transduction of naïve and immature T cell, thymocytes and HSCs, they might be interesting candidates for the use in the clinic and for LV production at large scale.

### 1.3.2) IS STABLE PRODUCTION POSSIBLE?

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As we previously explained, stable vector producing cell lines (VCL) have been developed in order to ensure a safer and less expensive LV production.

Although, this stable production is essential for clinical use, the cytotoxicity of numerous viral proteins prevent this potential development. For example, when VSV-G is stably expressed, the fusogenicity of this glycoprotein induces strong toxicity, which prevents its use in continuous HIV vector-producing cells (Ikeda et al. 2003).

Analyzing the cytotoxicity of our new LV pseudotypes, the BaEV/TR glycoprotein is the only one that could be stably produced considering the important fusogenicity induced by both BaEVRless and H/F glycoproteins. Thus, we can imagine that similarly to RD114/TR-LVs, a stable producing cell line would allow a stable production of BaEV/TR LV pseudotypes (Ikeda et al. 2003), (Strang et al. 2004), (Stornaiuolo et al. 2013) which is not the case for H/F and BaEVRless-LVs.

Finally, BaEV-LVs appear to be superior to H/F-LVs considering their potential adaptation for a clinical use notably in the context of LV production for the clinic. Compared to BaEVRLess-LVs, the BaEV/TR LV pseudotype constitutes a good compromise despite its slightly lower titer. Indeed, in comparison with the RD114/TR developed before (Sandrin et al. 2002) and optimized for clinical applications (Stornaiuolo et al. 2013), BaEV/TR-LVs present similar infectious titers and their production does not induce cytotoxicity. Additionally, the BaEV/TR-LV could be considered a better candidate for clinical development because it transduces much better HSCs and moreover succeeds in transducing the whole T cell lineage including naïve and immature cells and B lymphocytes.

## 2) WHAT ABOUT A POTENTIAL *IN VIVO* USE OF BAEV- AND H/F-LVS?

*In vivo* gene delivery using LVs has been employed for direct and stable gene modification. Local injection of LVs in neuronal cells (Blomer et al. 1997), retina cells (Miyoshi et al. 1997), liver hepatocytes and cardiomyocytes (Kafri et al. 1997), airway epithelium (Johnson et al. 2000) and kidney cells (Gusella et al. 2002) have been realized without inducing specific pathology. Of interest, bone marrow *in situ* injections of VSV-G-LVs have been reported to allow transduction of mouse HSCs (Worsham et al. 2006). Moreover, LVs have been used as genetic vaccine against cancer cells and infectious diseases (Di Nunzio et al. 2012), (Hu et al. 2011). In order to prevent off-target cell gene transfer and to improve vaccine efficiency, a specific targeting of antigen-presenting cells is necessary either by using the natural tropism of a viral envelope glycoprotein or by engineering specific targeting pseudotypes (Cire et al. 2014).

Considering these major advances in the field of gene therapy and immunotherapy, it would be interesting to optimize our LV pseudotypes for *in vivo* gene delivery. To achieve this, we should have to take into account the two major following challenges: LVs have to resist to the host immune and complement systems and a specific targeting is needed to prevent off-target cell gene transfer after systemic administration (Frecha et al. 2011).

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## 2.1) POTENTIALITIES OF BAEV- AND H/F- LVs FOR *IN VIVO* USE

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As developed above, VSV-G-LVs are sensitive to the human complement system that restrict its *in vivo* use in humans (Cire et al. 2014), (Frecha et al. 2011) except by a PEGylation procedure (Croyle et al. 2004).

Likewise RD114/TR-LVs, we demonstrated that both BaEV/TR- and BaEVRless-LVs are also resistant to the human complement system. This gives us an argument to develop *in vivo* gene delivery strategies using BaEV-LVs. Indeed, BaEV-LVs could survive in human because we have not developed an immune response against the baboon endogenous retrovirus. Nevertheless, considering *in vivo* gene delivery safety, we need to design modified BaEV-LVs in order to prevent the transduction of non-targeted cells.

Concerning H/F-LVs, our group generated this pseudotype using the envelope glycoproteins of the vaccine Edmonston measles virus (MV) strain. According to the World Health Organization, about 84% of the world's children are vaccinated against MV, this constitutes the major obstacle for the *in vivo* use of H/F-LVs. As the MV-mediated humoral immune response is directed specifically against the H protein, our group (Lévy et al. 2012) tried to mutate the dominant epitopes on this protein. Although LVs pseudotyped with this H mutant protein escaped inactivation by monoclonal antibodies, they still are neutralized by human serum. Novel H mutants were designed on the basis of newly emerged MV-D genotypes which are less sensitive to MV vaccination. These H glycosylated mutants partially escape to blood MV neutralizing antibodies during *in vitro* assays. Nevertheless, these mutated H/F LV pseudotypes have not yet been evaluated in primate models which are the only models that can mimic neutralization of the H/F-LVs vectors upon MV vaccination (Lévy et al. 2012). Likewise, Kneissl and co-workers (Kneissl et al. 2012) designed an H protein displaying a single-chain antibody (scFv) specific for the targeted receptors (Funke et al. 2008). These targeting LVs escape to neutralizing monoclonal antibody against MV and  $\alpha$ -MV antibody positive human plasma (Kneissl et al. 2012). Again, no *in vivo* evaluation was performed until now. In both cases, we might consider local injection an option to improve local transduction for example by intra-



thymic or intra marrow injection especially since target T cells and CD34+ cells respectively are highly permissive to these H/F-LVs.

In a nutshell, while BaEV-LVs would be easy to use *in vivo* regarding their resistance to the human complement system, it is not yet the case for H/F-LVs despite efforts of our team and other researchers.

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## 2.2) ENGINEERING OF THE LV SURFACE FOR DIRECT TARGETING AND *IN VIVO* APPLICATIONS

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### 2.2.1) ENGINEERING OF SPECIFIC CYTOKINE-DISPLAYING LVS FOR *IN VIVO* GENE TRANSFER

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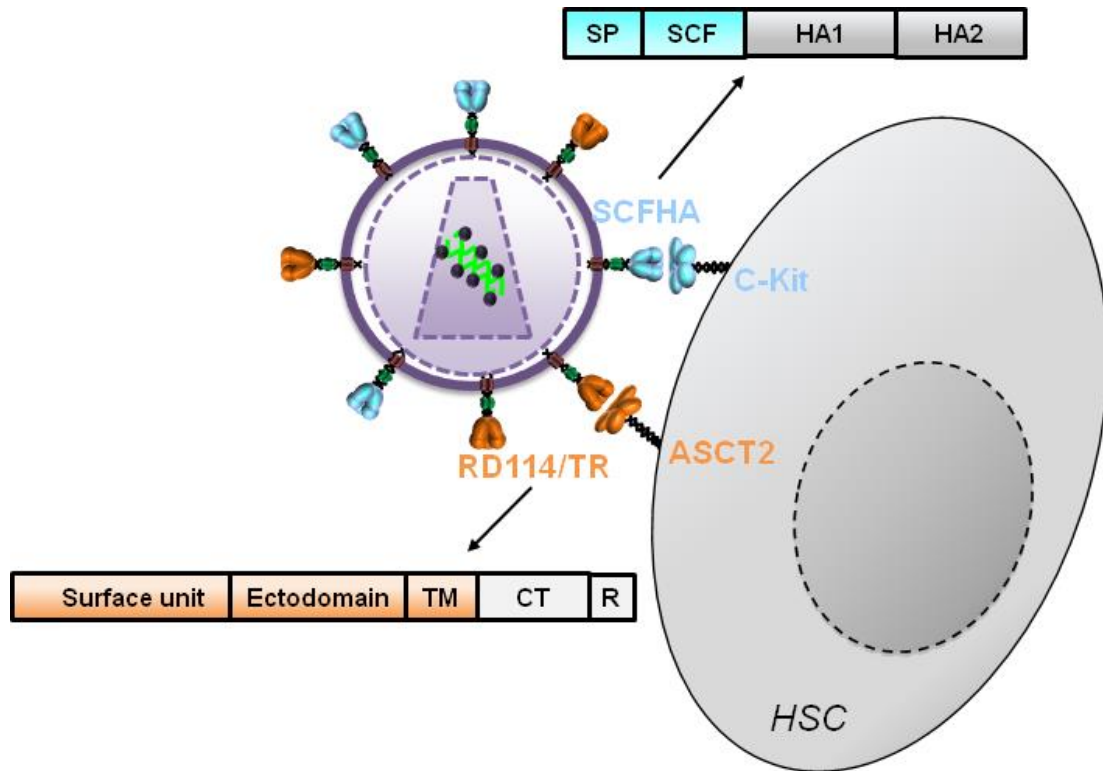
Our group designed several specific cytokine displaying LVs that could allow the specific targeting of HSCs, T and B lymphocytes (Verhoeyen et al. 2009), (Verhoeyen et al. 2008), (Szécsi et al. 2006), (Verhoeyen et al. 2005), (Verhoeyen et al. 2004), (Verhoeyen et al. 2003), (Maurice et al. 2002). Several strategies has been developed, firstly by fusing the surface subunit of the MLV envelope with a cytokine and codisplaying of an envelope glycoprotein on the LV surface (Verhoeyen et al. 2004), (Verhoeyen et al. 2003), (Maurice et al. 2002).

Of major interest for the *in vivo* targeting and activation of HSCs, our group designed the promising RDTR/SCFHA codisplaying vectors (Frecha et al. 2012). Indeed, SCF is a HSC-specific cytokine that has been shown to promote HSC survival without inducing loss of homing and engraftment. To achieve an efficient functional presentation on the vector surface, the cytokines were fused to the N-terminus of the haemagglutinin influenza glycoprotein HA (SCFHA) (Figure 26) (Szécsi; 2006). Thus, both cytokines needed to be displayed at the surface of LVs, together with an escorting fusion partner glycoprotein (Szécsi et al. 2006), (Verhoeyen et al. 2005). The fusion glycoprotein VSV-G in the VSV-G/SCF- co-displaying lentiviral vectors was firstly described (Verhoeyen et al. 2005) but it was unsuited for *in vivo* targeted gene delivery because of its complement sensitivity and its pantropic recognition of the LDL receptor (Amirache et al. 2014). Therefore, VSV-G was exchanged for the RD114 glycoprotein (Frecha et al. 2012) (Figure 26). The resulting RDTR/SCF -displaying LVs were far more efficient in transducing hCD34<sup>+</sup>

cells (up to 40%) than RDTR vectors in presence of rSCF and this in the absence of retronectin. In addition, vector doses can be decreased with the concomitant decrease of the risk of insertional mutagenesis while maintaining high transduction efficiency. Furthermore, RDTR/SCF-displaying lentiviral vectors selectively targeted transduction to 30-40% of the hCD34<sup>+</sup>-cells in cord blood (CB) mononuclear cells and in unfractionated BM of healthy and Fanconi anemia donors (Frecha et al. 2012). Moreover, this new LV pseudotype are ninetyfive- fold more selective for CD34+ cells as compared to T cells. Interestingly, the capacity of these LVs to allow *in vivo* gene transfer into human HSCs has been evaluated using beforehand humanized BalbC rag2<sup>-/-</sup>γc<sup>-/-</sup> mice. LVs were intrafemorally injected. Despite low level of transduced cells, mice analysis showed that RDTR/SCFHA-LVs specifically transduced bone marrow CD34+ cells which was not the case for RDTR-LVs. Since BaEV/TR-LVs allow recognition of both ASCT-1 and ASCT-2 receptors and confer 100% increase in transduction of stimulated CD34+ cells, it might be considered as a good candidate for co-display with SCFHA on LVs and might increase HSC targeted transduction level considerably as compared to the RDTR/SCFHA-LVs.

Following the same strategies, specific targeting of T and B cells could be imagined using for instance a codisplaying between BaEV/TR glycoprotein and IL-7HA or IL-2HA constructs respectively. Indeed, the superiority of BaEV-LVs as compared to RDTR-LVs for the transduction of HSCs, T and B cells, suggests that the cytokine co-displaying BaEV-LVs would have a really interesting potential for *in vivo* targeting of hematopoietic cells.

Of particular interest, the co-displaying of both H and F glycoprotein with a cytokine on the LV surface might be tried. Of note, the steric hindrance of the final structure may affect the infection capacity of the LV.



**Figure 26. Schematic representation of the LVs displaying the RD114 glycoprotein and SCF.**

SCF was fused to the N-terminus of the influenza HA glycoprotein, which allowed efficient functional incorporation on LVs (SCFHA). Because this chimeric HA glycoprotein demonstrated a reduced infectivity, it has been needed to coexpress an additional fusion competent glycoprotein, RD114. The cytoplasmic tail of RD114 was exchanged for that of the MLV glycoprotein, resulting in a mutant RD114/TR, which allowed efficient incorporation on HIV vectors as described previously. Both SCF and RD114/TR recognize their receptors on the HSC surface, C-kit and ASCT2 respectively.

### 2.2.2) LIGAND FUSED GLYCOPROTEINS TO RETARGET H/F-LVS

Direct targeting is based on the principle that fusion activation of a chimeric envelope should be triggered by the interaction of the ligand displayed on the vector surface with its specific receptor on the target cell. With this objective, the addition of natural ligands or single chain antibodies, (specific for a surface receptor or molecule) on the N terminus domain of retroviral envelope glycoproteins has been developed.

introduction of single chain antibodies in the H protein has allowed specific targeting of endothelial cells ( $\alpha$ CD105), B cells ( $\alpha$ CD20 and  $\alpha$ CD19), neurons, T cells ( $\alpha$ CD8), hematopoietic stem cells ( $\alpha$ CD133) and dendritic cells ( $\alpha$ MHC class II). This emphasizes the flexibility of this strategy, which is characterized by high specificity

since background transduction of non-targeted cells is low or non-existing (Ciré et al. 2014), (Buchholz et al. 2009), (Funke et al. 2008), (Buchholz et al. 1998), (Munch et al. 2011).

It might be tempting to speculate that we could combine this direct targeting strategy with cytokine display by inserting SCF or TPO into the hemagglutinin and in this way target the H-SCF/F-LVs to the immature HSCs.

### 3) BIOSAFETY AND IMPROVEMENTS OF BAEV AND H/F LV PSEUDOTYPES FOR CLINICAL PROSPECTS

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#### 3.1) HOW TO REGULATE AND TARGET THE TRANSGENE INTEGRATION?

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Numerous tools and LV designs are available in order to reduce the risk of insertional mutagenesis. Regarding the high level gene delivery into hematopoietic cells allowed by BaEV-LVs and H/F-LVs, we should try to target their integration into a specific genome site and reduce the mutual influence of both the integrated transgene and the chromatin regions.

First, the addition of insulators in the U3 deletion in the 3' LTR might increase the autonomy of the integrated transgene protecting both host cell genome and integrated transgene. Indeed, insulators can protect the transgene silencing or activation notably induced by the condensation state or the presence of enhancers in the adjacent chromatin. Moreover, insulators might prevent the background expression of the transgene notably by reducing the enhancer-related risk of insertional mutagenesis. Various design of insulators could be envisaged for optimizing BaEV- and H/F-LVs. For example, the HS4 core from the Beta-globin LCR has been used as an insulator resulting in longterm stable transgene expression in human hematopoietic targeted cells (Arumugam et al. 2007), The use of S/MARs (scaffold/matrix attachment regions) could also be considered because it resulted in increased transgene expression stability by mediating attachment of chromatin loops to viral matrix or scaffold proteins of the nuclear envelope (Park et al. 2001). Finally, we might improve LV design by introducing ubiquitously acting chromatin-opening elements (UCOEs); enhancer-less elements that appear to be very promising in

ensuring stable long-term transgene expression (Zhang et al. 2001). The A2UCOE element for example confers stable transgene expression in HSCs by blocking DNA methylation mediated silencing of different promoters (Zhang et al. 2010). Nevertheless, we must be cautious because the effects of the addition of insulators in the U3 region of the 3'LTR of SIN LVs is still discussed in particular about their genetic instability or their interference with reverse transcription, RNA processing (Arumugam et al. 2009), (Hanawa et al. 2009). For example, the generation of the truncated HMGA2 transcript described above (See Introduction Part I. 4.2.3) in the context of the B-thalassemia clinical trial was induced by a rearranged insulator element (Cavazzana-Calvo et al. 2010). Therefore, further investigation is needed to allow us to choose the optimal insulator design for an improvement of both biosafety and transgene expression (Schambach et al. 2013).

Secondly, since the pattern of integration of LVs in specific “hotspots” appears to be related to at least the binding of the integrase by the LEDGF cellular protein and the passage of the PIC through nuclear pores, numerous groups have developed some integrase based optimization of LVs. We could hope to increase the level of specific integration of our BaEV- and H/F-LVs by producing IDLVs and targeting the integration by using either ZFN (Lombardo et al. 2007), TALEN (Holkers et al. 2013) technologies. Recently engineered RNA guided DNA endonuclease (CRISPR/Cas) allow a targeted gene delivery which offer interesting prospects for the development of LV with a targeted integration (Gaj et al. 2013), (Kabadi et al. 2014). Since BaEV- and H/F-LVs confer high levels of transduction into HSCs they might be excellent tools to pseudotype integration deficient lentiviral vectors (IDLVs) carrying the ZFN, TALEN and CRISPR/CAS components in order to target the integration to specific sites in the host genome of hCD34+ cells. These potential advances in the field of LV gene delivery need to be extensively studied for a potential use in the clinic.

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### 3.2) OPTIMIZATION OF LV TARGETING BY SELECTIVE EXPRESSION

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In this thesis, we focused on the development of LV tool for hematopoietic gene delivery without designing them for a specific kind of hematopoietic targeted cell. In addition to LV surface modifications, a second strategy has been developed in vector targeting. LVs can be engineered not only to control cell entry (transductional

targeting) or for integration targeting but also to control expression of the vector's transgene in the transduced cells (transcriptional targeting).

The classic strategy used is to include tissue-specific promoters and enhancers in the transfer vector to drive expression of the transgene and to reduce the risk of side-effects related to aberrant expression profiles. Moreover, except in vaccine strategies where APCs are targeted, another interesting benefit of this limited expression is to exclude antigen presenting cells (APCs) from transgene expression. Indeed, if these APCs express the transgene, this might result in an efficient antigen processing, a swift immune response followed by elimination of transgene expressing cells by the immune system. Many laboratories have developed tissue-specific expression LVs notably including hematopoietic specific promoters (Table 9).

**Table 9. Overview of the major hematopoietic specific promoter developed for LV transcriptional targeting**

Target cell	Specific promoter	Ref.
<b>Hematopoietic cells</b>	WASp promoter	(Dupre et al. 2006), (Martin et al. 2005), (Charrier et al. 2007), (Frecha et al. 2008c)
<b>B cell lineage</b>	CD19 promoter, Ig kappa promoter	(Moreau et al. 2004), (Taher et al. 2008), (Laurie et al. 2007)
<b>T cell lineage</b>	CD4 promoter, CD40-L proximal promoter, Luria / Delta / V $\beta$ 5.1 / V $\beta$ 6.7	(Marodon et al. 2003), (Romero et al. 2011), (Yang et al. 2011)
<b>APCs</b>	HLA-DR $\alpha$ promoter	(Lesniak et al. 2005)
<b>Myeloid cell lineage</b>	SP146.gp91 <sup>phox</sup> Myeloid specific promoter	(Chiriaco et al. 2014), (Santilli et al. 2011)
<b>Erythroid cell lineage</b>	$\beta$ -globin promoter	(May et al. 2000)
<b>Megakaryocytes</b>	Alpha IIB promoter	(Shi et al. 2004)

Of importance, a HSC specific promoter would represent a valuable tool since a part of these cells can give rise to non-hematopoietic cells such as neurons or endothelial, skeletal and heart muscle cells. Therefore, the use of hematopoietic-specific promoters is essential such as used in the Wiskott Aldrich Syndrome gene therapy (WAS) (Martin et al. 2005). For WAS gene therapy, authors used a hematopoietic-specific promoter because of the risk of toxicity when using a transgene non-restricted expression especially since the endogenous WAS protein is strictly detected in hematopoietic lineages (Toscano et al. 2008).

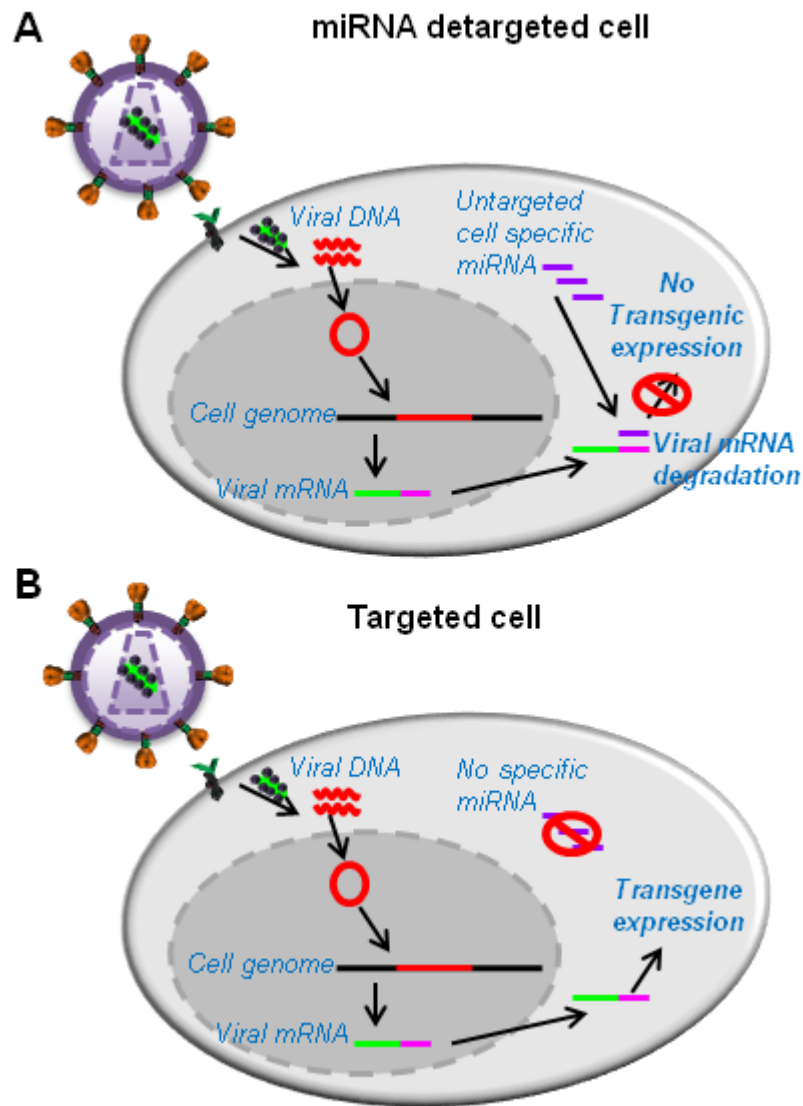
Therefore, we should develop BaEV- and H/F-LVs carrying transgenes under the control of the adapted either hematopoietic, T lineage specific or B lineage specific promoters in order to increase tissue specificity expression and to prevent the expression of the transgene by off-target cells.

Although the control of lentiviral expression by tissue-specific promoters appears to be a good option, this is not the perfect solution since it is sometimes difficult to reconstitute a tissue-specific endogenous promoter. Secondly, once the vector is integrated, the genomic surroundings such as enhancers or neighbouring strong promoters can influence the performance of the internal LV promoter.

In order to increase tissue-specificity of gene expression, a new strategy has been developed combining the microRNA post-transcriptional regulation with the lentiviral vector technology. miRNA are 22nt regulatory RNAs that act post-transcriptionally and influence cellular functions. Although many of miRNAs are ubiquitously expressed, some of them have a tissue-specific pattern (Kelly et al. 2009). Therefore, in order to prevent the expression of a transgene in hematopoietic cells and especially in APCs, some groups introduced in the transgene's 3'UTR perfect complementary target sequences of microRNA miR-142, highly specific for hematopoietic cells (Gentner et al. 2012). In this way, hematopoietic cells were detargeted for transgene expression and thus no immune response against the transgene was generated (Figure 27). Of importance, the introduction of these new targets for cellular miR-142 does not seem to perturb its natural activity. In contrast to miR-142, miR233 can be used to obtain a lymphoid specific expression pattern, important for immunotherapy (Gentner et al. 2012).

This approach requires less than 100 bases of vector sequence and as a consequence can be developed for BaEV- and H/F-LVs without impacting and limiting the transgene design. This strategy could allow a highly controlled transgene expression in particular for instance for the specific targeting of the different stages of thymic differentiation. Nevertheless, more studies are required to evaluate the consequence of modifying the natural miRNA targeting in presence of the new targets carried by the vector. Indeed, even if the insertion of multiple target sequence have been reported to reduce the loss of miRNA regulation, mutations of the miRNA

target sequence inserted in the vector can occur and must be investigated (Brown et al. 2009).



**Figure 27. Schematic representation of lentiviral vectors for RNA silencing.**

(A) In non-target cells, an efficient de-targeting of expression is achieved because they contain cell-specific miRNAs which bind to their corresponding miRNA binding sites inserted in the LV mRNA transcripts. (B) In targeted cells, these same miRNAs are absent or are expressed at significant lower levels and therefore transgene expression from the same LV encoding transcripts is efficient in these cells.



#### 4) IMPORTANCE OF MINIMIZING CD34+ STIMULATION AND HEMATOPOIETIC STEM CELL BASED GENE THERAPY

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Although it has long been known that resting human CD34+ cells engraft better than cells in the SG2M phases of the cell cycle (Gothot et al. 1998), (Jetmore et al. 2002), (Ahmed et al. 2004), (Uchida et al. 2011) , until now, VSV-G- and RD114/TR-LVs, two popular pseudotypes available for HSC gene delivery was not able to transduce CD34+ cells in the G0 phase. The work presented here proposes two novel LV pseudotypes that manage to transduce up to 70% of resting HSCs.

Therefore, the improved homing and renewal capacities of resting HSCs would make them better candidate for gene correction than stimulated HSCs. Also to study human hematopoiesis these BaEV- and H/F-LVs are valuable tool in order to study clonogenicity by the analysis of integration sites (LAM-PCR) or using barcode technology. Indeed, a complete follow-up of transduced unstimulated CD34+ cells could give us informations about their differentiation characteristics and their potential advantages for gene therapy applications.

The potentiality to increase transduced HSC engraftment using resting cells and the capacity of BaEV- and H/F-LVs to transduce next to 100% of mild cytokine stimulated CD34+ cells is an obvious advantage for gene therapy correction of multiple monogenetic diseases.

Moreover, the development of gene therapy trials using these new LV pseudotypes could be particularly interesting in the case of the Fanconi Anemia (FA). In this disease, patients develop severe congenital hematopoietic defects and cancers (Bagby et al. 2006), (D'Andrea et al. 2003). Current allogeneous HSCT treatment present limitations because it induce severe GVHD and FA-specific malignancies (Rosenberg et al. 2003), (Rosenberg et al. 2005). These complications could be prevented by autologous HSCT of patient's gene corrected cells (Tolar et al. 2011). However, cells from FA patients present numerous characteristics in disfavor of the commonly used VSV-G-LVs. First, a low number of CD34+ cells is available because of the disease physiopathology, then, these cells are very fragile and do not

survive to prolonged and strong cytokine stimulation transduction protocols (Haneline et al. 2003). Therefore, minimizing CD34+ cell stimulation combined with very efficient gene transfer allowed by BaEV- and H/F-LVs would be invaluable for FA gene therapy (Jacome et al. 2009), (Muller et al. 2008), (Tolar et al. 2012). Despite the H/F-LVs highest level of transduction, BaEV/TR-LVs would be more quickly and more effectively to the GMP requests due to their higher infectious titers and the low toxicity of the BaEV glycoprotein.

## 5) PERSPECTIVE TOWARDS TRANSPLANTATION OF PROGENITOR T CELLS AND CD34 CELLS FROM THE SAME DONOR IN GENE THERAPY

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As developed by Reimann and co-workers, the DL-4 culture system of CD34+ cells induces their differentiation in T progenitors. This could allow the co-transplantation of HSCs and early T progenitors offering an apparently safe way to reduce the long phase of immune system recovery detected when solely HSCs are transplanted (Reimann et al. 2012).

The application of this cotransplantation protocol in gene therapy procedures could allow a better adaptive immune system recovery. Indeed, autologous HSCT or T cell based gene therapy and immunotherapy in which gene correction provides a selective advantage for corrected cells is rare (Bigger et al. 2014). Thus, conditioning regimens are applied in order to suppress myeloid cells and the host immune response to allow a long-time adaptive immune system recovery.

The co-transplantation of corrected HSCs and T progenitors could accelerate the enhancing of T cell lineage reconstitution and thus it could prevent the infection related post transplantation morbidity. In case of allogenic HSCT, induced GVHD is a major concern, it consists in the targeting of host organs by donor T cells. Zakrzewski and colleagues (Zakrzewski et al. 2006), (Zakrzewski et al. 2008) demonstrated that the co-transplanted T progenitors derived from the CD34+ donor cells do not cause GVHD because they pass through the host tolerance selection process by engrafting in the thymus. Of importance, they showed that these cells offer to the host recipient

an increased antimicrobial resistance and a Graft-versus-Tumor (GVT) in cancer immunotherapy.

As a consequence, by developing co-transplantation of BaEV- or H/F-LV corrected HSC and derived T progenitors, we could propose safer gene therapy and immunotherapy procedures. Moreover, some co-transplantation assays such as we developed in NSG mice could allow to better understand the mechanisms of co-transplanted cell engraftment. Indeed, as we suggested with our preliminary results in NSG mice, we could follow separately the fates of transplanted CD34+ cells and T progenitor cells for instance by using different fluorescent proteins encoded by the vector pseudotyped either with BaEV- or H/F glycoproteins.

## 6) B CELL GENE DELIVERY BY BAEV-LVS AND IMMUNOTHERAPY EXPECTATIONS

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Transgene expression in B cells is of particular interest as B cells have the potential to induce specific immune activation and tolerance, which could improve genetic vaccination against cancer or autoimmune diseases (Lei et al. 2005), (Melo et al. 2002), (Stripecke et al. 2000).

One of the major goals of cancer immunotherapy is to increase the immunogenicity of tumor cells. Thus, vaccination strategies using autologous tumor cells manipulated *ex vivo* might be considered as a new approach for patients with B-cell malignancies, especially for those patients who are not responding to current treatment regimens (Moskowitz et al. 2006).

Autoimmune diseases represent failure of self-tolerance in populations of circulating B and T cells (Melo et al. 2002), (Lei et al. 2005b). Current treatments are based on immunosuppression and do not induce the cure of patients. Thus, novel approaches that can induce tolerance are required. B-cell gene therapy is an important option for induction of potent tolerogenic APCs (El-Amine et al. 2000), (Gourley et al. 2002), (Xu et al. 2004). The clinical relevance of this B-cell gene therapy approach has been demonstrated by tolerance induction for targeted antigens in experimental models of several autoimmune disease (Xu et al. 2004),

(Agarwal et al. 2000), (Song et al. 2004). In these models, this gene therapy induced protection, delayed the onset of the disease or even reversed the ongoing clinical course.

Of importance, primary B cells are the most potent antibody producing cells. Indeed, the programming of B cells to make a predefined protective antibody would provide a continuous supply of antibodies *in vivo* that might reduce the viral load in patients (Luo et al. 2005). A novel strategy requiring efficient B-cell gene transfer may facilitate production and the identification of monoclonal neutralizing antibodies by allowing efficient transduction of primary patient memory B cells with BCL6 and BCL-XL genes inducing immortalization (Kwakkenbos et al. 2010).

Stable gene transfer in human B cells may thus allow the engineering of improved genetic vaccination strategies against cancer, infectious or autoimmune diseases (Li et al. 2006), (White et al. 2000). As we developed above, BaEV-LVs display interesting characteristics and could be optimized in order to be employed for clinical applications. Furthermore, the ability of BaEV-LVs to efficiently and stably transduce B cells allow a current development to our group to develop HCV immunotherapy (Fusil et al. work in progress).

To conclude, although numerous steps of optimization are needed to allow the use of both BaEV-LVs in the clinic, this work proposes an extensive characterization of a new promising tool for delivering genes into the major hematopoietic cell populations.

## 7) VIRAL VECTORS AND GENE THERAPY

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In this work, we focused on lentiviral vectors, nevertheless, a lot of other viral vectors have been developed. Each viral vector has a precise scope, for instance, Herpesvirus derived vectors present the best transgene capacity reaching 150kb versus 8kb with lentiviral vectors. However, their transgene expression appears not very stable. Adenovirus derived vectors are non-integrative and are easy to produce at high titers, they can infect most of cell types but their high immunogenicity can induce a severe humoral immune response. Such as lentiviral vector, adeno-

associated viral vectors AAV are integrative and present a low immunogenicity. Although they are easy to produce and they allow a long term gene expression, they present a limited transgen capacity. All these vectors constitute a pool of interesting tools for developing new therapies in order to improve human health.

## EPILOGUE

---

Cette thèse propose de nouveaux outils, prometteurs à mes yeux pour la mise en place de nouveaux protocoles de thérapie génique et d'immunothérapie. Ce travail propose ici de détailler ces outils, depuis leur optimisation jusqu'à leurs champs d'applications. Ce n'est peut-être pas l'étape la plus glorieuse mais pourtant...

Pourtant il me semble que ce travail apporte une pierre à l'édifice du transfert de gènes et des thérapies qui y sont liées. L'émulation ressentie lors des congrès et depuis la publication du brevet et de l'article dans Blood me montrent que cette étape était nécessaire et que ces vecteurs répondent à un besoin dans le domaine. Tel un entraîneur envers ses compétiteurs, je nourris beaucoup d'espoir envers ces nouveaux vecteurs, j'espère qu'ils franchiront chaque étape vers leur usage clinique et surtout qu'ils contribueront à la guérison de certains. Si cela venait à échouer, ils constitueront au moins une base à la réflexion et permettront le développement de nouveaux outils encore plus performants.

Certes, j'aurai aimé pouvoir développer ces vecteurs jusqu'à leur utilisation thérapeutique, aboutissement plein et entier de ce travail. Néanmoins, j'ai ressenti un réel plaisir à développer des outils globaux que plusieurs spécialistes pourront adapter aux pathologies qui les concerne.

Cet épilogue ne constitue pas une page de publicité à laquelle seuls ceux qui se seront attachés à lire cette thèse jusqu'à la fin auront accès. Il faut garder en tête les quelques mots d'Albert Einstein : « *L'homme et sa sécurité doivent constituer la première préoccupation de toute aventure technologique* », par conséquent il me semble que dans cette aventure nous n'en sommes qu'au début.

Enfin, pour tout avouer, toujours dans cette métaphore de l'entraîneur, j'ai un favori, un poulain que j'aimerai par-dessus tout voir réussir, pour des raisons scientifiques mais aussi pour un attachement personnel purement irrationnel, bon vent BaEV/TR...



## APPENDIX

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1) ENVELOPE, PLEASE; AND THE AWARD GOES TO...  
INSIDE BLOOD COMMENTARY BY D.B. KOHN

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● ● ● GENE THERAPY

Comment on Girard-Gagnepain et al, page 1221

## Envelope, please. And the award goes to...

Donald B. Kohn and Roger P. Hollis UNIVERSITY OF CALIFORNIA, LOS ANGELES

In this issue of *Blood*, Girard-Gagnepain et al report a potentially important discovery for improved gene transfer to human hematopoietic stem cells (HSCs) for gene therapy of blood cell diseases: a novel retroviral envelope to package (pseudotype) vectors for enhanced gene delivery to human HSCs.<sup>1</sup>

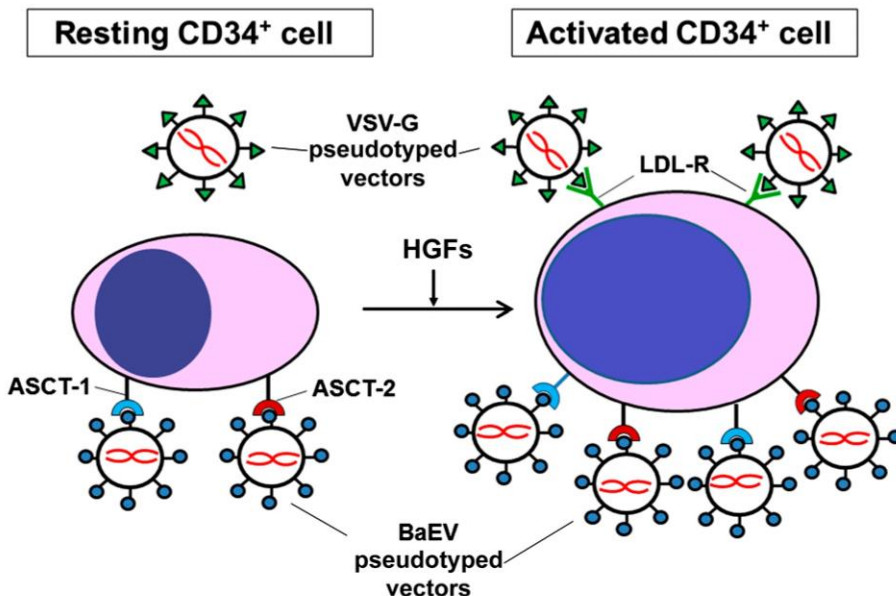
**A**utologous transplantation using gene-corrected HSCs is achieving a growing number of clinical successes for the treatment of primary immune deficiencies, lysosomal storage and metabolic disorders, and

hemoglobinopathies.<sup>2,3</sup> Despite the gratifying progress, it remains challenging to achieve optimal gene transfer to human HSCs, and 10- to 100-fold higher amounts of vectors are typically needed for their gene modification

compared with transduction of cell lines, mesenchymal stem cells, and many other target cell types. This is perhaps most relevant to efforts toward gene therapy of hemoglobinopathies, where the large, complex human  $\beta$ -globin gene cassettes needed for high-level, erythroid-specific expression lead to lower vector titers and limit gene transfer efficacy, in contrast to what can be achieved with vectors carrying simple cDNA and small promoter elements.

The relative restriction of gene transfer to human HSCs has been known for a long time, requiring activation of HSCs for effective transduction,<sup>4</sup> typically achieved by a prestimulation culture period of 24 to 48 hours in medium with a mixture of early-acting hematopoietic growth factors (HGFs), such as *ckit* ligand, *flt-3* ligand, and thrombopoietin, prior to vector exposure. The mechanisms by which prestimulation increases gene transfer have been attributed to moving the largely quiescent, G<sub>0</sub> phase HSCs to more active states, allowing higher levels of vector genome reverse transcription. Prestimulation induces stem cell mitosis to allow  $\gamma$ -retroviral vectors to access the chromosomes, because they lack the nuclear import signals present in lentiviral vectors, which lets the latter transduce nondividing cells and largely supplant the use of  $\gamma$ -retroviral vectors. More recently, blocks to transduction by retroviral restriction factors, innate intracellular inflammatory response pathways, or endosomal trapping have been identified as potential roadblocks, with reports suggesting they may be at least partially surmounted with proteasome inhibitors, rapamycin, or other small molecules. Always, the manipulations used to augment gene transfer efficiency (eg, extended culture with HGFs) need to be balanced with the potentially adverse effects from these approaches on HSC capacity, with induction of differentiation likely to limit the longevity of engraftment of the gene-corrected cells.

Part of the search for methods for improved transduction has involved assessments of



Transduction of human CD34<sup>+</sup> HSCs by pseudotyped vectors. Resting CD34<sup>+</sup> cells display the ASCT-1 and ASCT-2 proteins that serve as receptors for the BaEV envelope glycoprotein, and retroviral or lentiviral vectors pseudotyped with the BaEV envelope can bind to the receptors and transduce the cells. Resting CD34<sup>+</sup> cells lack expression of the LDL-R that serves as the receptor for VSV-G-pseudotyped vectors and thus are not effectively transduced by them. Activated CD34<sup>+</sup> cells, prestimulated with HGFs, such as *ckit* ligand, *flt-3*-ligand, and thrombopoietin, express the LDL-R and can bind and be transduced by VSV-G-pseudotyped vectors. The levels of the ASCT-1 and ASCT-2 receptors increase further in activated CD34<sup>+</sup> cells, and transduction by the BaEV-pseudotyped vectors also increases.

different envelopes to pseudotype the vectors for optimal target HSC binding and entry. Initially, envelopes from retroviruses were used that conferred tropism to human cells, such as the murine retrovirus amphotropic envelope, the gibbon ape leukemia virus envelope, or the feline leukemia virus RD114 envelope. The envelope glycoproteins are essentially nontoxic, which allows them to be incorporated into stable “packaging cell lines,” and these pseudotypes have been most commonly used for  $\gamma$ -retroviral vectors in clinical trials. However, the use of vectors with these envelope pseudotypes requires surface expression of the specific cellular protein(s) that they exploit as receptors on target cells, typically amino acid or mineral transporters that may require cell activation to be induced.

The vesicular stomatitis virus (VSV-G) protein was identified as having some advantages over retroviral envelopes for pseudotyping retroviral and lentiviral vectors, including the wide species tropism it confers (from human down to zebrafish). The physical hardiness of VSV-G-pseudotyped virion allows vector concentration to high titers without significant loss of biological activity (in contrast to retroviral envelope proteins that are thought to be more fragile, leading to their loss during ultracentrifugation).<sup>5</sup> However, there has been a love-hate relationship with the VSV-G envelope, as it is moderately fusogenic; this causes cytotoxicity and makes it difficult to derive stable packaging lines that produce VSV-G pseudotyped virion, necessitating vector production by cumbersome transient transfection methods (although a first vector produced from a stable VSV-G packaging cell line has entered clinical trials).<sup>6</sup> Also, VSV-G-pseudotyped vectors can be cytotoxic to their target cells, such as HSCs, especially when used at high concentrations or with impure preparations.

Investigators in Lyon, France, reported earlier this year that the VSV-G cellular receptor, only recently identified as the low-density lipoprotein receptor (LDL-R),<sup>7</sup> is minimally expressed on human HSCs; induction of LDL-R expression turns out to be one of the major mechanisms by which prestimulation with growth factors augments HSC transduction.<sup>8</sup> They now report studies using a novel envelope pseudotype derived from the baboon endogenous retrovirus (BaEV) that appears to present an important advance for the production of vectors for gene

transfer to human HSCs.<sup>1</sup> Vectors packaged with the BaEV envelope were shown to use 2 cellular proteins, the neutral amino acid transporters ASCT-1 and ASCT-2, as receptors, and basal expression of these receptors on freshly isolated human HSCs allowed moderately effective gene transfer without prestimulation, in contrast to the strict requirement for prestimulation for transduction by VSV-G-pseudotyped vectors (see figure). Although the titers measured for the BaEV-pseudotyped vectors were ~20-fold lower than the VSV-G pseudotypes, they were more effective when used at equivalent amounts (adjusted for virion p24 content). Using both in vitro and in vivo serial xenografts in NOD/SCID/ $\gamma$  (null) (NSG) mice as assays of human HSC transduction, the BaEV-pseudotyped vectors led to significantly higher levels of gene transfer to human HSC than VSV-G-pseudotyped vectors: up to 30% of NSG-engrafting cells without prestimulation and up to 90% with prestimulation, with vector present in all lineages of human blood cells produced. Effective transduction could be achieved using fewer HGFs than are typically used, which may contribute to better preservation of stem cell function.

Of course, the xenograft models are still surrogates for the long-term reconstituting HSCs in clinical transplants, and additional studies will need to be performed to verify and extend these findings, both in nonhuman primate models (the BaEV packaged vectors were shown to effectively transduce macaque CD34<sup>+</sup> cells) and eventually in clinical trials. However, the identification of the unique properties of the BaEV envelope holds the prospect of improving

gene production methods, by allowing the development of stable packaging cell lines, as well as increasing the efficiency of gene transfer to stem cells while better preserving their function by minimizing ex vivo manipulation. This newly characterized envelope may be the award winner for Best Envelope in a Pseudotyping Role needed to take HSC gene therapy to the next level of effectiveness.

*Conflict-of-interest disclosure:* The authors declare no competing financial interests. ■

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## ● ● ● HEMATOPOIESIS & STEM CELLS

Comment on Lopez et al, page 1232

# Hematopoietic ontogeny in the axolotl

David L. Stachura and David Traver UNIVERSITY OF CALIFORNIA, SAN DIEGO

In this issue of *Blood*, Lopez et al undertake the heroic task of characterizing the blood-forming system of the axolotl (*Ambystoma mexicanum*), an aquatic salamander that provides an excellent model for tissue regeneration and scar-free wound healing.<sup>1</sup> Commonly referred to as the “Mexican walking fish,” axolotls are not fish at all, but rather neotenic salamanders that retain many larval traits throughout their lifespan because they do not undergo a typical juvenile to adult metamorphosis. This retention of larval traits is associated with the profound ability of the axolotl to regenerate many of its tissues, including limbs, spinal cord, heart, and even parts of its brain.<sup>2-6</sup>

2) CHAPTER LENTIVIRAL VECTORS (TAYLOR AND  
FRANCIS GROUP): DESIGN AND APPLICATIONS. GENE  
AND CELL THERAPY: THERAPEUTIC MECHANISMS  
AND STRATEGIES

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# 1 Lentiviral Vectors

## *Design and Applications*

*Anais Girard and Els Verhoeyen*

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## 1.1 LENTIVIRAL VECTOR FEATURES

In order to accomplish efficient gene transfer, numerous tools have been developed. Indeed, the simple addition of naked or complexed DNA on certain cell types results in extremely poor gene transfer. Some vehicles have been developed on the basis of existing viruses such as retrovirus, herpes virus, adenovirus, and adeno-associated virus. Retroviral vectors allow a stable integration into the host genome and therefore allow long-term expression via the use of their viral reverse transcriptase (RT) and integrase (IN) [1]. Moreover, these vectors allow this long-term gene transfer without transferring any viral genes. The absence or the poor preexisting immunity in the recipients *in vivo* is also an essential feature of retroviral vectors. These murine leukemia virus (MLV)-based vectors transduce only actively dividing cells, and therefore important gene therapy targets including liver, brain, heart, hematopoietic progenitors, or skeletal muscles cannot be transduced unless they are induced to proliferate.

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However, nondividing cells are important targets for gene therapy and for resolving questions in basic research. Lentiviral vectors (LVs), which are derived from immunodeficiency viruses, appeared to be the better candidates because they are able to infect nonproliferating lymphocytes and macrophages. Indeed, in contrast to the oncoretroviruses, lentiviruses contain a preintegration complex (PIC) (Gag, Vpr, IN) with nuclear localization signals that allow its transport into the nucleus during interphase [2–4]. The aim of this chapter is to provide an overview of the LVs starting from the understanding of basic virology of human immunodeficiency virus (HIV), of which they are derived, through to the many applications of these powerful tools. The provided references give examples or more detailed explanation/information.

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Lentiviruses are complex retroviruses studied since the early 1980s, when the AIDS pandemic emerged due to the current well-known HIV type 1 (HIV-1). *Lentivirus* is a genus of the family Retroviridae that cause slow (lentus, slow in Latin) progressive human and animal diseases. They are enveloped RNA viruses. Five serogroups were identified: bovine, equine, feline, ovine/caprine lentiviruses, and the primate lentiviruses, HIV and simian immunodeficiency virus (SIV). Most of the LVs are derived from the primate lentivirus group, which includes the HIV types 1 to 3, the SIV, and the simian AIDS retrovirus SRV-1. Because the majority of the LVs are based on HIV-1, we will use it

as an example for the following reasons: (1) similar features are present in the vector systems derived from other lentiviruses and (2) the HIV-1-derived vector design is the most advanced.

### 1.1.1 BASICS IN LENTIVIROLOGY AND HIV-1 [4]

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In order to understand the manipulation of LVs, having a good comprehension of the viral components is important.

#### 1.1.1.1 Lentiviral Genomic Structure and Proteins

HIV-1 has a single-stranded positive RNA genome of approximately 9 kb and encodes at least nine viral proteins. It is important to distinguish the *cis*- and *trans*-acting components of the HIV genome. The *trans*-acting components are composed of nine open reading frames (ORFs). The three major ORFs encode the gag, pol, and env polypeptides and are also characteristic for MLV-based vectors. The six others encode lentivirus-specific genes such as *Tat* and *Rev*, the viral regulatory proteins, and the four accessory proteins: Vpr, Vpu, Nef, and Vif. The *cis*-acting elements consist of the long terminal repeats (LTRs), the Rev responsive elements (RRE), the splicing sites, and the packaging signal ( $\Psi$ ) (Figure 1.1a).

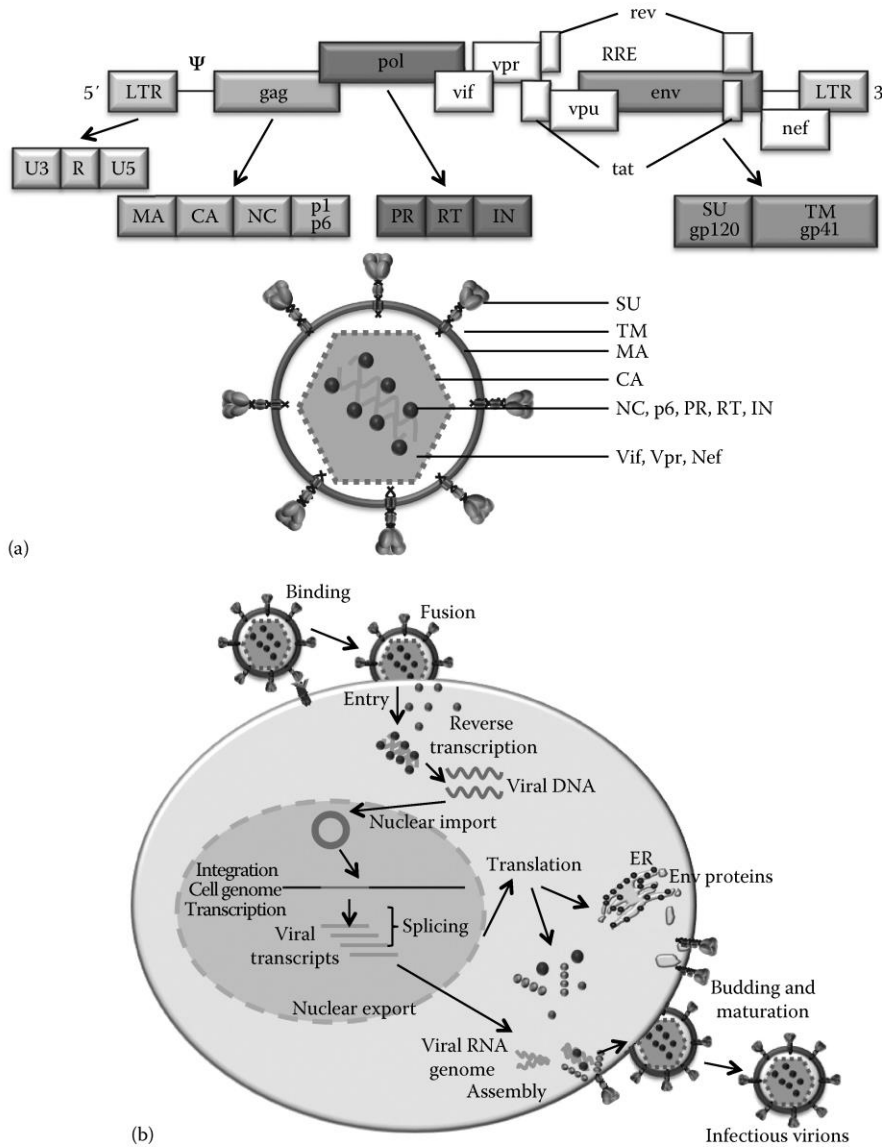
##### 1.1.1.1.1 Gag, Pol, and Env Are the Three Major Structural Proteins of HIV-1

The gag precursor protein (p55) is involved in budding of the viral particle from the infected cell membrane by recruiting two copies of viral genomic RNA and other viral components. During the viral maturation, p55 is cleaved by the viral protease into matrix (MA) (p17), capsid (CA) (p24), nucleocapsid (NC) (p9), and P6 (Figure 1.1a). The MA protein stabilizes the viral particle and facilitates the nuclear import of the viral genome, allowing HIV-1 to infect nondividing cells. The CA protein interacts with p55, which favors viral replication. The NC protein recognizes the packaging signal of HIV and also facilitates reverse transcription. The p6 polypeptide allows the incorporation of Vpr into viral particles due to its interactions with p55 and Vpr. In addition, it is required for the efficient release of viruses from the cell.

The gag-pol fusion (F) protein (p160) is produced by a ribosomal frame shifting event. During viral maturation, the pol polypeptide is digested in protease (PRO, p10), RT (p50), RNase H (p15), and IN (p31) (Figure 1.1a). The protease is required during viral maturation for Gag and Gag-pol precursors' cleavage. The RT allows the polymerization of a double-stranded

AQ5





**AQ6** **FIGURE 1.1** Schematic representation of the HIV-1 viral genome, virion structure, and viral life cycle. (a) The HIV-1 viral genome encodes structural (*gag*, *pol*, *env*), regulatory (*tat*, *rev*), and accessory (*vpr*, *vif*, *vpu*, *nef*) genes flanked by two LTRs. (b) Inner and outer viral membranes are coated with MA and envelope (*Env*) proteins, respectively. The two viral ssRNAs, the RT, the IN, and protease (PR) and the accessory proteins are surrounded by CA. (c) During cell entry, CA proteins are uncoated and the two ssRNA viral genomic strands (marked in green) and viral enzymes are released into the cytoplasm. Viral RNA is converted by RT into proviral dsDNA (marked in red) and imported into the nucleus. Then the IN inserts the dsDNA into the host genome. During the early infection phase, only spliced viral mRNAs encoding *tat*, *nef*, and *rev* are exported to the cytoplasm. Then, the Rev protein allows the export of unspliced or singly spliced (*vif*, *vpr*, *vpu*, *env*) mRNA. Once every viral component is synthesized, viral genome and proteins are assembled at the plasma membrane and new HIV particles are released from the host cell. Just after the virus budding, the PR induces virus maturation, which renders the viral particles infectious.

DNA (dsDNA) copy of the HIV-1 genome with the help of the RNase H that removes the original RNA template from the first retrotranscribed DNA strand. The IN allows the integration of the proviral DNA into the host genomic DNA via its exonuclease, endonuclease, and ligase activities.

The Env polypeptide (gp 160) is cleaved after glycosylation in gp41 that contains the transmembrane domain

(TMd) and in gp120 the surface (SU) domain, which is linked to gp41 by noncovalent interactions [5].

*1.1.1.1.2 Tat and Rev: The Viral Regulatory Proteins of HIV-1*

Tat is a transcriptional transactivator essential for viral replication. It is an RNA binding protein that recognizes

AQ7 the transactivation response element (TRE) at the 5' end of HIV-1 RNA and strongly activates transcription from the HIV LTR.

Rev is also a RNA binding protein that recognizes the RRE allowing the transition from the early to the late phase in HIV infection. Of note, this binding facilitates the export of unspliced viral RNA to the cytoplasm using snRNAs and the ribosomal 5s RNA.

#### 1.1.1.1.3 *Vpr, Vpu, Nef, and Vif: The Viral Accessory Proteins of HIV-1*

Vpr is a member of the PIC and plays a role in the infection of nondividing cells by HIV-1 by facilitating the nuclear localization of this PIC. Vpu facilitates the particle assembly by triggering the degradation of intracellular Env-CD4 complexes. It also enhances virus release from the infected cell surface. Vif is necessary for the viral replication in peripheral blood lymphocytes and macrophages by inactivating the host antiviral factor APOBEC3G [6]. Nef has three major activities: (1) it decreases the cell surface expression of CD4 and thus (2) facilitates viral budding, and (3) it perturbs T cell activation and stimulates the HIV-1 virus infectivity [7].

#### 1.1.1.1.4 *cis-Acting Elements*

The LTRs contain three elements: U3, R, and U5. The U3 region, at the 5' end of each LTR, has binding sites for multiple transcription factors and includes most of the *cis*-acting DNA elements. The central region, R, contains a specific sequence (100 bp) where transcription starts, whereas polyadenylation occurs just at the end of this region. The U5 region contains the Tat binding site. HIV genome transcription is allowed by a single promoter in the 5' LTR. The 3' LTR mediates the polyadenylation that stabilizes the transcripts. Duplication of LTR elements occurs during reverse transcription just before integration when the copied 3' LTR U3 subregion is transferred to the 5' LTR.

The packaging element ( $\Psi$ ) is an important feature, consisting of two short sequences flanked at each side of the major splice donor site, which provides a structured RNA element and allows the recognition and packaging of genomic RNA into the CA just before assembly. As discussed previously, RRE plays a role in the export of unspliced RNA from the nucleus.

#### 1.1.1.1.5 *Nonhuman Lentiviruses*

The nonhuman lentiviruses used for vectorization are bovine lentiviruses [8], equine lentiviruses [9], feline lentiviruses [10], ovine/caprines lentiviruses [11], and the primate SIV lentiviruses [12]. Of importance, most of the proteins of these lentiviruses are conserved, except for some variations in the accessory proteins (e.g., Vif and Tat). Additional genes have been discovered in these viruses; however, the function of some of these other accessory proteins has not been elucidated. Furthermore, these accessory proteins are not required for *in vitro* replication and gene delivery and are thus not included in the LV design. These alternative LV systems are considered safer by the public because they

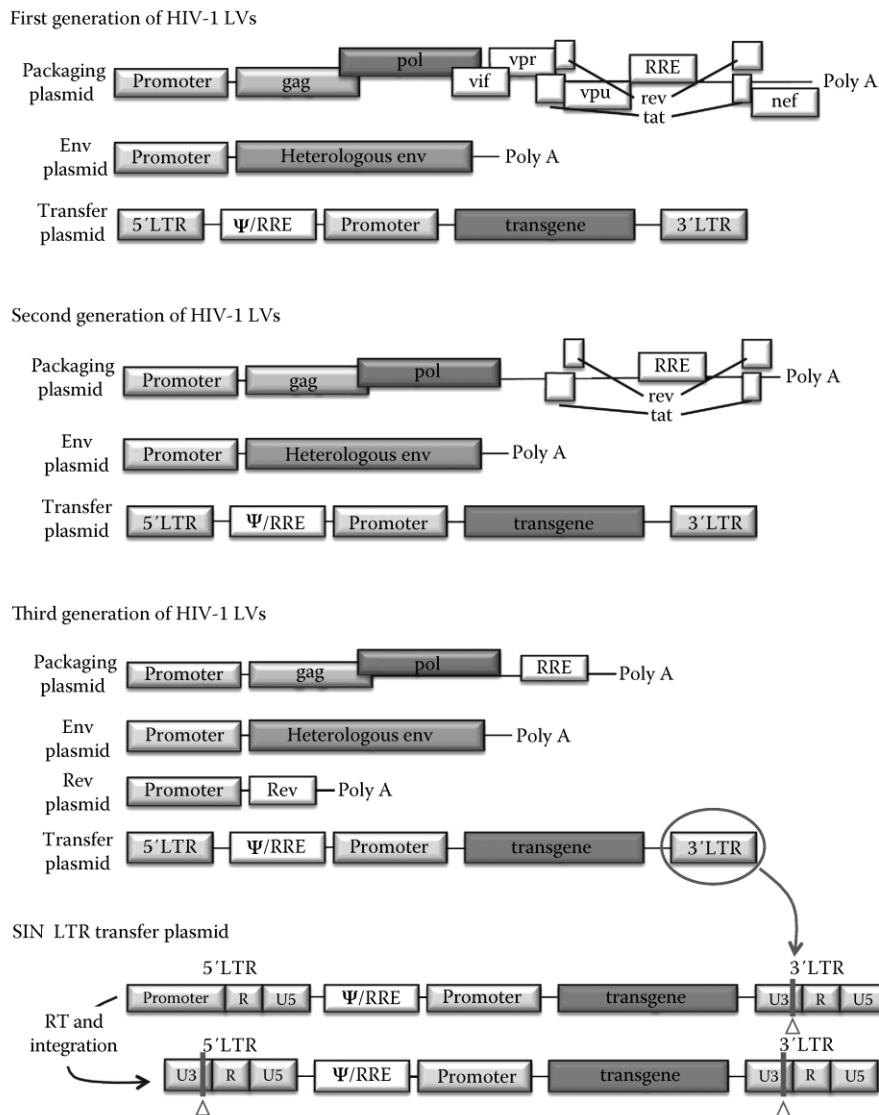
are not derived from a human pathogen; however, this issue is still under debate. Nonhuman primate models have been invaluable for developing hematopoietic gene therapy strategies in humans because they closely mimic human biology. HIV-based lentiviral gene transfer into macaque cells is inefficient due to the presence of restriction factors in simian cells causing an intracellular block [13]. Because SIV-derived vectors are not subjected to this restriction [14], the latest generation self-inactivating (SIN) SIV vectors have been developed for preclinical testing in nonhuman primate models [15].

#### 1.1.1.2 **Lentiviral Life Cycle (Figure 1.1b)**

The global mechanisms involved in the HIV-1 infection cycle are reiterated in this paragraph in order to appreciate the essential differences between lentiviruses and LVs. The natural HIV infection cycle begins by the attachment of the SU (gp120) part of its envelope glycoprotein to its primary receptor CD4 and its co-receptor CXCR4 on T lymphocytes or CCR5 on activated T lymphocytes, monocytes, macrophages, and dendritic cells (DCs). A conformational change of TM follows this event and leads to viral and host cell membrane fusion. Upon cell entry, CA proteins are uncoated resulting in the release of the viral RNA genome and the MA, RT, IN, and Vpr proteins. The viral RT converts the positive ssRNA into dsDNA, then the proviral DNA is imported into the nucleus and integrated into the host genome by use of its viral IN. During the early phase of infection, only spliced mRNAs encoding tat, rev, and nef can be produced and exported to the cytoplasm. Then, the late phase of infection is initiated by the action of Tat, Rev, and accessory protein. The complete viral genome is exported by rev into the cytoplasm and proteins are assembled at the plasma membrane. Just after viral budding, the multimerization of Gag and GagPol activates the protease that induces the maturation of the virus by structural rearrangements.

#### 1.1.2 **FROM LENTIVIRUSES TO HIV-1 LENTIVIRAL VECTORS (FIGURE 1.2)**

The first LVs were developed to better understand viral replication, and they were used as a tool for anti-HIV-1 drug screening. These early HIV-1 LVs were replication-competent viruses that carried transgenes. However, the current and main goal of LV use is to deliver a gene or a set of genes into a target cell in the absence of replication. Because nonreplicating LVs were considered safer, their design was achieved by separation of viral sequences on several expression units and identification of the minimal viral components to engineer an efficient gene transfer vector. The first LVs consisted of a plasmid coding for the proviral DNA deleted for the *env* gene and another plasmid encoding the *env* gene. LVs could be produced by transcomplementation without inducing a second round of infection simply because they lacked the *env* gene. In these first LVs, transgenes replaced *env* or *nef* sequences and thus their expression was under the control of the promoter in the 5' LTR.



**FIGURE 1.2** Schematic representations of HIV-1 derived vectors. The first generation of HIV-1 vectors is composed of (1) a packaging plasmid containing all the viral protein encoding genes except for the envelope, (2) an envelope plasmid that encodes a heterologous envelope glycoprotein, and (3) a transfer vector encoding the 5' and 3' LTRs, the packaging signal ( $\Psi$ ), and RRE sequences and the transgene driven by an internal promoter. In the second generation of LVs, all the accessory proteins are excluded from the packaging plasmid. In the third generation of LVs, regulatory proteins (tat and rev) are excluded from the packaging plasmid and an additional fourth plasmid encoding Rev is added. In SIN LTR transfer vectors, the 5' LTR U3 subregion is replaced by a strong promoter. The integrated transfer vector lacks the complete U3 sequence, which reduces the risk of RCL generation or the activation of proto-oncogenes after vector integration since this U3 region contains enhancer sequences.

**1.1.2.1 Self-Inactivating Lentiviral Vectors: An Important Safety Measure [16,17]**

HIV-derived LVs integrate a transgene into the host genome flanked by the two HIV-1 LTRs. This implies that, if replication-competent lentiviruses (RCLs) are produced or if transduced cells are coinfecting by a wild-type virus, new infectious viral particles can be produced. Moreover, cellular genes might be activated by the enhancer and promoter activity of integrated LTR. If this integration occurs near to a proto-oncogene, this can have a dramatic result in the

induction of oncogenesis. Highly relevant in this context is that if a part of the 3' LTR U3 subregion is deleted, the same deletion is transferred into the 5' LTR during LTR duplication, and this event results in elimination of its promoter/enhancer function. As a consequence, the SIN approach was applied to LVs by deletion of the 3' LTR TATA box, SPI1, NF-kB, and NFAT binding sites. This SIN modification is still currently maintained in all LV generations because it strongly reduces or even ablates the risk of RCL propagation. It also avoids the mobilization of integrated vectors by

a wild-type helper virus and diminishes insertional activation of cellular oncogenes and transcriptional modifications induced by intact LTRs.

### 1.1.2.2 First Generation of HIV-1 Vectors

In order to reduce the risk of generating RCLs with pathogenic abilities, Naldini et al. [18] introduced a first-generation packaging vector with three separated plasmids. The first plasmid contained a packaging construct expressing HIV-1 gag, pol, Tat, Rev, and accessory proteins under the control of a strong mammalian promoter. The Env plasmid encodes a viral glycoprotein that allows the host cell recognition by the LV, virus–cell membrane fusion, and LV cell entry. The process of displaying a heterologous viral envelope glycoprotein at the LV surface is called pseudotyping. Several existing strategies for LV pseudotyping will be developed separately in this chapter.

Both packaging and env encoding plasmids do not contain the packaging signal and LTRs in order to prevent their assembly into the particle and to reduce RCL production resulting from recombination between homologous sequences on the different plasmids. The third plasmid also called the transfer vector contains the transgene cassette and all of the essential *cis*-acting elements such as LTR,  $\Psi$ , and RRE without encoding the HIV-1 proteins. An internal promoter was added to control transgene expression in the host cell (Figure 1.2).

The separation of the different LV functions on three plasmids reduces the risk of RCL formation because this requires at least two recombination events. Moreover, the use of an ectopic envelope glycoprotein rather than the HIV-1 env by eliminating homologous sequences between the env and the transfer vector plasmids also decreases the risk of recombination.

### 1.1.2.3 Second Generation of HIV-1 Vectors

In order to increase the LV safety, a second generation of LVs was developed by deleting the accessory proteins. Indeed, although these proteins are essential for HIV-1 virulence, they can be deleted without affecting the gene transfer role of LVs. Therefore, in this second generation of LVs, the packaging vector encoding plasmid only contained gag, pol, tat, and rev and was devoid of all other HIV-1 genes [1,19].

### 1.1.2.4 Third Generation of HIV-1 Vectors [20]

This third generation of LVs was developed in 1998 in order to increase LV safety by preventing the role of HIV regulatory genes. The independence for Tat regulation was obtained by replacing the 5' LTR U3 subregion with a strong viral promoter from the human cytomegalovirus (CMV). Since then, other promoters are also in use. The envelope encoding plasmid relies on the same design used for the second LV generation. Tat and rev sequences have been removed from the packaging plasmid; thus, they carry only the gag and pol sequences. The transfer vector has adapted a SIN configuration, and therefore, the transgene is driven by an internal heterologous promoter (Figure 1.2). Moreover, a fourth plasmid expressing Rev was introduced to conserve its functions including the

nuclear export of unspliced RNAs and the transcript stability. With this third generation of LVs, at least three recombination events are necessary to generate an RCL. Even if an RCL would be produced, Tat, accessory proteins, and active LTRs would not be present in the resulting viruses. Because of their high infectious titer, high-level transduction, and important biosafety features, these third-generation vectors are currently predominant. Nevertheless, a fourth generation of LVs has been developed to further improve biosafety for clinical applications. Several approaches have been evaluated such as the development of Rev-independent vectors by replacing the RRE with heterologous viral sequences known to facilitate the export and stability of unspliced viral RNAs. However, the titer decrease induced in these vectors is one of their great disadvantages [21]. Gag–pol “codon optimization” has been performed in order to eliminate HIV-1 gag–pol homologous regions between the packaging plasmid and the transfer vector. This approach consists of introducing a mutation in the wobble codon position into most of the gag–pol codons in the packaging construct thereby conserving the primary amino acid sequence [22]. Despite these numerous improvements in terms of biosafety, most laboratories still use the third generation of LVs, except when clinical use is envisaged.

## 1.1.3 MODIFICATIONS THAT ENHANCE TRANSFER VECTOR PERFORMANCE

In addition to the biosafety modifications, optimizations of transfer vector performance in terms of expression level and stability have been evaluated through the modification of *cis*-acting elements.

### 1.1.3.1 Modifications to Improve Transduction

During HIV-1 RT, the DNA synthesis starts from the poly-purine tract (PPT) and the central PPT (cPPT). The plus strand overlap obtained is called the central DNA flap (99 nucleotides), which is known to play a role in enhancing HIV-1 provirus nuclear import. In the field of lentivector technology, it is now common knowledge that the introduction of this *cis*-acting cPPT element in the transfer vector plasmid highly increases the vector transduction efficiency in certain cell types [23–25] notably in hematopoietic stem cells (HSCs).

### 1.1.3.2 Modifications to Increase Transgene Expression

Transcriptional and posttranscriptional steps are vital for gene expression regulation. It appeared very useful to interfere with the posttranscriptional step in order to optimize transgene expression. The woodchuck hepatitis virus posttranscriptional regulatory element (WPRE), a *cis*-acting element known for its ability to increase by two- to fivefold the amount of unspliced RNA, was included in the transfer vector design. As expected, the introduction of WPRE in LVs significantly increases transgene expression in host cells [26,27]. However, biosafety modifications such as a mutation of the ORF of the WHV X gene in the WPRE sequence proved

necessary because of its involvement in oncogenesis [28]. Posttranscriptional regulatory elements from other viruses also proved interesting to improve vector design.

### 1.1.3.3 Modifications to Prevent Silencing of the Transgene

MLV-based retroviral vectors revealed an unwanted property: transgenes can be transcriptionally silenced. Although the LVs contain less CpG islands than the MLV vectors, this silencing was also observed. The use of SIN vectors had largely contributed to reduce this unwanted feature. However, in certain cases, the addition of some positive regulatory elements that isolated the proviral expression cassette from the chromatin and made the transgene more accessible to transcription factors proved useful. For this purpose, DNase hypersensitive regions could be used with the well-known example of the incorporation of the beta-globin locus control region (LCR) to prevent the beta-globin transgene silencing in hematopoietic host cells [29]. Moreover, some insulators protected the transgene from the activation or the silencing of adjacent chromatin. The HS4 core from the beta-globin LCR has been used as an insulator resulting in long-term stable transgene expression in human hematopoietic targeted cells [30]. The use of scaffold/MA attachment regions (S/MARs) also resulted in increased transgene expression stability by mediating attachment of chromatin loops to MA or scaffold proteins of the nuclear envelope [25]. Finally, ubiquitously acting chromatin opening elements (UCOE) are enhancerless elements that appear to be very promising in ensuring stable long-term transgene expression [31]. For example, the A2UCOE element confers stable transgene expression in HSCs by blocking DNA methylation-mediated silencing of different promoters [32]. An example of an advanced gene transfer vector design is depicted in Figure 1.3.

### 1.1.3.4 Lentiviral Vectors Encoding for Multiple Genes

It may be necessary to express multicistronic vectors notably for expressing both a transgene of interest and a resistance gene or a reporter gene such as green fluorescent protein (GFP) to sort out the transduced cells. Two strategies have been developed to express multiple transgenes. In order to understand these methods, it is important to understand that the packaging capacity of LVs is limited to approximately 9–10 kb. Larger inserts have been introduced but this was at a cost to vector stability and titer.

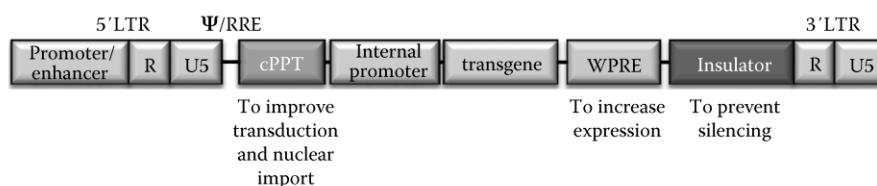
The introduction of multiple expression cassettes in LVs has proven possible. Firstly, independent gene expression was achieved using different promoters or a central bidirectional promoter located between two transgenes in opposite orientation [33,34]. Secondly, it is possible to express multiple genes from a single expression cassette encoding a polycistronic transcript by use of internal ribosome entry sites (IRES) that link several cDNA sequences [35,36]. Nevertheless, often, a difference in expression level is induced with a stronger expression of the first gene in certain cell types. However, this phenomenon depends on the two cDNAs that are introduced. The use of picornaviral self-cleaving 2A peptides that link multiple genes is a valid alternative and is now widely used [37,38]. Nevertheless, the protein from the cDNA placed in front of the 2A peptide obtained from the polycistronic transcript retains a few amino acids encoded by the 2A peptide, which might affect protein stability and function in some cases.

## 1.2 OPTIMIZATION OF LENTIVIRAL VECTOR TARGETING BY SURFACE MODIFICATIONS AND PSEUDOTYPING

LV biosafety, change in tropism (more cell-specific or expanded tropism), and stability were obtained by replacing the HIV-1 envelope on the LV surface by a heterologous viral envelope glycoprotein, a process called pseudotyping. The molecular basis of envelope glycoproteins is shown in Figure 1.4a.

### 1.2.1 OBTAINING LVs AT A HIGH INFECTIOUS TITER WITH AN EXPANDED TROPISM

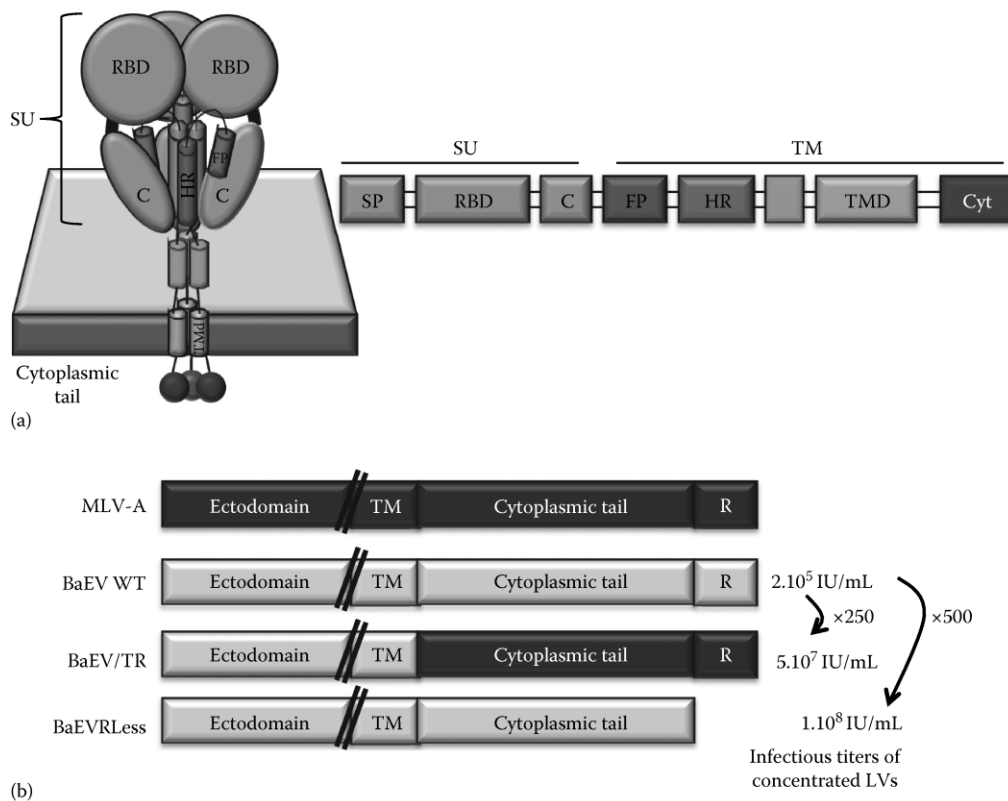
It is clear that the HIV-1 envelope only recognizes the human CD4 molecule on the cell surface; therefore, its display on the LV surface will limit gene transfer to very restricted cell types (CD4+ T cell and macrophages). Since it was used for MLV retroviral vector pseudotyping with success, the vesicular stomatitis virus envelope glycoprotein G (VSV-G) was an attractive candidate for LV pseudotyping. The VSV-G-LV pseudotype proved stable, and by using ultracentrifugation, concentration could be produced at higher titers than those obtained with other envelopes. However, only very recently, the receptor of VSV-G has been elucidated. The attachment of the VSV-G glycoprotein to the ubiquitously expressed low-density lipid (LDL) receptor provides the VSV-G pseudotyped LVs with a “pantropic” cell recognition



**FIGURE 1.3** Optimizing the LV transfer vector. The cPPT sequence improves transduction efficiency, while the WPRE sequence increases the transgene expression. Additional integration of an insulator sequence prevents transgene silencing.

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**FIGURE 1.4** Structure and optimization of envelope glycoproteins. (a) The sequence encoding the viral envelope glycoprotein is composed of two entities: (1) the SU (surface) subunit contains the signal peptide (SP), the receptor binding domain (RBD), and the SU carboxy terminal domain (C); (2) the TM (transmembrane) subunit contains the ectodomain (F peptide [FP] and heptad repeat [HR]), an anchor domain, and a cytoplasmic tail containing the fusion inhibitory peptide R. (b) Mutations of the cytoplasmic tail of the TM subunit can improve envelope glycoprotein incorporation on the LV resulting in increased LV titer. The replacement of the cytoplasmic tail of the BaEV wild-type glycoprotein by the one of the MLV glycoprotein allowed a 250-fold increased titer, and the deletion of the R fusion inhibitory peptide of the BaEV wt glycoprotein improved the infectious titer by 500-fold.

ability [39]. Thanks to this envelope, numerous cell types have been transduced from human HSCs to rat neurons and other nondividing cells. Currently, most LVs are pseudotyped with this envelope. Nevertheless, its sensitivity to the human complement system prevents its potential use in vivo. Moreover, cytotoxicity can be observed with high concentrations of VSV-G pseudotyped LVs. Due to their pantropic cell recognition, safety concerns exist for the in vivo use of this pseudotype in the clinic because it can easily transduce antigen presenting cells (APCs), which might induce a strong immune response in the recipient patient [40].

## 1.2.2 USE OF NATURAL TROPISM OF GLYCOPROTEINS

### 1.2.2.1 Natural Tropism of Parental Viruses and Pseudotyping

Due to the limitations of VSV-G pseudotyped LVs detailed previously, more selective tropisms were achieved by using glycoproteins from other enveloped viruses characterized by their own tropism. Many heterologous viral gps can be incorporated into lentiviral or MLV retroviral particles and

mediate infectivity. These include viral surface glycoproteins derived from lyssaviruses [41], arenaviruses [42], hepadnaviridae [43], paramyxoviridae [44], orthomyxoviruses [45], filoviruses [46], and alphaviruses [47]. For instance, human airway targeting is possible by LVs pseudotyped with surface glycoproteins derived from the Ebola or the influenza viruses. In contrast, LVs pseudotyped with glycoproteins from hepatitis C virus (HCV), baculovirus, or Sendai virus can target hepatocytes, while LVs pseudotyped with rabies glycoproteins target neurons [40] (Table 1.1). Likewise, the cat endogenous retroviral glycoprotein (RD114) [14] and more recently the baboon endogenous retroviral (BaEV) glycoprotein were shown to be very attractive candidates for hematopoietic progenitor cell targeting. LVs have recently been pseudotyped with the hemagglutinin (HA) protein and the F protein from the Edmonston vaccinal strain of measles virus (MV-LVs). This particular pseudotype has acquired remarkable properties for immune cell transduction. These newly developed MV-LVs conserved the parental MV tropism because they allowed transduction of T cells, B cells, and DCs, the primary targets of MV. Importantly, they allowed transduction

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**TABLE 1.1**  
**Pseudotyping of LVs with Heterologous Envelope Glycoproteins Relying on the Natural Tropism of These Glycoproteins**

Vector Pseudotypes	Targeted Cells/Tissues	References
Rabies virus	Neurons	[126–128]
Mokola virus	Neurons/retinal pigment epithelium	[126,129]
LCMV	Glioma/neuronal stem cells	[130,131]
Ross river virus	Glial cells	[132]
Ebola virus	Airway epithelium/skin	[133–135]
MuLV	Skin	[135]
Baculovirus GP64	Hepatocytes	[136]
HCV	Hepatocytes	[137]
Sendai virus F protein	Hepatocytes	[138]
HA influenza	Retinal epithelium	[139]
GALV (modified)	Hematopoietic cells	[14,140,141]
RD114 (modified)	Hematopoietic cells	[14,66]
MV (modified)	B and T lymphocytes	[44,48,56,58]
BaEV (modified)	Hematopoietic cells	Unpublished data

of resting T and B cells, which are not permissive for the polytropic VSV-G pseudotyped vectors, and this happens without changing the T and B cell phenotype [44,48,49] (Figure 1.5). Moreover, MV-LVs allow efficient gene transfer in both patient and healthy plasma cells [50] and other malignant B cells such as B-CLL and MZL, for which classical VSV-G-LVs performed very poorly [51]. In addition, an improved transduction of immature DCs could be established without inducing maturation [44,48,52,53] (Figure 1.5).

### 1.2.2.2 Glycoprotein Optimization for Pseudotyping of Lentiviral Vectors

Although the use of the natural tropism of heterologous surface glycoprotein is very interesting for cell targeting, the surface display of certain wild-type glycoproteins with a heterologous LV vector core was very inefficient. For instance, this is the case for the gibbon ape leukemia virus (GALV) glycoprotein, the cat endogenous retroviral envelope, RD114, and the baboon retroviral envelope (BaEV) LV pseudotypes. When LVs are produced with these wild-type glycoproteins, the resulting viral particles are not highly infectious due to a defect of glycoprotein incorporation on the lentiviral core. The replacement of the cytoplasmic tail of these glycoproteins by one of the MLV gp (which incorporate very well on the lentiviral core) resulted in a stronger incorporation on the LV surface with up to 500-fold increased infectious titer [14,54,55]. Other cytoplasmic tail modifications can be performed such as the deletion of the R fusion inhibitory peptide from the cytoplasmic tail of the BaEV glycoprotein, which allowed a comparable increased infectious titer (Figure 1.4b). Furthermore, co-incorporation of H and F glycoproteins from MV on the LV surface required deletions in the cytoplasmic tails of these glycoproteins [44]. Importantly, these cytoplasmic tail modifications do not impair the host cell range of the initial viral glycoprotein (Table 1.2).

## 1.2.3 LIGAND-FUSED GLYCOPROTEINS TO RETARGET LENTIVIRAL VECTORS

### 1.2.3.1 Direct Targeting Strategy

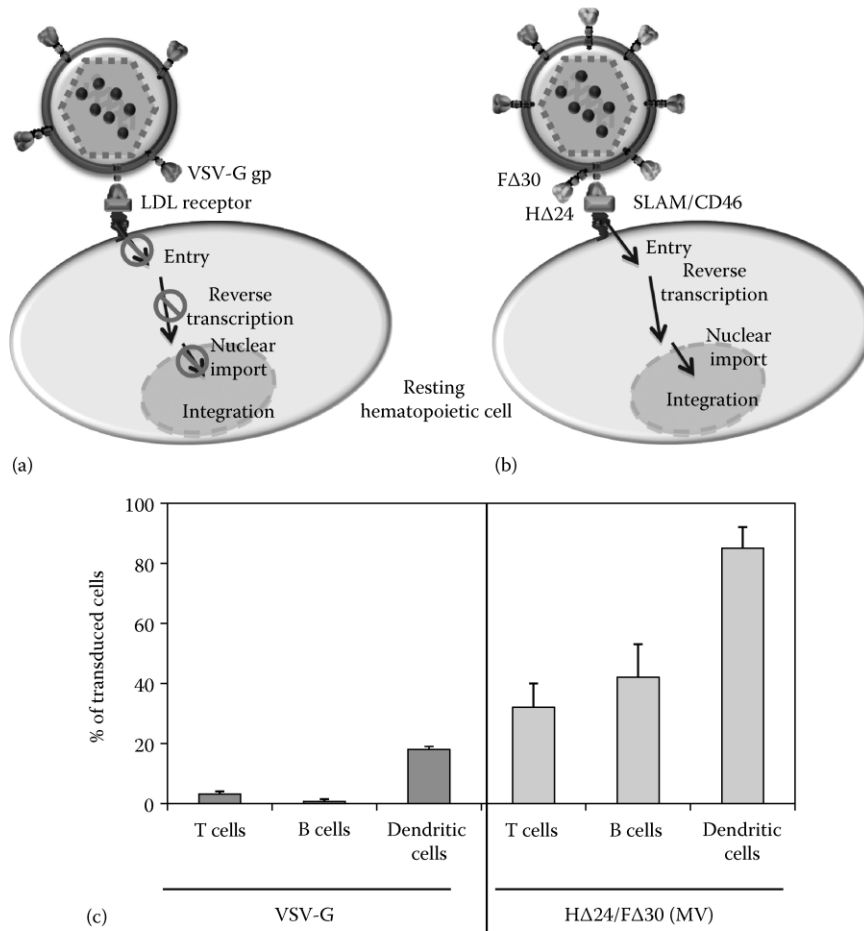
Direct targeting is based on the principle that fusion activation of a chimeric envelope should be triggered by the interaction of the ligand displayed on the vector surface with its specific receptor on the target cell. Therefore, the addition of natural ligands or single chain antibodies (specific for a surface receptor or molecule) on the N terminal domain of retroviral envelope glycoproteins has been evaluated. Unfortunately, although specific binding was achieved, the vector–cell membrane fusion was perturbed. As a consequence, a novel strategy has been designed based on the separation of the two functions, cell binding and virus–cell fusion, into two separate glycoproteins [56]. In this case, redirection of the glycoprotein, which confers binding to the target cell, renders the fusion gp “untouched” and functional. For instance, Morizono and Chen [57] designed an LV pseudotype able to transduce melanoma tumor cells using this principle. Indeed, they co-pseudotyped LVs with the Sindbis virus env F protein E1 and a mutated E2 noncovalently linked to a specific monoclonal antibody directed against melanoma antigen. However, an endocytic uptake of the LV particles is required with this system to activate the membrane fusion with the E1–E2 proteins.

A similar strategy was applied using the H and F MV envelope proteins, which confer receptor binding and membrane fusion, respectively. The modifications of the cytoplasmic tail of the H and F proteins were necessary to allow an efficient co-incorporation on the LV surface as previously mentioned. In order to accomplish retargeting, the natural CD46 and signaling lymphocyte activation molecule (SLAM) receptor binding residues of the H protein were mutated and H was fused to targeting ligands such as epidermal growth factor (EGF) or single chain antibodies specific for antigens expressed on the target cell surface [58]. This direct targeting strategy proved very successful. Meanwhile, introduction of single chain antibodies in the H protein has allowed specific targeting of endothelial cells (aCD105), B cells (aCD20 and aCD19), neurons, T cells (aCD8), and HSCs (aCD133). This emphasizes the flexibility of this strategy, which is characterized by high specificity since background transduction of nontargeted cells is low to nonexistent [56,58–61].

### 1.2.3.2 Indirect Targeting Strategies by Host Range Restriction

For a particular subset of targeted cells, indirect targeting strategies could be used. The basis for host range restriction is the introduction of modifications into the envelope glycoproteins to prevent them from infecting nontargeted cells. The first strategy is based on inverse targeting that exploits receptor-mediated virus neutralization. This is possible by displaying several ligands at the N terminus of the retroviral envelope glycoprotein in order to inhibit infectivity on cells expressing the targeted receptor. For instance, it was shown that amphotropic vectors displaying EGF or





**FIGURE 1.5** Infectivity of VSV-G and MV gp pseudotyped LV pseudotypes for resting hematopoietic cells. (a) VSV-G-LVs are not able to enter efficiently into quiescent T or B cells through their recently identified LDL receptor. And even upon entry, the RT activity and nuclear import is inefficient resulting in very low proviral integration efficiency. These hurdles can only partially be overcome by addition of nucleosides and exogenous ATP. (b) MV gp-LVs manage to enter into quiescent T and B cells and RT activity is detected without activating these cells, followed by efficient nuclear import of the proviral DNA. This results in proviral DNA integration into the resting T and B cell host genome. (c) Comparison of transduction efficiencies in unstimulated T cells, B cells, and DCs for VSV-G-LVs and MV-LVs. The transfer vector encodes for the GFP reporter gene, which allows detection of transduction efficiency (% GFP+ cells) in T and B cells and DCs by flow cytometry analysis.

**TABLE 1.2**  
Differences between VSV-G and MV gp Pseudotyped LVs for Hematopoietic Cell Transduction

Target Cell Type	References	Prestimulation/Activation	LV Pseudotype			
			VSV-G		HΔ24/FΔ30	
			Transduction Performance	Vector Dose	Transduction Performance	Vector Dose
T cells	[44,52]	aCD3/aCD28	Efficient	Low	Efficient	Low
		rIL7	Efficient	High	Efficient	Low
		None	Very poor	High	Efficient	Low
B cells	[48,52]	CD40L/IL-2 +stroma	Efficient	High	Efficient	Low
		SAC/IL-2	Not detected	High	Efficient	Low
		None	Not detected	High	Efficient	Low
DCs	[53]	Immature monocytes-derived DCs (MDDCs)	Poor	High	Efficient	Low
		LPS matured MDDCs	Very poor	High	Poor	Low

stem cell factor (SCF) could efficiently bind to EGF or c-kit receptor (SCF receptor)-positive target cells, respectively, without infecting them. Inverse targeting could be very interesting in order to minimize the risk of inadvertent transduction of cancer or other contaminating cells such as APCs [40].

### 1.2.3.3 Strategies for Selective Targeting of Tissues

Pseudotyping strategies for specific vector-mediated target cell activation have been developed. They consist of an interaction of a specific ligand displayed on the LV surface with its specific receptor thereby inducing the stimulation of the target cells. It was shown that gene transfer into stimulated target cells is specifically enhanced. Initially, MLV retroviral vectors in which the MLV glycoprotein was designed to display the hepatocyte growth factor [62] or the interleukin-2 [63] proved to confer selective targeting.

Importantly, although LVs are able to transduce nondividing cells, some important targets of gene therapy such as resting HSCs, monocytes, and resting T lymphocytes resist gene transfer by classical LVs. In response to this problem, new LV pseudotypes have been engineered. A T cell activating polypeptide consisting of a single chain antibody variable fragment derived from the anti-CD3 OKT3 antibody, which is known to activate the T cell receptor (TCR), was fused to the SU subunit of the MLV envelope glycoprotein [64]. Although the LVs displaying anti-CD3 were able to activate T cells, they did not induce a significant transduction level. Therefore, an “escorting” VSV-G glycoprotein was coexpressed on the LV surface and these codisplaying vectors allowed 100-fold higher gene transfer than in nonactivated T cells. Because a phenotypic shift to memory cells was observed in these anti-CD3-LV activated T cells, other ligands have been evaluated such as interleukin-7 (IL-7), allowing conservation of the T cell phenotype while activating them transiently to allow efficient gene transfer [65].

The difficulty in transducing unstimulated HSCs with classical LVs is well established in the field. To overcome this hurdle, thrombopoietin (TPO) or SCF, two early-acting cytokines that stimulate HSCs, were fused to the N terminus of the influenza HA glycoprotein [55,66,67]. These constructs allowed a better co-incorporation of the VSV-G or RDTR envelope F glycoproteins and the cytokine-displaying glycoproteins on the LV surface. These LVs are very promising for selective targeting. In complete human cord blood, the RDTR/SCF codisplaying LVs transduced hematopoietic progenitor cells (CD34+ cells) at low vector doses with a 95-fold selectivity compared to T cells. Moreover, a selective long-term transduction of c-kit+ HSCs in vivo was observed in humanized mice [66].

## 1.3 OPTIMIZATION OF LENTIVIRAL VECTOR TARGETING BY SELECTIVE EXPRESSION

In addition to LV surface modifications, a second strategy has been developed in vector targeting. LVs have been engineered not only to control cell entry (transductional targeting) but also to control expression of the vector's transgene in the transduced cells (transcriptional targeting).

### 1.3.1 CONTROL OF TRANSGENE EXPRESSION WITH TISSUE-SPECIFIC PROMOTERS

The classic strategy used is to include tissue-specific promoters and enhancers in the transfer vector to drive expression of the transgene and to reduce the risk of side effects related to aberrant expression profiles. Moreover, another interesting benefit of this limited expression is to exclude APCs from transgene expression. Indeed, transgene expression by transduced APCs may result in efficient antigen processing and a swift immune response followed by elimination of transgene expressing cells by the immune system.

Many laboratories have developed tissue-specific expression vectors including neuro-specific, DC-specific, angiogenesis-specific, hepatocyte-specific, and hematopoietic-specific promoters [40]. For instance, a hepatocyte-specific expression had been obtained by replacing the constitutively active CMV promoter by the albumin gene promoter (ALB) [68]. After a systemic administration in immunocompetent mice of this type of liver-specific LV, the expression of the transgene was limited to hepatocytes with a reduced immune response. Furthermore, although HSCs appear to be an attractive target for correcting immune disorders, the real human HSCs have not been perfectly identified and are a subset of the hCD34+ cells present in the bone marrow. Some of these cells can give rise to nonhematopoietic cells such as neurons or endothelial, skeletal, and heart muscle cells. Therefore, the use of hematopoietic-specific promoters seems to be essential such as those used in the Wiskott–Aldrich syndrome (WAS) gene therapy [69]. In this study, investigators used a hematopoietic-specific promoter due to the risk of toxicity when using a transgene nonrestricted expression because the endogenous WAS protein is strictly detected in hematopoietic lineage [70].

### 1.3.2 CONTROL OF TRANSGENE EXPRESSION THROUGH miRNA-MEDIATED SILENCING

Although the control of lentiviral expression by tissue-specific promoters appears to be a good option, this is not the perfect solution because it is sometimes difficult to reconstitute a tissue-specific endogenous promoter. Secondly, once the vector is integrated, the genomic surroundings such as enhancers or neighboring strong promoters can influence the performance of the internal LV promoter.

In order to increase tissue specificity of gene expression, a new strategy has been developed combining the microRNA posttranscriptional regulation with the LV technology. miRNAs are 22nt regulatory RNAs that act posttranscriptionally and influence cellular functions. Although many of miRNAs are ubiquitously expressed, some of them have a tissue-specific pattern [71]. Therefore, in order to prevent the expression of a transgene in hematopoietic cells and especially in APCs, some groups introduced in the transgene's 3'UTR perfect complementary target sequences of microRNA miR-142, highly specific for hematopoietic cells [72]. In this manner, hematopoietic cells were detargeted for transgene expression

and thus no immune response against the transgene was generated (Figure 1.6a). Of importance, the introduction of these new targets for cellular miR-142 does not seem to perturb its natural activity. In contrast to miR-142, miR233 can be used to obtain a lymphoid-specific expression pattern important for immunotherapy [72].

A potential concern using this method is that miRNA regulation could be lost upon mutation of the miRNA target sequence inserted in the vector. Nevertheless, the insertion of multiple target sequences reduces this risk. Moreover, more studies are required to evaluate the consequence of modifying the natural miRNA targeting in presence of the new targets carried by the vector.

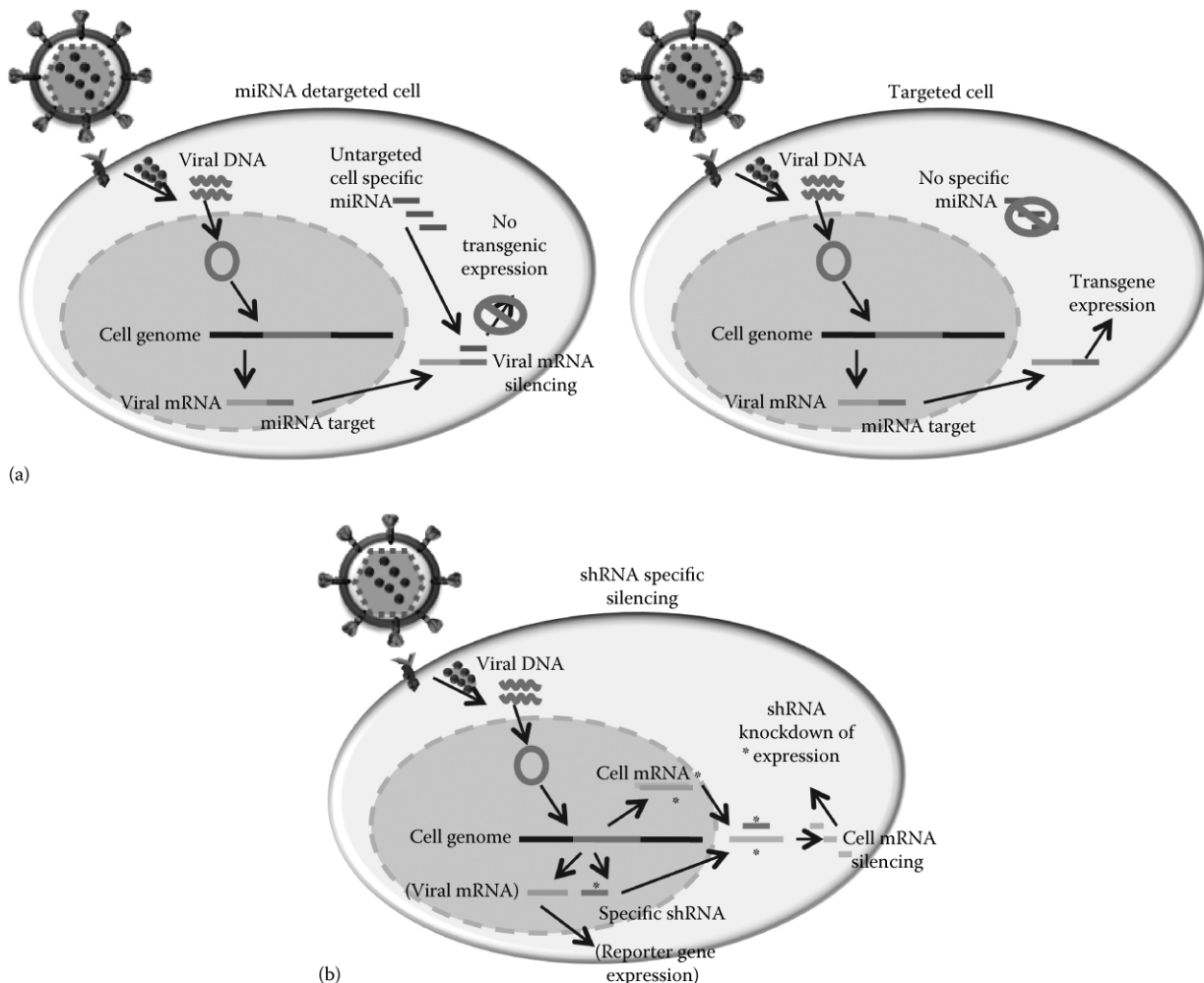
This approach requires less than 100 bases of vector sequence and as a consequence can be developed for LVs and

other viral vectors without impacting and limiting the transgene design. This strategy could allow a highly controlled transgene expression in different cells or even according to the differentiation state of the cell.

## 1.4 LENTIVIRAL VECTOR DESIGNS FOR REGULATED INTEGRATION AND REGULATED EXPRESSION

### 1.4.1 REGULATION OF INTEGRATION OF LENTIVIRAL VECTORS

Many genomic integration regulation strategies have been developed since the concerns raised by the clinical trial that used MLV retroviral vector for X-linked severe combined



**FIGURE 1.6** Schematic representation of LVs for RNA silencing. (a) In nontarget cells, an efficient detargeting of expression is achieved because they contain cell-specific miRNAs that bind to their corresponding miRNA binding sites inserted in the LV mRNA transcripts. In targeted cells, these same miRNAs are absent or are expressed at significant lower levels, and therefore transgene expression from the same LV encoding transcripts is efficient in these cells. (b) The lentiviral transfer vector codes for shRNA, specific for a gene of interest, under the control of a Pol II promoter. Upon expression, the shRNA recognizes a particular sequence of a host cell mRNA and will silence its expression. To identify the transduced cells, these LVs often carry a reporter gene such as GFP.

immunodeficiency (X-SCID) [73,74]. Although this work demonstrated a successful gene therapy in humans, some of the patients developed leukemia due to insertion into a proto-oncogene. Later, it was elucidated that this was caused by the MLV vector integration profile [73,74]. Today, the knowledge of LV integration tropism has advanced enormously and pointed out the preferential insertion of the provirus in transcriptionally active sites of the cell genome. Indeed, for instance, in hematopoietic cells, the insertion pattern of LVs appeared to be quite different than that obtained with MLV retroviral vectors. It is not surprising that the latter one resulted in cell transformation because it integrates in and around transcriptional start sites. Nevertheless, both MLV retroviruses and lentiviruses integrate semirandomly into the genome, and thus insertional mutagenesis cannot be excluded. This justifies the numerous strategies that have been evaluated to target gene transfer in a specific “safe harbor” in the cell genome and of which some are described in the succeeding texts.

AQ14 Of importance are the major interest of SIN vector design in reducing potential risks of insertional mutagenesis and the role of insulators in case of integration in condensed chromatin regions as discussed previously. Therefore, we will focus on the other strategies that have been developed in order to decrease the risks induced by random integration of LVs.

#### 1.4.1.1 Integration-Deficient Lentiviral Vectors for Nonproliferating Cells

For nondividing target cells, integration of deficient LVs could represent an alternative approach to reduce insertional mutagenesis [75,76]. The product of reverse transcription of LV is a linear dsDNA with LTRs at both ends. Classic LVs with a functional IN usually can be integrated into the cell genome as proviruses. But the proviral linear DNA can also be circularized by nonhomologous end joining that produces 2-LTR circles and by homologous recombination between the LTR or by ligation of RT products that gives 1-LTR circles. In order to exclusively obtain these episomal forms, some mutations are performed in the IN encoding gene. Because IN has a pleiotropic function in the viral cycle, such as in virion morphogenesis, RT, nuclear translocation, and integration, only one type of mutation can specifically affect the cleavage and integration functions. These class I mutations are created by substituting one of the three amino acids of the catalytic triad (D64, D116, and E152 for HIV-1 IN), and most investigators use the well-described D64V mutant. Some groups tried to mute the attachment sites at the end of the provirus but these mutations appeared less efficient than those obtained with IN class I mutations. In order to optimize the action of these mutations, a pharmacological treatment against IN or its cofactors is possible by using raltegravir or elvitegravir, which are strand-transfer inhibitors [77].

Integrase-deficient lentiviral vectors (IDLVs) are far more developed for clinical applications such as retinal dystrophy gene therapy with interesting results obtained in rodents and dogs [78,79] or immunization against HIV and tumors. Although these IDLV vectors appear to be attractive

candidates for transgene delivery into quiescent tissues and for gene repair, their interest for long-term transgene expression must be confirmed. Moreover, the high level of nonreplicative episomal molecules in transduced cells could assure a high level of transgene expression while preventing insertional mutagenesis and RCL generation. Many investigators are currently trying to develop IDLVs with replicating episomes that may provide a stable transgenesis in proliferating tissues while maintaining their biosafety characteristics.

#### 1.4.1.2 Site-Directed Integration of IDLVs

The targeted integration of LVs can be performed by using specific nucleases and nonintegrating LVs.

##### 1.4.1.2.1 Zinc Finger Nuclease Strategy

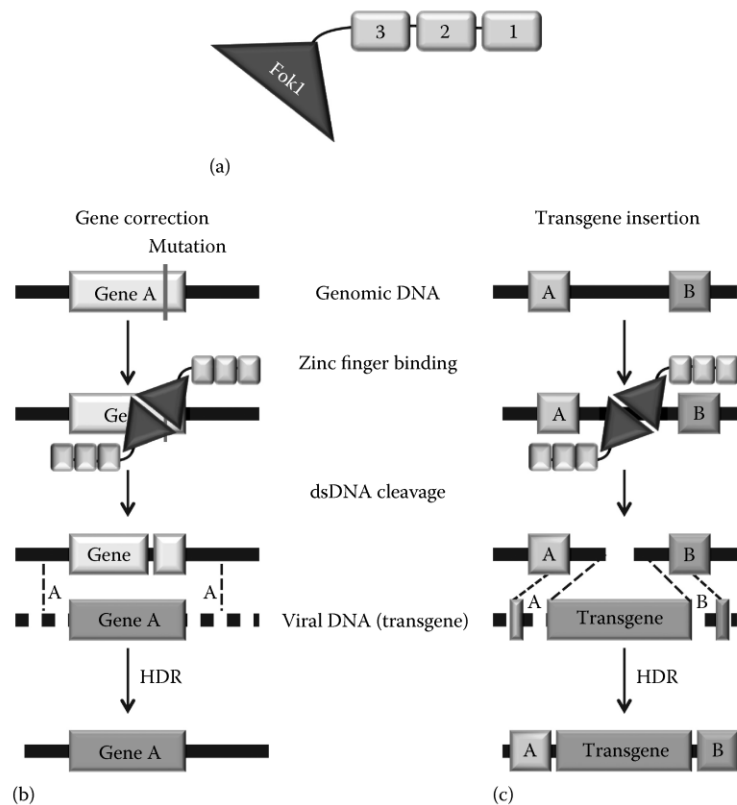
The zinc finger nucleases (ZFNs) are hybrid restriction enzymes that link the cleavage domain of FokI to a designed zinc finger protein (ZFP) [80–82]. They are used to modify the eukaryotic genome from simple sequence editions to targeted integration of entire genes. ZFNs introduce a double-strand break at the chosen site in the host genome, and if an exogenous repair template is available, it may be incorporated into the genome by homology directed repair (HDR). The targeting of the cleavage event is mediated by the ZFP domain that can be engineered to recognize a wide range of DNA sequences (Figure 1.7).

To introduce the ZFN technology into a target cell, Naldini and colleagues used IDLVs successfully [82]. The IDLV episomal accumulating proviral DNA is competent for both transient expression of the ZFNs and a transgene substrate for homologous recombination. A 10- to 100-fold increase in site-specific integration was observed when the ZFNs are co-delivered. In this study, authors managed to correct specifically the IL2RG gene mutation, involved in the X-SCID disease, at its endogenous genomic site.

The choice of the integration locus appears to be very important because it may influence the ZFN accessibility, the permissiveness to HDR, and the maintenance of transgene expression. Although these results are very promising and have provided a large step forward toward improved biosafety by reducing the risks of insertional mutagenesis, a careful safety assessment is required to validate potential off-target effects of ZFNs.

##### 1.4.1.2.2 TALE Nucleases Strategy

In parallel to ZFNs engineering, many studies are focused on artificial enzymes able to generate dsDNA breaks at pre-defined genomic sequences. Indeed, since the recent discovery of the potential of bacterial transcription activator-like type III effectors (TALEs) to recognize specific DNA sequences, many groups have developed some specific TALEs [83]. The DNA binding domain of TALEs displays a tandem array of 15.5–19.5 repeat units with 34 residues each. A 2-amino acid hypervariable polymorphism at position 12 and 13 is often the only feature difference among the repeats. The TALE nucleases (TALENs) operate similarly to ZFNs by assembly of a pair of TALENs at a given



**FIGURE 1.7** Gene editing using designed ZFNs. (a) Schematic representation of a ZFN with three specific DNA binding domains linked to the FokI nuclease. (b) Targeted endogenous gene correction or (c) transgene addition in a well-defined genomic locus, which is driven by the activation of the ZFN nucleases encoded by LVs by ZFN dimerization, cleavage, and then homologous directed repair (HDR) with the corresponding homologous sequences flanking the correcting transgene encoded by a second LV.

DNA sequence consisting of two half target sites separated by a spacer sequence. Nevertheless, the integration seems to be more independent from the chromatin context, and the simple DNA binding code used suggests that TALENs could bind a wider range of DNA sequence with a more predictable manner than obtained with ZFNs that might reduce the off-target effects.

HIV-1-based LVs or IDLVs bearing TALEN sequences could serve as TALEN delivery platforms and could be safer than the ZFNs sequences. However, extensive comparisons between the two systems are required to make definite conclusions.

Interestingly, another genomic targeting system made its appearance based on the bacterial CRISPR/CAS system, which is popular because of its easy design.

## 1.4.2 REGULATABLE LENTIVIRAL VECTORS

### 1.4.2.1 Self-Deleting Vectors

For a toxic gene of interest, a transient expression of the transgene might be necessary. For instance, it had been shown that LVs that deliver the Cre recombinase induced a reduction in cell proliferation due to an accumulation in the G2/M cell cycle phase of the transduced cells. Indeed, some “pseudo”

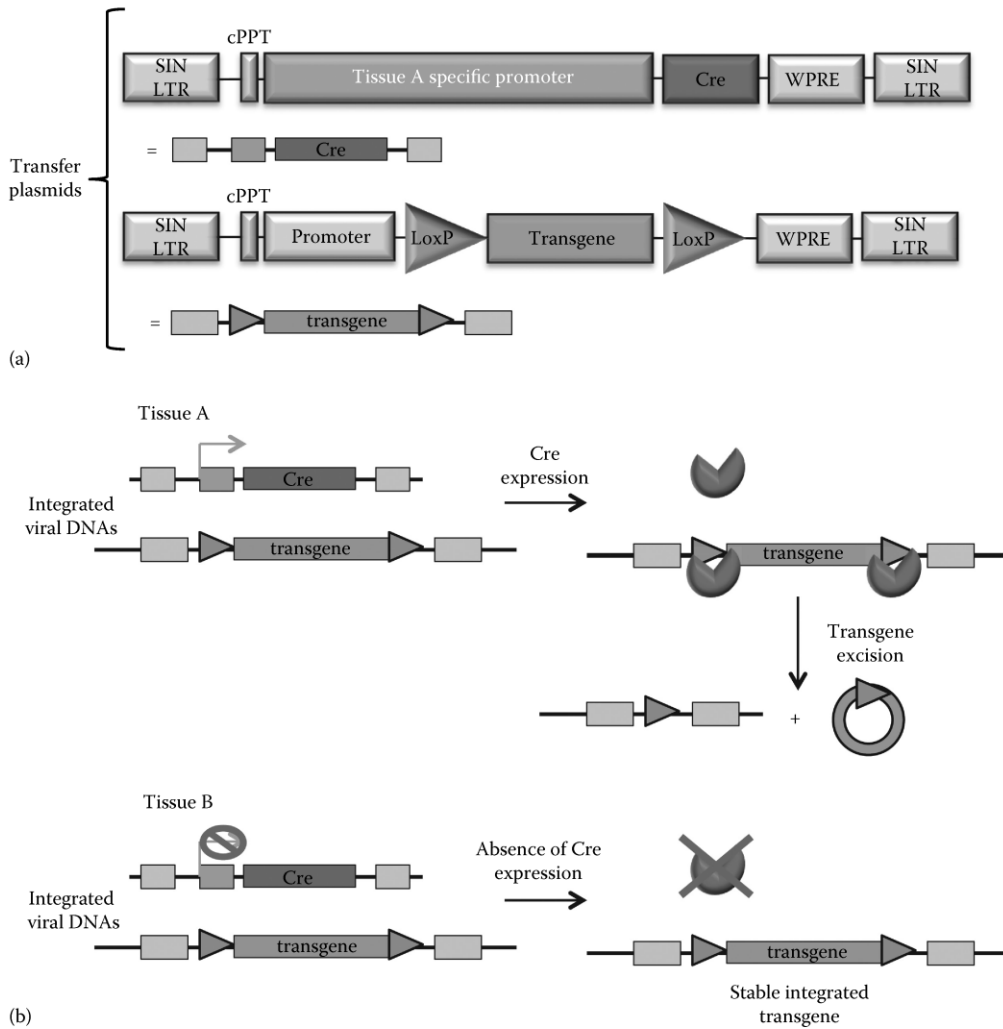
lox sites are present in the human and mouse genomes and were shown to be Cre-mediated recombination substrates. In order to limit this side effect, Verma and colleagues [84] generated LVs that carry the Cre recombinase, which is itself subject to the Cre-mediated excision. As a consequence, the Cre expression remains transient and is restricted only during the time needed for recombination. They designed an LV that carries the Cre cDNA and 2 loxP sites flanking the transgene. After integration and Cre expression, Cre-mediated recombination of the two loxP sites leads to the deletion of the integrated transgene sequence except for the flanking LTR sequences and one loxP site (Figure 1.8). These investigators showed an efficient recombination of loxP sites in neurons, hepatocytes, and hematopoietic progenitors avoiding the Cre cytotoxicity by this self-deleting mechanism.

This concept of self-excising LVs can be used for the short-term expression of toxic genes by designing a vector containing both the toxic gene of interest and Cre [85], for instance, in a bicistronic Cre self-deleting vector [85].

### 1.4.2.2 Inducible Vector Systems

For numerous applications, a controlled transgene expression is preferred or needed. The reverse tetracycline-controlled transactivator (rtTA)-regulated system has been widely used

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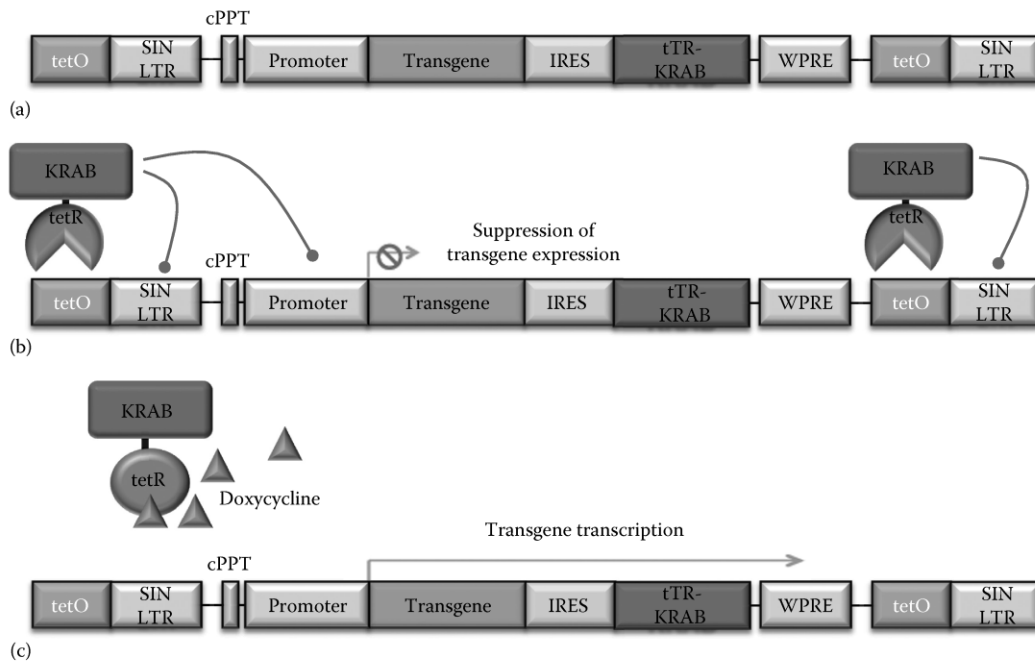


**FIGURE 1.8** Self-deleting LVs using a tissue-specific Cre/loxP excision system. (a) Schematic representation of two possible transfer plasmids. The Cre recombinase expression depends from a tissue-specific promoter A and the transgene sequence is flanked at both ends with LoxP sequences. (b) After integration of both proviral sequences, the Cre recombinase expression induces the transgene excision in the tissue A in which the promoter A is active. The absence of Cre expression in the nontarget tissue B prevents transgene excision and thus allows stable expression of the transgene.

for inducible gene expression. This system is based on an F protein between the bacterial Tet repressor (TetR) with the activating domain of herpes simplex virus VP16 resulting in a chimeric transcription factor, the transactivator, tTA. This tTA can interact with a TetO-binding site containing promoter for tetracycline-induced gene silencing (Tet-Off system) or activation (Tet-On system).

LVs have been converted in inducible LVs by inserting an inducible expression cassette between the first promoter (e.g., the constitutively active CMV promoter) and the gene of interest. The inducible cassette contains the tTA at its 5' end and an inducible promoter containing a minimal promoter and seven copies of the Tet operon (TetO) at its 3' end. The tTA gene expression is driven by the first promoter (e.g., CMV promoter). After the transduction, the constitutively expressed tTA binds to the inducible promoter and induces a

high level of transcription of the gene of interest. In contrast, the addition of tetracycline or its more stable variant doxycycline induces conformational changes that prevents tTA binding to the tetO and represses the transgene expression. Many improvements in inducible LV design discussed previously were introduced, notably the use of SIN LVs, specific promoters, or insulators in order to reduce the background expression of the transgene because some leakage of expression is detected in the repressed state. A binary vector system, consisting of a gene transfer vector encoding the tTA and an additional vector encoding for a tetracycline responsive element (TRE) and the transgene, allowed tight regulation of transgene expression. However, for gene therapy purposes, a single vector approach is much better adapted; therefore, a self-regulating single transfer vector has been developed with the insertion of TRE in both LTRs [3].



**FIGURE 1.9** Example of a doxycycline-regulatable LV system using the tTR-KRAB F protein. (a) Schematic representation of the Tet-On vector design. The KRAB-based repressor, the tetO sequences, the SIN LTR, the IRES, and WPRE elements are represented. (B+C) Mode of action of the Tet-On vector. (b) In the absence of doxycycline/tetracycline, the tTR-KRAB F protein binds to tetO and inhibits high transgene expression. (c) In the presence of doxycycline, tTR-KRAB does not bind to tetO allowing transgene expression to occur.

In order to reduce the background expression of the transgene, Szulc et al. [86] have designed an F protein between the tetR and KRAB known to lead to heterochromatin formation. The chimeric protein tetR-KRAB epigenetically silences 2–3 kb of surrounding sequence when it is recruited by a tetO site. This configuration prevents most of the background expression observed in the initial inducible vector design [87] (Figure 1.9).

In addition, the importance of inducible lentivectors for the field led to further improvements of LV design [88]. All of the improvements in LV design are summarized in Figure 1.10.

## 1.5 PRACTICAL ASPECTS OF LENTIVIRAL VECTOR PRODUCTION

Because the theoretical aspects of lentivectorology are detailed earlier, the following section is dedicated to reviewing methods available for producing and characterizing LVs. Figure 1.12 shows the entire LV production process.

### 1.5.1 PRODUCTION OF LENTIVIRAL VECTORS

#### 1.5.1.1 Transient Vector Production

Transient production of LVs is the most commonly used method because it allows production of LVs quickly with suitable titers for numerous laboratory applications. The human embryonic kidney (HEK) 293T cells or its derived

clones are the preferred producing cells. Separate plasmids classically encoding for the packaging sequence, the transfer vector, and the envelope (Figure 1.11) are introduced in the producer 293T cells by different transfection methods. Because of the cytotoxicity of the transfection process during LV production, it became clear that the less the plasmid is transfected, the more the production is efficient. Moreover, a silencing of certain tissue-specific promoters can be observed, and in this case, the choice of the cell line used for titer determination is very important. Once plasmids are in the producer cell, vector RNA and proteins can be produced and assembled followed by budding of the viral particles from the cell (Figure 1.11). This production process is called transient because of the restricted time (2–3 days) of vector production (Figure 1.12). Indeed, after this time point, producer cells suffer from the toxicity of certain viral proteins (e.g., gagpol, VSV-G) and cannot produce LVs efficiently [1]. The transient transfection method is still the most utilized method for vector production for lab use and even for clinical trials.

#### 1.5.1.2 Production by Stable Packaging Cell Lines

To better guarantee safe LV production, needed for clinical applications, stable vector producing cell lines (VCLs) have been developed. They are engineered to stably express the different vector components (packaging, transfer, and envelope sequences) by using transfection or transduction methods to introduce them into the producer cell. In addition to

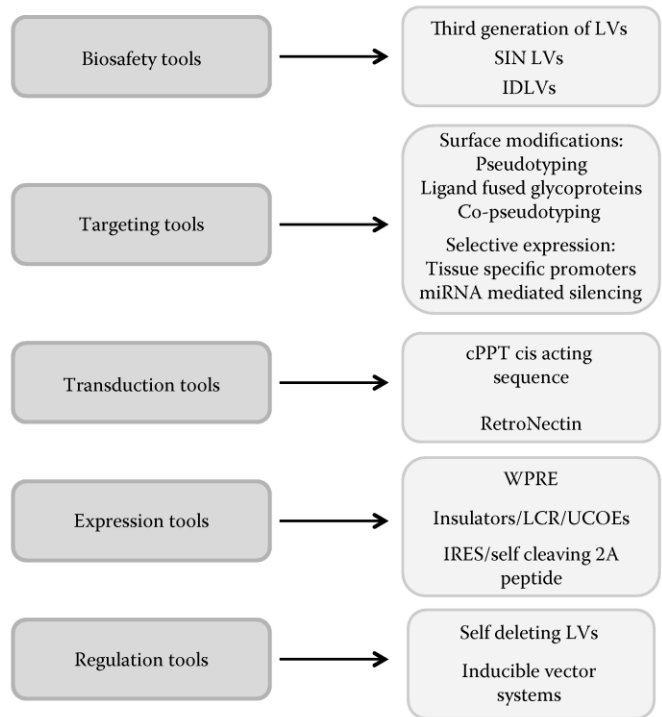


FIGURE 1.10 Several improvement steps in LV design.

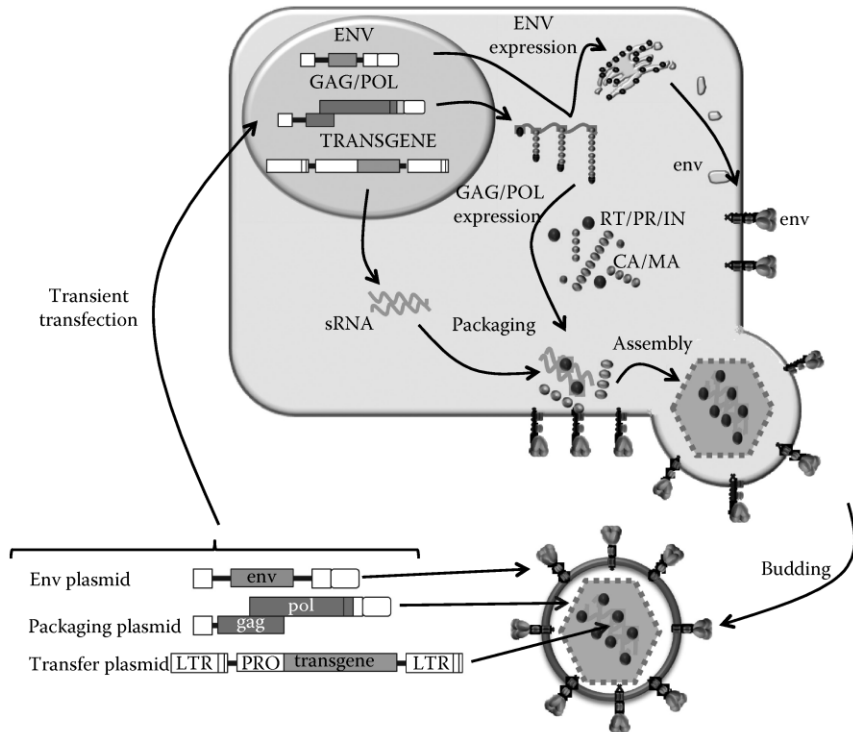
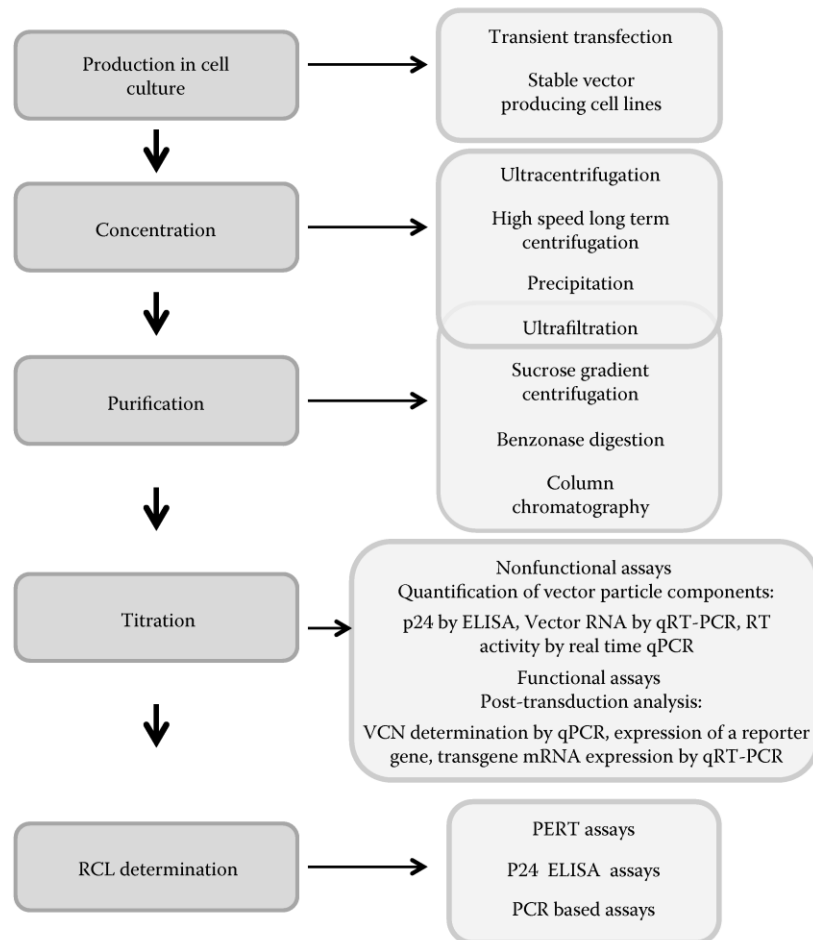


FIGURE 1.11 Transient production of LVs. For transient transfection, plasmids encoding for the packaging sequence, the transfer vector, and the envelope glycoprotein (+/- the rev protein depending on the LV generation) are transfected into 293T cells using a transfection reagent. These 293T cells efficiently produce vector particles at 36 until 72 h after the transfection. After 72 h, a severe drop in infectious titer is detected.





**FIGURE 1.12** LV production process for clinical applications.

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being a reproducible source of LVs, stable production prevents plasmid DNA contaminations and homologous recombination of transient transfected plasmids, which reduces the risk of RCL formation. Because of these advantages of stable LV production, many groups have invested in the production of stable VCLs but with moderate success. Although this stable production is essential for clinical use, the cytotoxicity of numerous viral proteins and the fusogenicity of viral envelopes such as the VSV-G glycoprotein prevented the development of these stable VCLs or induced a strong titer reduction, too low for clinical use. Moreover, production of a stable cell line is cumbersome because a new cell line for each therapeutic vector must be established, and before moving the vector into the clinic, an extensive evaluation to prove absence of RCLs is required. The STAR cell line has been created by the Collins' group [89,90] by transduction of the necessary sequences, and this cell line is able to produce high titer of retrovirus glycoprotein pseudotyped LVs for more than 3 months. Although this advance does not solve the VSV-G stable production concerns, it holds great promise for LV stable production notably for RD114 envelope pseudotyped LVs [91].

### 1.5.2 CONCENTRATION AND PURIFICATION OF LENTIVIRAL VECTORS

After production, there are numerous protocols that allow concentration and purification of LVs. The producer cell supernatant is collected and filtrated to remove cellular debris. Subsequently, the vector containing supernatants are concentrated.

The most commonly used method is the ultracentrifugation of the viral supernatant at  $1-3 \times 10^5g$  for 1–4 h at  $4^\circ C$ . Then, the viral pellet is resuspended in an isotonic buffer over several hours. Another step of ultracentrifugation can be applied through a sucrose cushion to remove soluble protein contamination. A 10- to 100-fold titer increase is noted after this concentration and purification step. The purity of these preparations is sufficient for most uses in the laboratory.

Nevertheless, several LV pseudotypes do not tolerate these high ultracentrifugation forces. In addition, the pelleting of cellular vesicles and debris is a side effect of this method and is a concern in terms of immune response induction. As a consequence, other methods have been developed such as high-speed long-term centrifugation at  $10^4g$  for 8–12 h at



4°C, precipitation with calcium phosphate, or ultrafiltration by using a 100 kDa molecular mass cutoff filter [1].

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For the production of LVs used in the clinic, the purification process is well established according to the rules of good manufacturing practice (GMP) [92,93]. Directly following the supernatant harvest, the supernatant is first clarified by filtration. The benzonase nuclease is added in the presence of Mg for 6 h at 37°C in order to degrade most of residual nucleic acids. The clarified and benzonase-treated supernatant is ultrafiltered and diafiltered (by a diafiltering [DF] buffer filtration) and centrifugated for 16–20 h at 6000g. Then, the resulting viral pellet is reconstituted in DF buffer and centrifuged for 1–2 min for removal insoluble material. The majority of the LV production is aliquoted and stored at –80°C; however, some samples are tested for sterility and titration and undergo quality–safety control. The overall yield of this type of manufacturing production is approximately 30%.

### 1.5.3 TITRATION OF LENTIVIRAL VECTORS

It is essential to determine the infectious titer of a vector preparation. Many methods have been developed, but unfortunately, a standard protocol in titration does not exist [94]. We provide an overview of possible methods and their limitations below.

#### 1.5.3.1 Titration by Measuring the Quantity of Vector Particle Components

This type of titration is based on a nonfunctional assay by quantifying the content of a vector preparation by measuring the lentiviral CA antigen (p24 for HIV-1 LVs) by ELISA or the vector genomic RNA content by real-time reverse-transcription PCR (qRT-PCR). Finally, some users measure the RT activity using real-time PCR (qPCR) quantification. The major concern with these methods is that they detect both infectious and noninfectious vector particles and largely overestimate the LV titer. Moreover, the titer obtained with these methods does not take into account the nature of the vector construct with the addition of *cis*-acting sequences or the use of a tissue-specific promoter, a certain envelope gp, and the targeted cell type, which all can influence the titer.

#### 1.5.3.2 Titration by Functional Assays

Different methods for functional titration are available. The first assay involves the isolation of genomic DNA from the target cells after their transduction. Then the total number of integrated vector copy numbers (VCN) per cell is determined by qPCR, which is normalized to the number of target cells (determined by an endogenous cellular gene used as a reference). However, the VCN does not provide information for the transgene expression in the target cell, which might vary according to the genomic integration sites of the provirus.

When a reporter gene is included in the transfer vector (GFP, antibiotic resistance, surface display of ligands at the target cell surface), the direct measurement of transgene expression by transduced cells appears to be the more

suitable method to determine the “true” infectious LV titer. In absence of reporter elements to measure the transgene expression, qRT-PCR on transgene mRNA in transduced cells is reported to correlate to titers obtained using reported genes in most cases. Of note, if a tissue- or cell-specific internal promoter is used, the cells used for titering should support expression driven by this specific promoter.

### 1.5.4 LV BIOSAFETY DETERMINATION

In pursuit of biosafety, the detection of RCLs is a necessary step in the testing of LV preparations, and many assays for RCL detection have been developed.

RCL detection is performed on the supernatant of LV-infected target cells that have been cultured for 2 weeks to avoid contamination of the original LV used for the infection. The product-enhanced reverse transcription assay (PERT), a PCR-based method, is known for its high sensitivity. It consists of using an RNA template as a substrate for RT activity. If a reverse transcript is identified by qPCR, it means that RT is present in a sample contaminated by RCL. Every RCL that presents a functional RT can be detected by this method [95,96]. Another frequently used method consists in detecting and measuring CA amounts by detection of p24 using ELISA. Absence of RCLs is shown when serial passaging of supernatant from infected cells is devoid of p24 [97]. However, these methods are based on the principle that RCLs have a usual viral form. As a consequence, there is no current standard for RCL determination and the methods are evaluated case by case in the context of clinical trials. However, of note, if a third or fourth LV vector generation was used, RCLs were seldom or not detected using the available methods.

## 1.6 LENTIVIRAL VECTOR APPLICATIONS IN BASIC RESEARCH AND GENE THERAPY

LVs are able to transduce a wide range of host cells including both dividing and nondividing cells. Their success in preclinical and clinical studies makes them popular tools for gene transfer. Several possible applications for LVs in fundamental research and in the clinic are reported in the following texts; however, of course, this list is not exhaustive.

### 1.6.1 LENTIVIRAL VECTOR APPLICATIONS IN FUNDAMENTAL RESEARCH

#### 1.6.1.1 Gene Silencing Vectors

RNA interference (RNAi)-mediated gene silencing is an interesting technology to study gene functions or to restrict overexpressed or aberrantly expressed genes in specific cell types in some diseases. In mammalian systems, small interfering RNAs (siRNAs) can be expressed endogenously from pol III promoters as small hairpin RNAs (shRNAs) or from pol II promoters as a part of a microRNA molecule [98,99].

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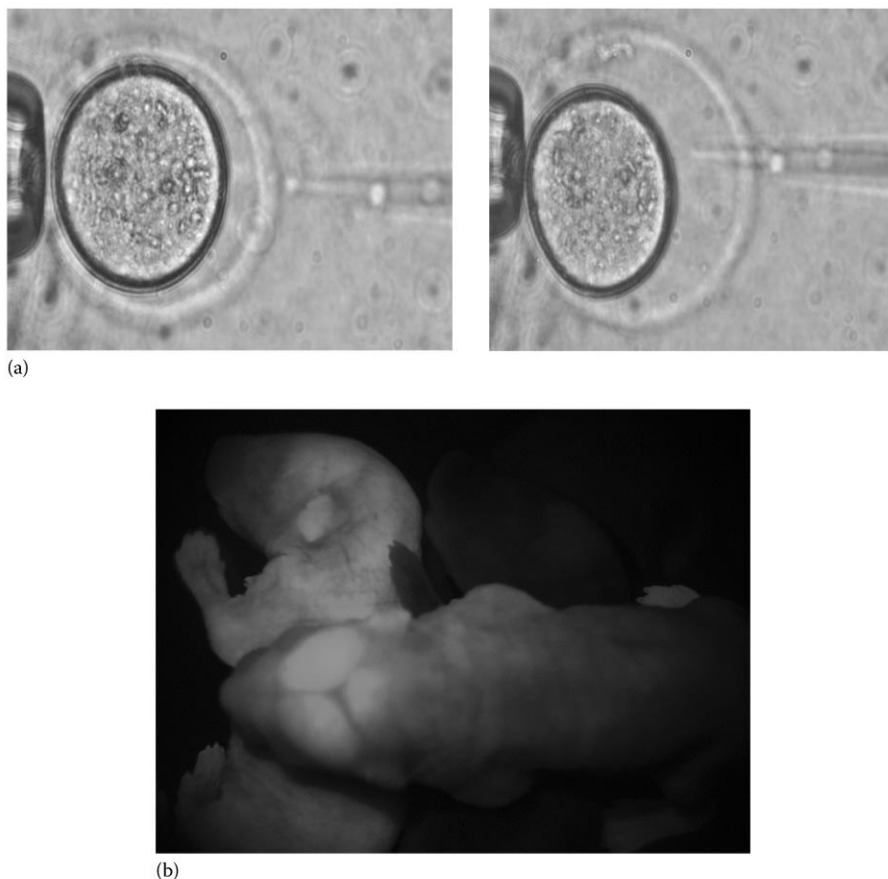
Ideally, a gene silencing lentivector would contain both a reporter gene and the shRNA silencing cassette driven by a pol III promoter (e.g., H1, U6). The interfering RNA target sequence should be ideally 19–23 bases long and several different shRNAs must be evaluated in order to identify the most effective one (Figure 1.6b). Indeed, if the objective is to knockdown a certain gene, only the sequences showing the highest efficiency should be selected. Since overexpression of shRNAs can result in off-target effects, a control shRNA in the context of the same LV backbone and reporter gene should be included. For instance, scrambled sequences, nontarget sequences, or mutated sequences are used as a shRNA control.

Because LVs have a large packaging capacity, they can harbor the shRNA combined with a regulated expression system. For instance, a Tet-regulatable shRNA cassette in addition to the TtA sequence was included in the same LV and proved functional [100]. This gene silencing LV technology can lead to better understanding of cellular biology (e.g., in cancer cells) as it provides a clear knockdown phenotype for cells and transgenic animals.

### 1.6.1.2 Lentiviral Vector-Mediated Transgenesis and In Vivo Cell Lineage Tracking

Many investigators have shown that LVs can efficiently transduce embryonic stem (ES) cells. Verma and colleagues [84] already showed that in contrast to MLV retroviral vectors, for which expression is silenced in ES cells, LV expression is maintained in human and murine ES cells. Moreover, embryos at the morula stage can be transduced and the resulting progeny expresses the transgene. Likewise, transgenes introduced by LVs in ES cells are transmitted to the germ line. Therefore, authors concluded that LVs are very attractive tools for generating transgenic animals. Many transgenic animals have been generated using LVs including mice [101], rats [102], cats [103], rabbits [104], pigs [105], and even chickens [106] (Figure 1.13). These transgenic animals can either harbor a specific tissue reporter gene such as GFP or can present a specific knockdown in order to study gene function or to produce a disease preclinical model [107].

Moreover, LV-mediated stable gene transduction can be exploited in vivo in order to follow targeted cells. For instance,



**FIGURE 1.13** (See color insert.) Generation of transgenic mice by LV injection into fertilized oocytes. (a) LV perivitelline injection of fertilized oocytes. (b) The injection of the fertilized oocytes with LVs encoding for GFP under an ubiquitous promoter resulted after transfer into a recipient female in newborn mice expression GFP in all tissues (upper left); wild-type mice are shown for comparison (upper right).

Gallo et al. [108] developed an LV for tracking cardiomyocyte differentiation from human ES cells in order to better understand cardiac muscle differentiation.

### 1.6.1.3 Cellular Reprogramming

The induced pluripotent stem (iPS) cell generation relies on the introduction of defined pluripotency-associated factors such as OCT4, SOX2, KLF4, and cMyc in somatic cells [109]. LVs have been used in order to introduce these multiple factors in fibroblasts, keratinocytes, and HSCs in order to reprogram them [1]. After reprogramming, the MLV retroviral vector encoding the factors mentioned earlier was spontaneously silenced. Nevertheless, the risks of reactivation of the pluripotency-associated factors once integrated into the genome of these iPS cells led laboratories to develop other technologies such as protein transfer or privileged IDLVs for gene transfer of these factors [110].

### 1.6.1.4 Lentiviral Vector-Mediated Immune Modulation

LVs can efficiently transduce APCs including macrophages or DCs. These properties can be used for vaccines against infectious diseases and tumors.

The LV-mediated expression of specific antigens in DCs induce both CD4+ and CD8+ T cell responses leading to cellular immunity and humoral response [111,112].

In order to efficiently present transduced antigens on DCs and generate an antigen-specific immune response, LVs must transduce but also stimulate DCs. Both cell entry and reverse transcription were shown to affect the activation of DCs in vitro and in vivo. Contrary to adenoviruses, LVs are able to transduce DCs in a persistent, nontoxic, and nonimmunogenic manner. Positive results have been obtained in LV-transduced DCs for use as immunity boosting vaccines, and numerous researchers are focused on the development of an HIV-1 vaccine using LV immunization. Unfortunately, until now, the extreme ability of HIV-1 to escape the immune system prevented the production of an efficient vaccine [113]. Of interest, DCs transduced by LVs expressing tumor-associated antigens such as melanoma antigens or ovalbumin are able to activate cytotoxic T lymphocyte responses and provide protection against tumor challenges. This strategy can efficiently lead to tumor regression [114].

IDLVs are also attractive candidates for immunization because of their ability to maintain durable gene expression in nonproliferating or slowly proliferative cells such as certain DC subtypes [115].

An important anticancer strategy based on the transfer of tumor-specific TCR genes into patient T cells has been proven successful in the clinic. More recently, a radically different strategy consisting of introducing a coding sequence for a chimeric antigen receptor (CAR) conferred the desired specificity for a cancer antigen to T cells. Indeed, ongoing clinical trials have described durable rejection of previously refractory B cell malignancy in patients after CD19-directed CAR gene therapy of autologous T cells [116].

## 1.6.2 LENTIVIRAL VECTORS IN GENE THERAPY: STATE OF THE ART

For the moment, ex vivo gene therapy strategies using the correction of autologous target cells in vitro followed by reinfusion into the patient is favored [117]. Although numerous gene therapy trials have been performed using MLV retroviral vectors, the first LV-based clinical trial was initiated only 10 years ago. Indeed, Levine et al. [118] performed a phase I clinical trial for HIV therapy using a conditional replicating VSV-G pseudotyped LV coding for an antisense sequence complementary to the HIV envelope. Five subjects with chronic HIV-1 infection were enrolled in the trial and autologous CD4+ T cells were transduced with the anti-HIV therapeutic vector and reinfused back into the patients. An increase of CD4+ T cells, normally depleted in AIDS patients, and a decrease of viral load were observed, and until now, insertional mutagenesis was not detected. Further follow-up is required to generate conclusions concerning the safety and the efficacy of this therapy; however, these initial results were promising for further clinical applications of LVs.

Although the MLV-based retroviral vectors were successful for HSC-based gene therapy, the “safer” LVs are gaining interest in the gene therapy field (Table 1.3). For instance, Cartier et al. [119–121] obtained promising results for treating X-linked adrenoleukodystrophy (ALD) by ex vivo correction of autologous HSCs employing LVs encoding the wild-type *ABCD1* gene. This disease is indeed characterized

**TABLE 1.3**  
**Gene Therapy Clinical Trials Using LVs**

Disease	Gene to Correct	LV	Number of Treated Patients	Benefits for the Patient	References	AQ21
X-linked ALD	<i>ABCD1</i>	Third-generation LV	2	Cerebral demyelination ceased	[119–121]	
$\beta$ -Thalassemia	$\beta$ -globin		1	Transfusion independence—hemoglobin level stabilized (8.5–9 g/dL)	[122,123,142]	
Metachromatic leukodystrophy	<i>ARSA</i>	VSV-G	3	Neurodegeneration ceased	[124]	
WAS	<i>WASP</i>	HIV-1 pseudotype	3	Protection from bleeding and severe infections—eczema resolution	[125]	

by a deficiency in the ALD protein, an adenosine triphosphate binding cassette transporter encoded by the *ABCD1* gene. This defect in ALD protein causes a severe brain demyelination in boys, and the only currently available treatment to halt the neurodegeneration is an allogeneic HSC transplantation. In this trial, 14–16 months after infusion of autologous corrected cells into the patients, the progressive cerebral demyelination ceased as compared to patient that did not undergo transplant (Table 1.3).

The phase I/II clinical trial of  $\beta$ -globin gene therapy for  $\beta$ -thalassemia major that was initiated in 2007 also showed very promising results [122,123].  $\beta$ -Thalassemia major is characterized by absence or strong decrease in  $\beta$ -globin chains of hemoglobin, which triggers the precipitation of unpaired  $\alpha$ -globin chains in erythroid precursor cells. This phenomenon leads to a perturbed erythropoiesis, and thus a decreased production of red blood cells and a decrease of the hemoglobin levels. In this trial, a SIN-LV containing large elements of the  $\beta$ -globin LCR and chromatin insulators in the LTRs was designed and used to transduce autologous HSCs of one patient. Three years after starting this treatment,  $\beta$ -globin-corrected cells are still detected, and the patient is transfusion independent and his or her blood hemoglobin level stabilized. However, of note, a dominant integration clone appeared but this did not lead to leukemia to date.

Very recently, Biffi et al. [124] and Aiuti et al. [124,125] provide very encouraging results on the HSC-based gene therapy for the correction of metachromatic leukodystrophy or WAS. Metachromatic leukodystrophy is an autosomal recessive lysosomal storage disease caused by mutations of the *ARSA* gene encoding for the arylsulfatase enzyme. This enzyme deficiency leads to an accumulation of the enzyme substrate in oligodendrocytes, microglia, Schwann cells, and macrophages and in certain neurons of the central nervous system. This results in a widespread demyelination and neurodegeneration. Today, no efficient treatment for this disease exists.

The WAS is caused by a mutation in the *WAS* gene encoding for the WASP protein, which regulates the cytoskeletal architecture. A deficit in WASP induces an X-linked primary immunodeficiency characterized by infections, thrombocytopenia, eczema, autoimmunity, and lymphoma. Currently, the only treatment for patients is allogeneic HSC transplantation. In 2010, clinical trials were initiated using LVs encoding *ARSA* (6 patients) and *WASP* (10 patients). In both clinical trials, no clonal dominance or preferential integration pattern was observed. Although a long-term follow-up of these patients is required, the initial results in three patients for each of these two trials are very encouraging with regard to efficacy.

Because LVs integrate into the host cell genome, they can still perturb endogenous gene expression. Therefore, a careful biosafety assessment must be performed. However, their ability to stably introduce a therapeutic gene in multiple different target cells and their favorable integration pattern predict that they might become powerful gene therapy tools

in the near future. In parallel, safer LV vectors are being designed that will extend their applications to other genetic diseases and give hope to many patients suffering from these incurable diseases.

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3) UNITED STATES PATENT APPLICATION PUBLICATION:  
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The present invention concerns a pseudotyped viral vector particle for transferring biological material into cells, wherein said vector particle comprises at least:—a chimeric envelope glycoprotein which comprises or consists in a fusion of the transmembrane and extracellular domain of a baboon endogenous retrovirus (BaEV) envelope glycoprotein and the cytoplasmic tail domain of a murine leukemia virus (MLV) envelope glycoprotein; or—a modified BaEV envelope glycoprotein wherein the cytoplasmic tail domain is devoid of the fusion inhibitory R peptide.

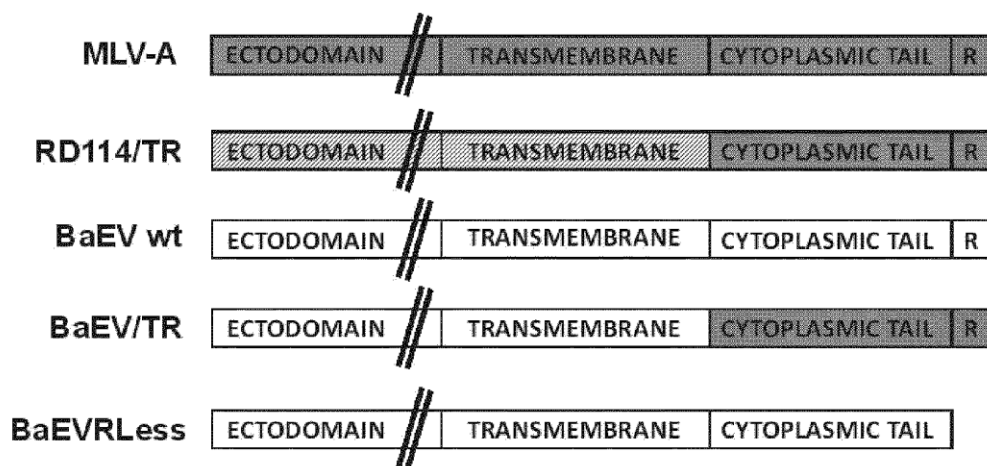


FIG.1

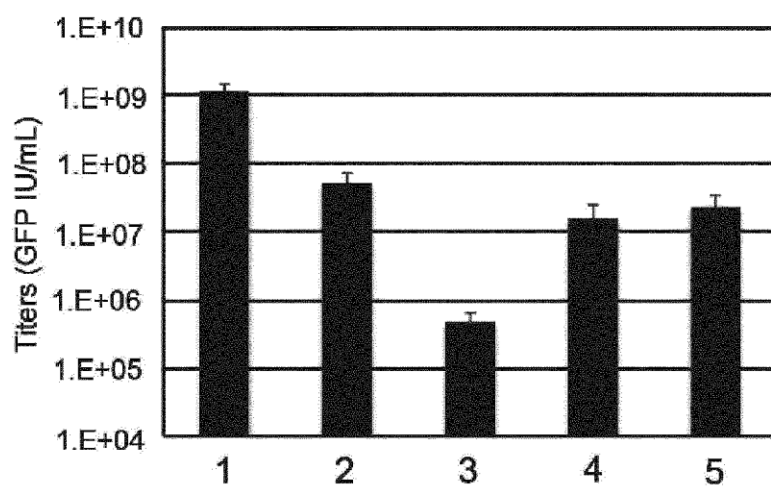
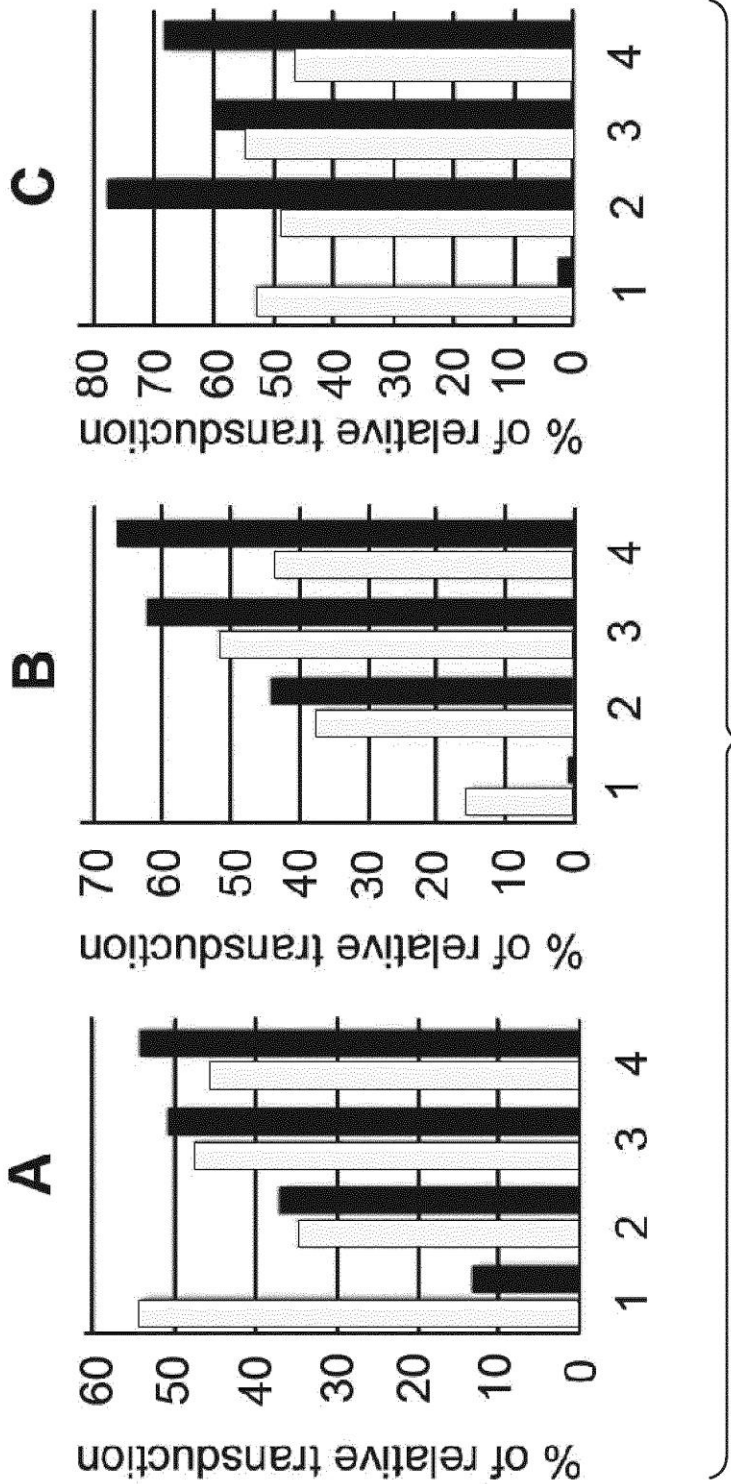


FIG.2



**FIG.3**

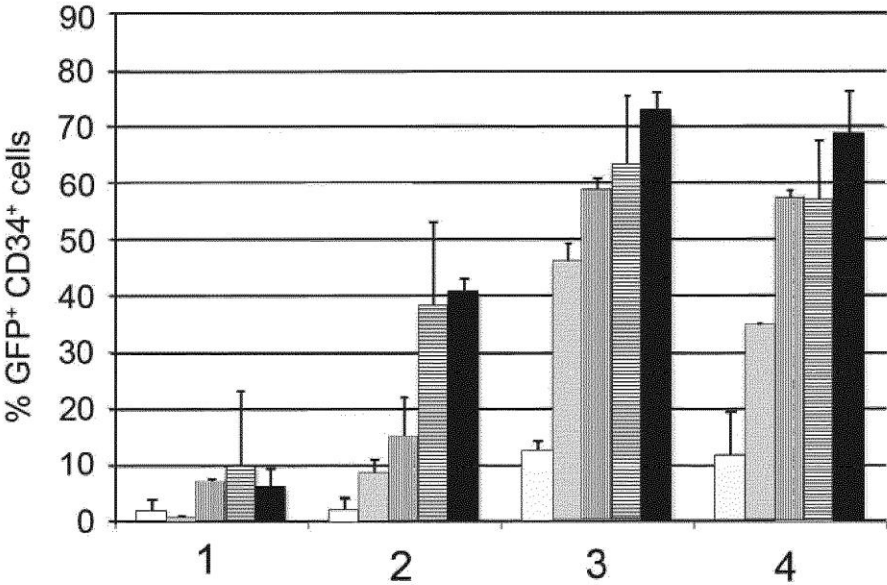


FIG.4

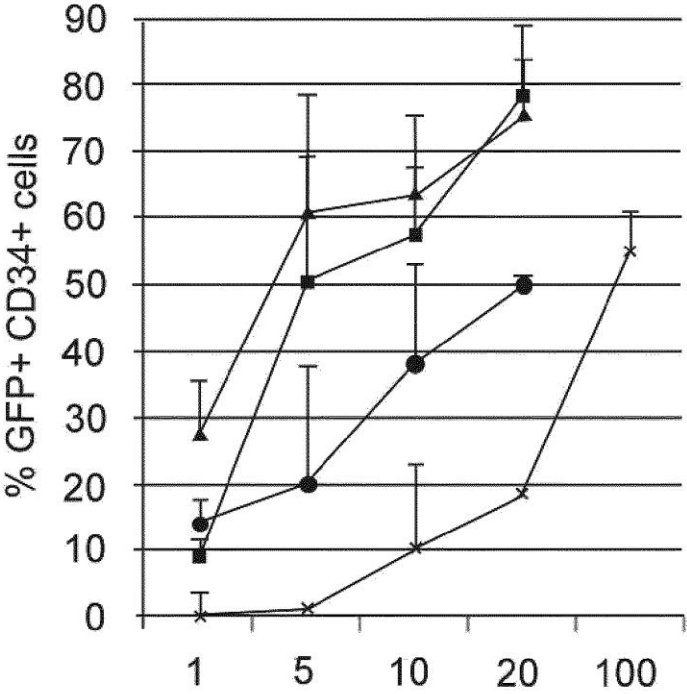
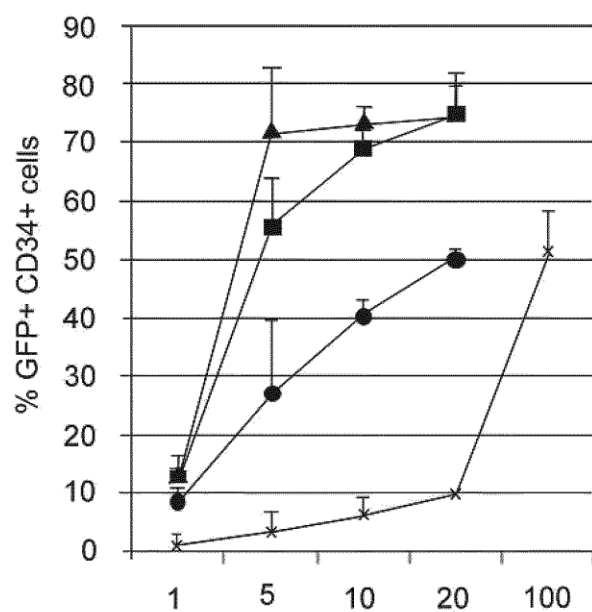
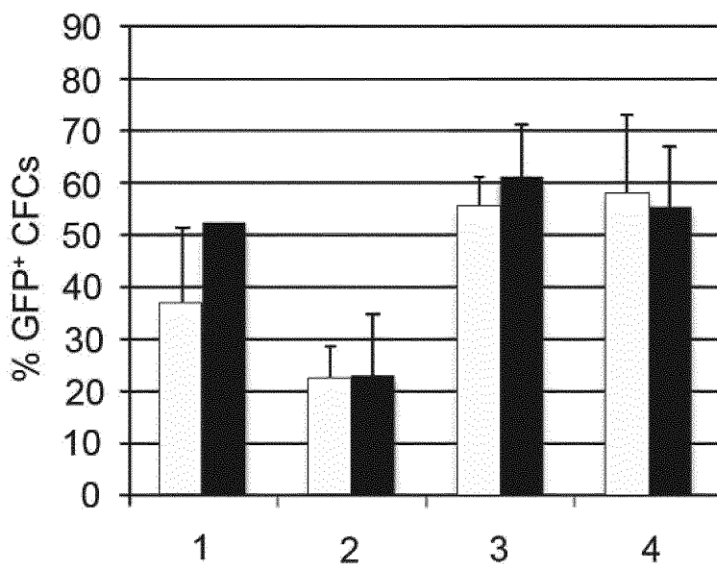


FIG.5

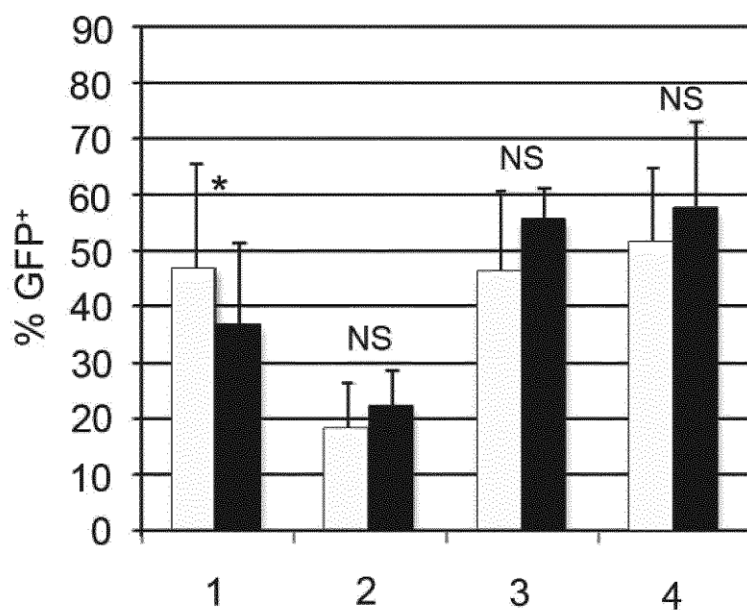


**FIG.6**

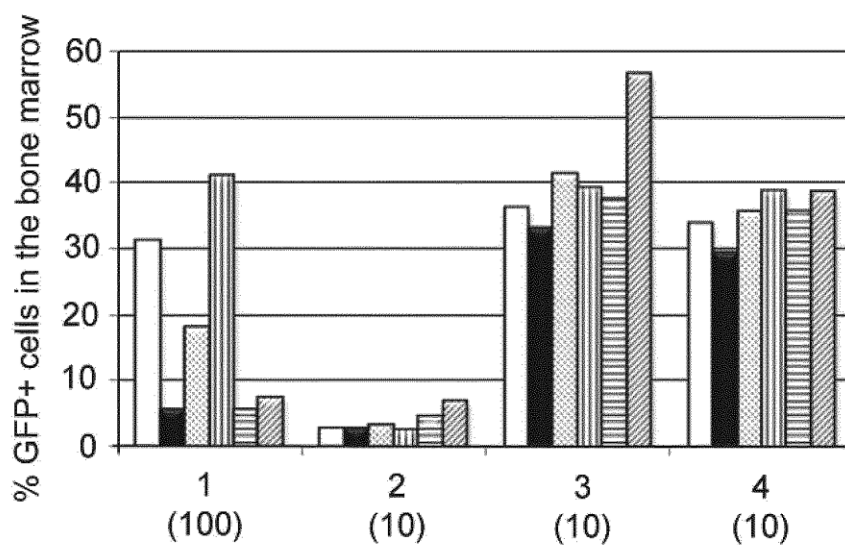


**FIG.7**

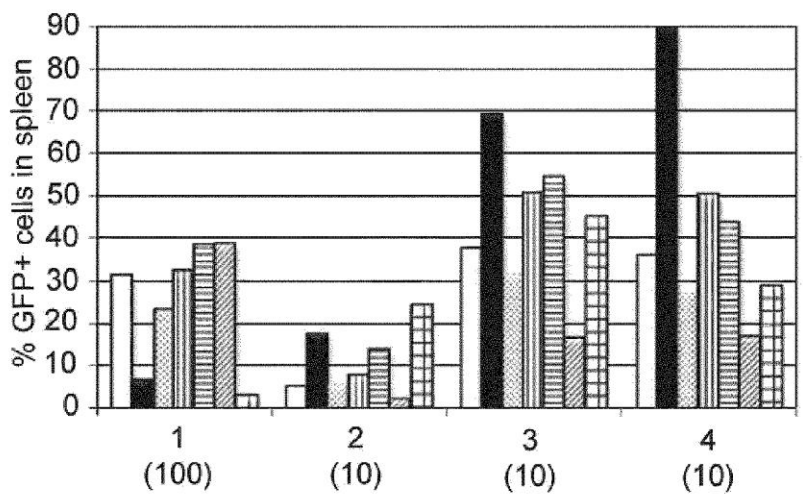




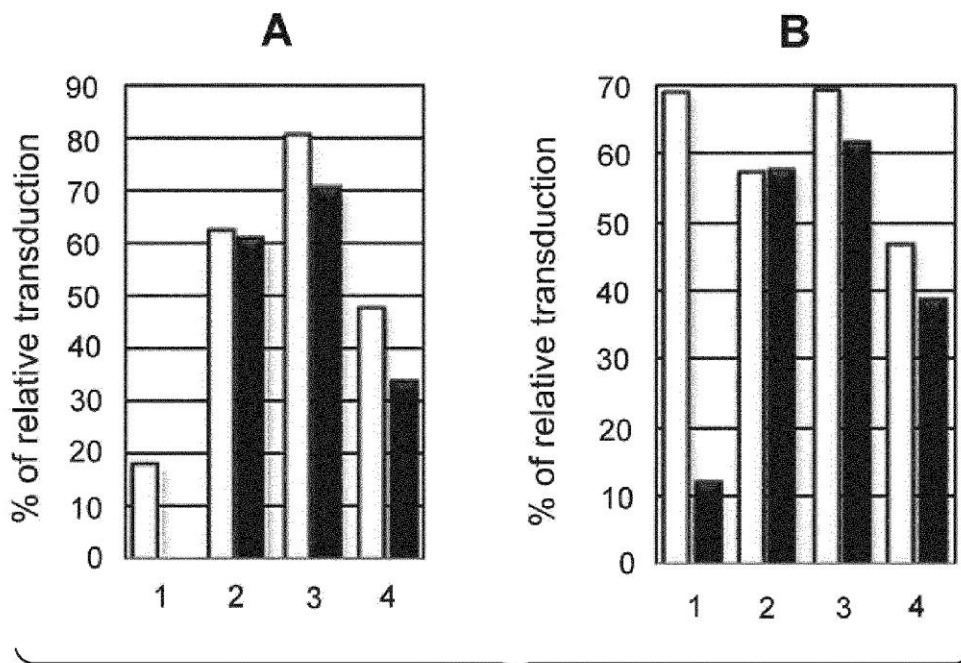
**FIG.8**



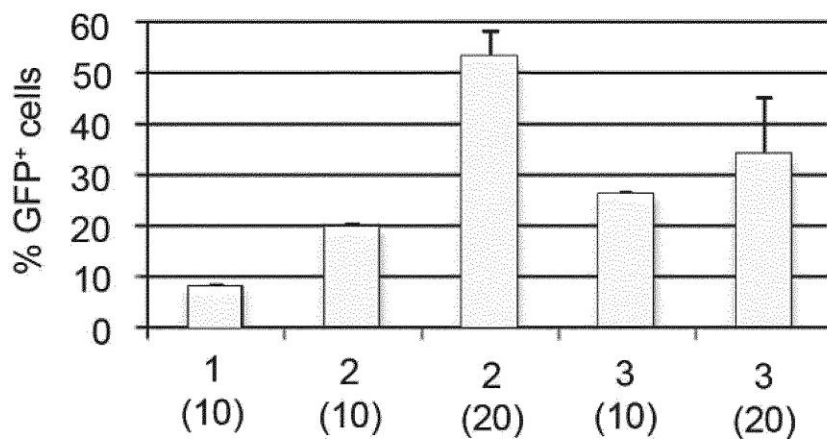
**FIG.9**



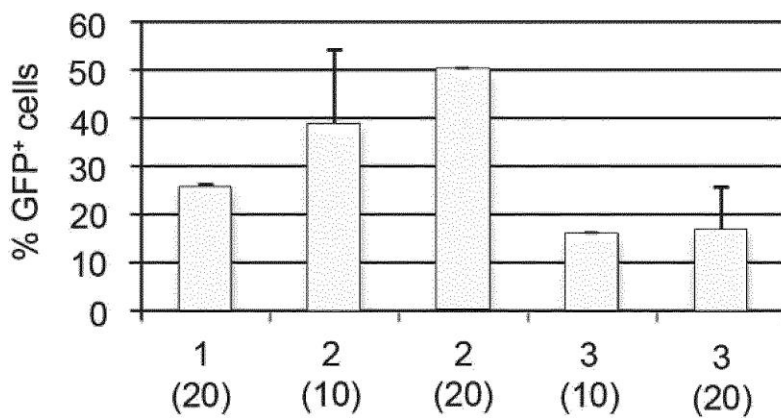
**FIG.10**



**FIG.11**



**FIG.12**



**FIG.13**

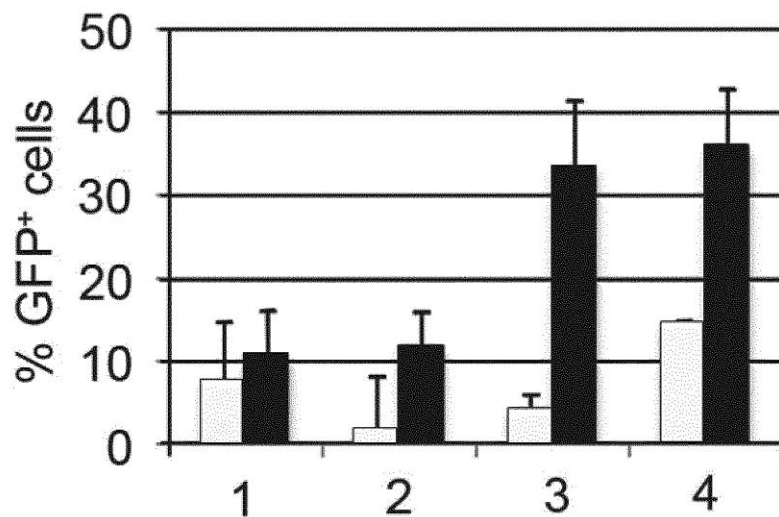


FIG.14

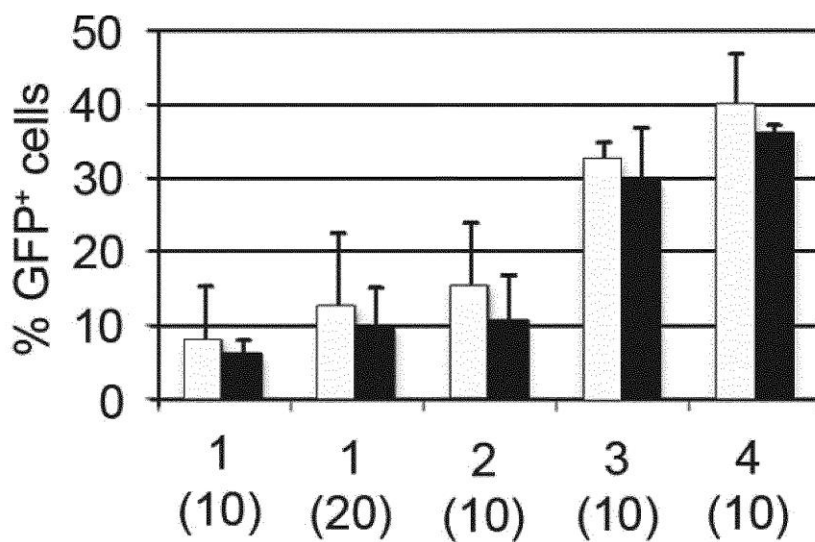
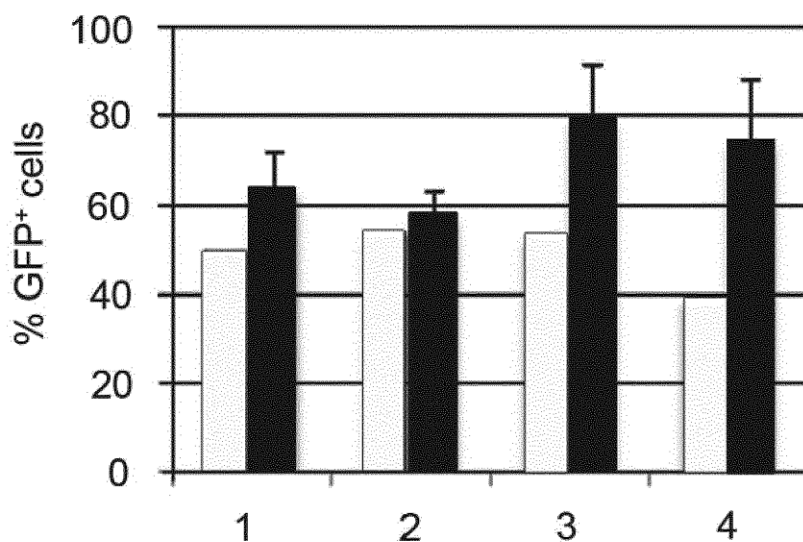
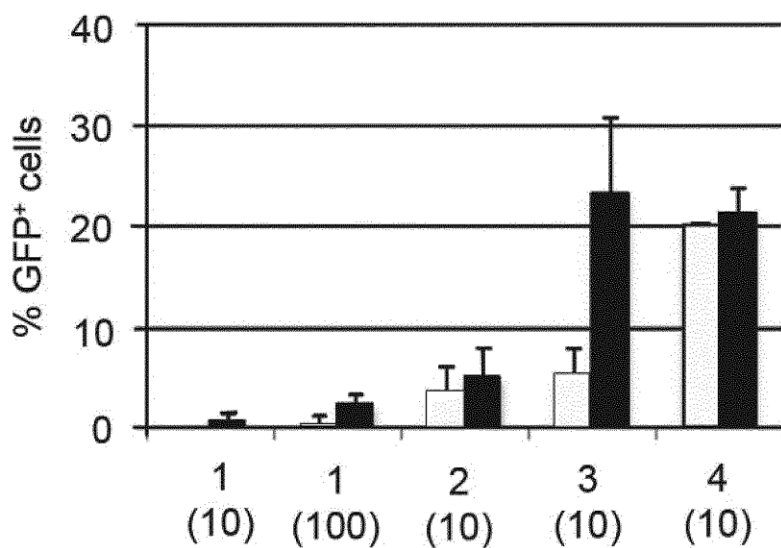


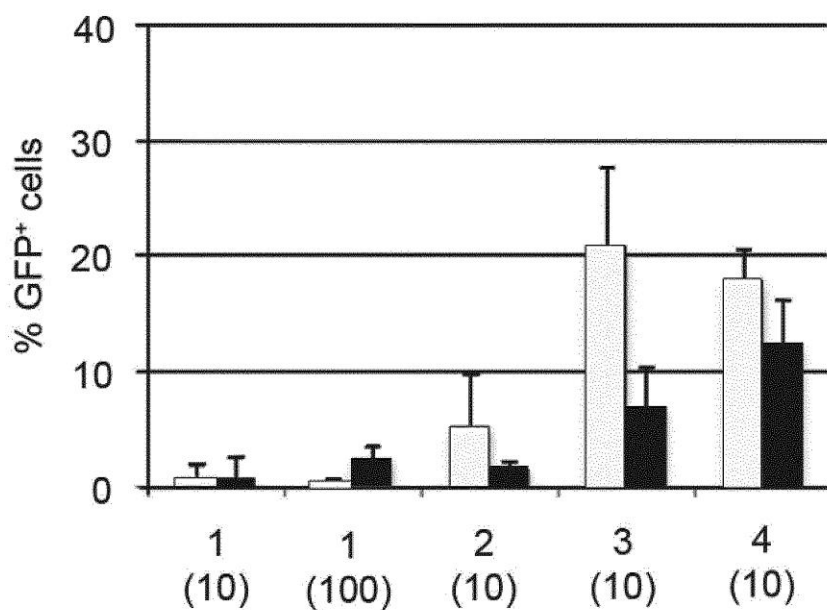
FIG.15



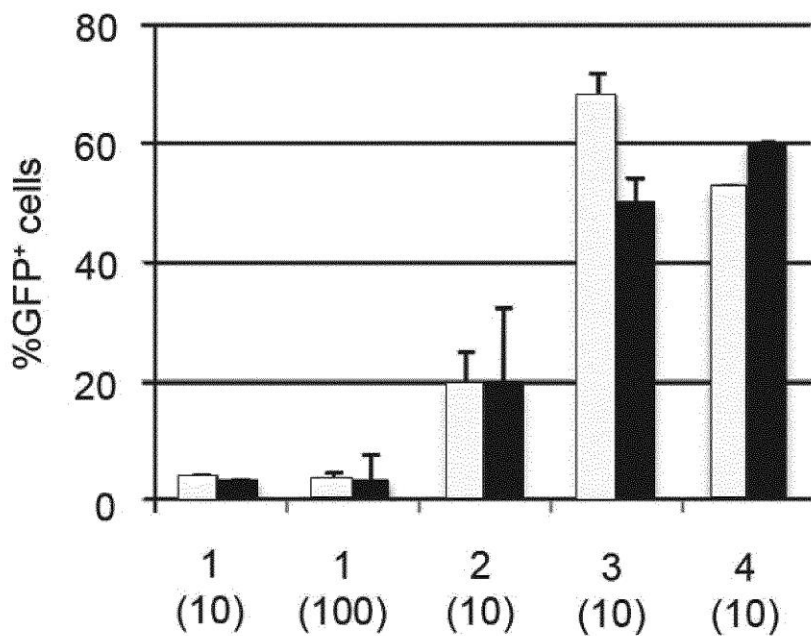
**FIG.16**



**FIG.17**



**FIG.18**



**FIG.19**

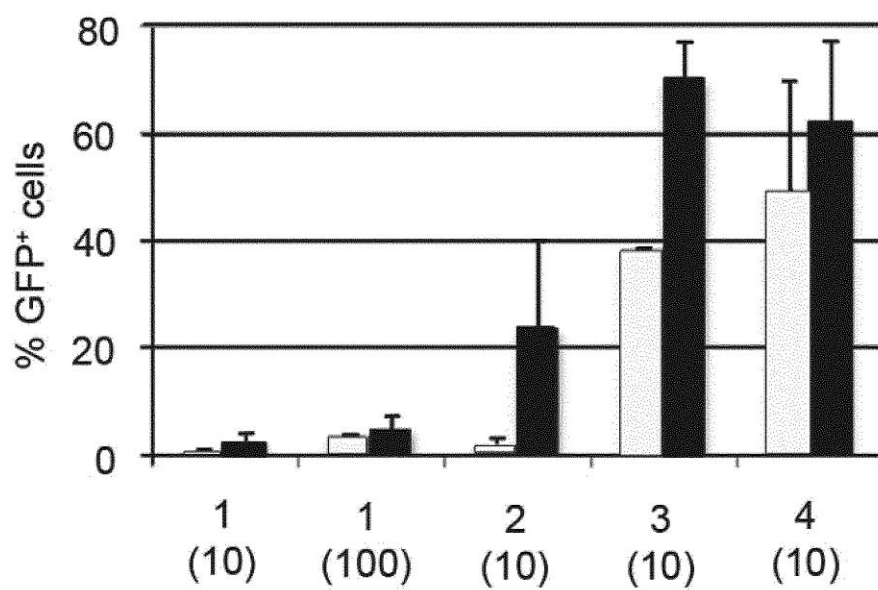
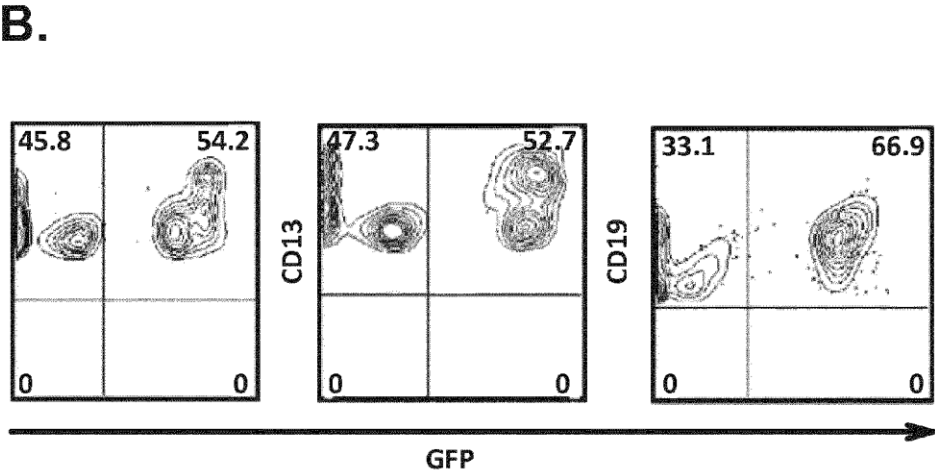
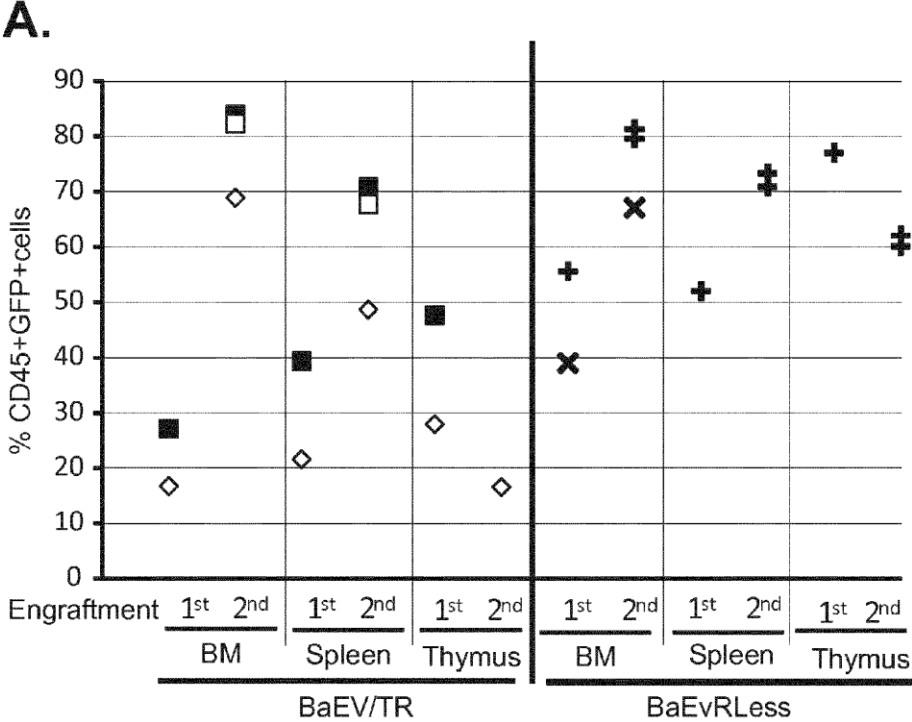
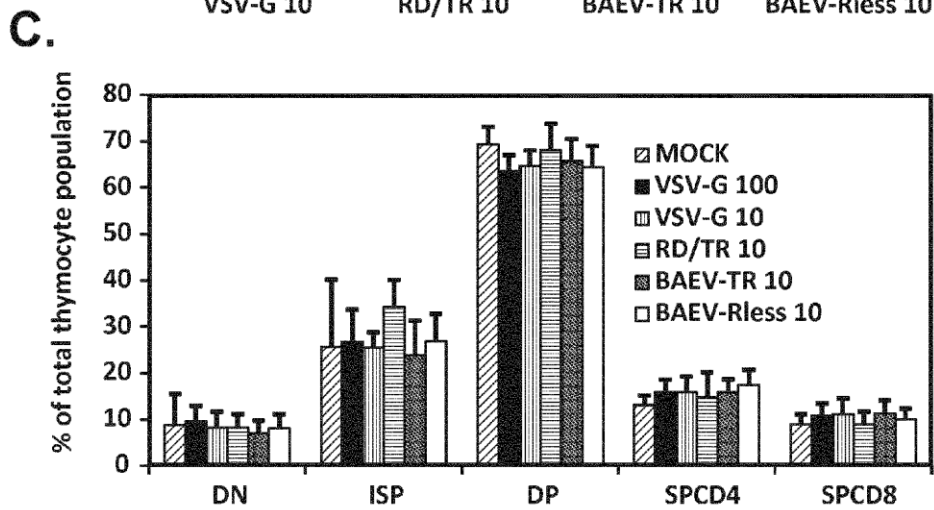
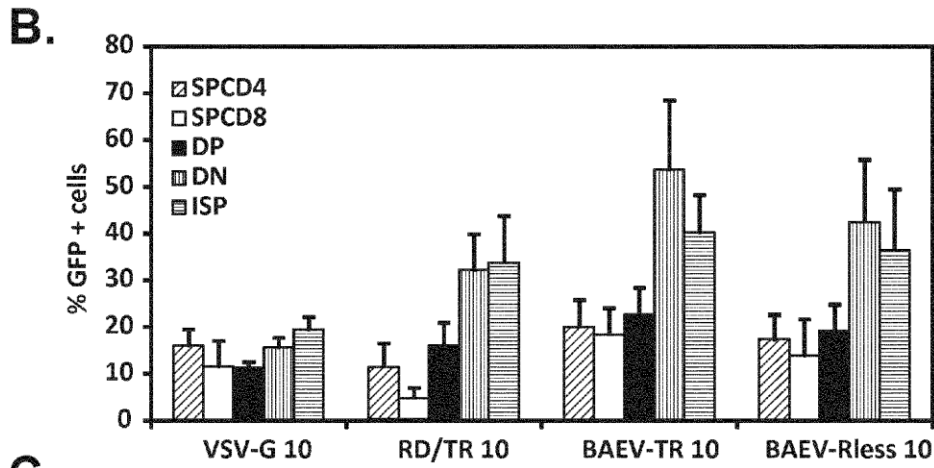
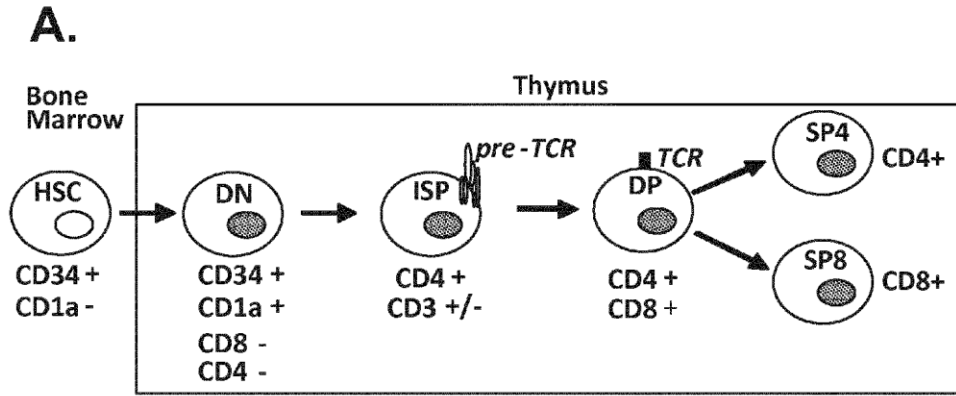


FIG.20

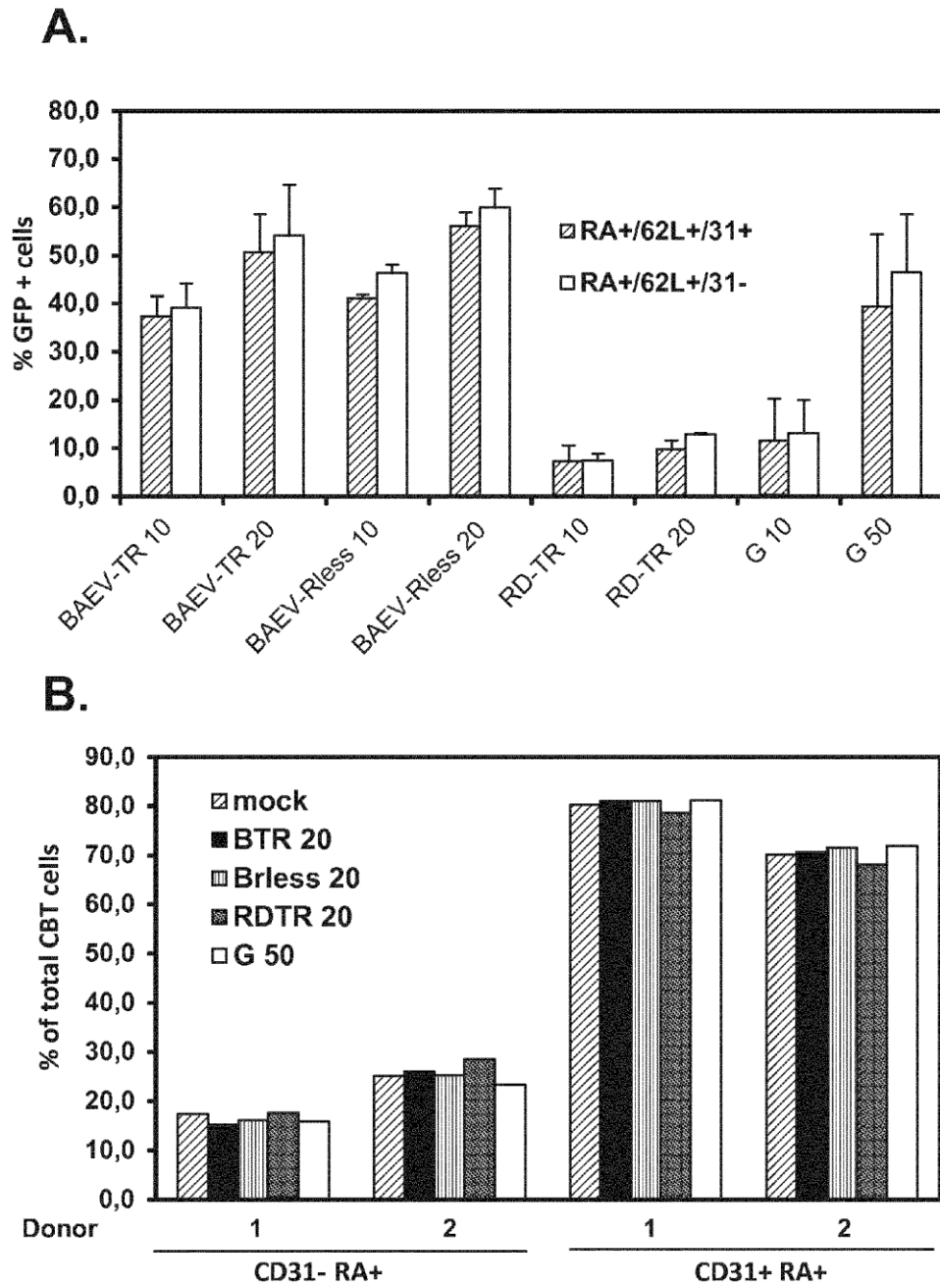


**FIG.21**





**FIG.22**



**FIG.23**

### LENTIVIRAL VECTORS PSEUDOTYPED WITH MUTANT BAEV GLYCOPROTEINS

**[0001]** The present invention concerns pseudotyped lentiviral vectors enabling efficient gene transfer of hematopoietic cells.

**[0002]** Gene therapy holds promise for the cure of many inherited and acquired diseases as evidenced by the success in treatment of X-linked severe combined immunodeficiency (SCID-X1), adenosine desaminase (ADA) deficiency and chronic granulomatous disease. Recently, the successful treatment of patients suffering from X-linked adrenoleukodystrophy (ALD) using lentiviral vectors was reported. In this trial, hematopoietic stem cell (HSC) based gene therapy was able to stop progression of the disease in two patients suffering from this fatal demyelinating disease of the central nervous system. Importantly, for the correction of all these 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. Since HSCs answer to these criteria they represent 'the' attractive candidates for gene therapy applications.

**[0003]** However, a major barrier in lentiviral vector transduction of HSC is that 75% of HSC are residing into the G<sub>0</sub> phase of the cell cycle and are not very permissive for classical lentiviral vector transduction. This limitation hampers the application of conventional lentiviral vectors for HSC gene therapy, as they do not allow efficient gene transfer into a subpopulation of quiescent (G<sub>0</sub>) HSCs (Sutton et al. (1999) *J. Virol.* 73:3649-3660). To overcome this limitation, many studies using lentiviral vectors for hCD34<sup>+</sup> cell transduction employed a high vector input and the presence of very strong cytokine cocktails (TPO, SCF, Flk-3, IL-6, IL-3) in order to induce HSC cycle entry. Very often, retronectin, a fragment of fibronectin, is used to colocalize vector particles and targeted cells or multiple administrations of vector are applied to achieve high gene transfer rates in HSCs. However, an undesirable effect of extended cytokine stimulation is a decrease of the multi-potentiality and long-term engraftment of human HSCs. Moreover, a too high vector dose poses the risk for multi-copy integration, and in particular for insertional mutagenesis.

**[0004]** There is therefore a need for viral vectors enabling transduction of quiescent HSCs and limiting the risk of multi-copy integration.

**[0005]** Efficient gene transfer into quiescent T and B lymphocytes for gene therapy or immunotherapy purposes may allow the treatment of several genetic dysfunctions of the hematopoietic system, such as immunodeficiencies, and the development of novel therapeutic strategies for cancers and acquired diseases. Lentiviral vectors (LV) are not able to transduce some particular quiescent cell types such as resting T and B cells (Bovia et al. (2003) *Blood* 101:1727-1733; Verhoeven et al. (2003) *Blood* 101:2167-2174). In T cells, completion of reverse transcription, nuclear import and subsequent integration of the genome of lentiviral vectors pseudotyped with the envelope glycoprotein of the vesicular stomatitis virus (VSV-G) do not occur efficiently unless they are activated via the T-cell receptor or by survival-cytokines inducing them to enter into the G<sub>1b</sub> phase of the cell cycle. Lentiviral transduction of B cells is another matter since even B-cell receptor (BCR)-stimulation inducing proliferation is not sufficient to allow efficient transduction with VSV-G pseudotyped lentiviral vectors (VSV-G-LVs).

**[0006]** There is therefore an important need for viral vectors enabling transduction of resting T and B cells.

**[0007]** In order to deliver genes by a lentiviral vector in hematopoietic cells, an "entry" envelope protein allowing an efficient vector-cell fusion needs to be presented at its surface. The process of incorporating a heterologous envelope glycoprotein on the core of a lentiviral vector is called "pseudotyping". For a long time, VSV-G associated with viral cores derived from HIV-1 has been used. Nevertheless, there are disadvantages in using VSV-G-LVs. Toxicity is associated with long-term expression of VSV-G which makes generation of stable cell lines difficult. In addition, VSV-G-LVs are sensitive to human complement, which makes them unsuited for in vivo use. Only high VSV-G-LVs doses (multiplicity of infection=MOI of 50-100) allow efficient hCD34<sup>+</sup> cell transduction increasing the risk of multi-copy integration and thus genotoxicity (Di Nunzio et al. (2007) *Hum. Gene Ther.* 18:811-820). Human beings may also develop strong immune responses against VSV-G which may reduce the efficacy of a second administration of VSV-G-LVs. All these evidences limit ex vivo and in vivo use of VSV-G-LVs.

**[0008]** The chimeric envelope glycoprotein comprising the extracellular and transmembrane domains of the RD114 feline leukemia virus envelope glycoprotein fused to the cytoplasmic tail (designated TR) of the murine leukemia virus A (MLV-A) envelope glycoprotein, called herein after RD114/TR and described in the international application WO 03/091442) appeared to be a quite good alternative envelope. Indeed, this pseudotype is not sensitive to the human complement system which makes it attractive for in vivo applications. RD114/TR-LVs allow efficient transduction of human and macaque CD34<sup>+</sup> cells. However, transduction levels stay way below those of VSV-G-LVs and titers of RD114 are much lower than for VSV-G-LVs. Additionally, RD114/TR glycoprotein cannot enter efficiently into murine cells. So this receptor specificity limits the potentialities of work with this LV since the majority of preclinical disease models eligible for gene therapy are mice models.

**[0009]** The present invention arises from the unexpected finding by the inventors that lentiviral vectors pseudotyped with either a chimeric envelope glycoprotein which comprises or consists in a fusion of the transmembrane and extracellular domain of a baboon endogenous retrovirus (BaEV) envelope glycoprotein and the cytoplasmic tail domain of a murine leukemia virus (MLV) envelope glycoprotein; or a modified BaEV envelope glycoprotein wherein the cytoplasmic tail domain is devoid of the fusion inhibitory R peptide, display transduction properties particularly suitable for gene transfer into hematopoietic cells, including HSC and resting T and B cells. Indeed, these new LV pseudotypes can transduce very efficiently and stably hCD34<sup>+</sup> cells up to 70% at low vector doses and upon mild cytokine stimulation. BaEV-LVs outperformed by far VSV-G and RD114/TR-LVs for the transduction of macaque and human CD34<sup>+</sup> cells. Additionally, the inventors showed that BaEV-LVs transduced very early progenitor hCD34<sup>+</sup> cells including HSCs since these cells were able to reconstitute an immunodeficient mice model and high level of transduced blood cells were found in several hematopoietic tissues and in those tissues in the different blood cell lineages. Importantly BaEV-LVs were able to transduce IL-7 prestimulated memory and naive T cells at high levels. Moreover, in contrast to VSV-G-LVs and RD114/

TR-LVs, these BaEV-LVs also allowed high level transduction of resting and BCR stimulated B cells without induction of a phenotypic switch.

**[0010]** The present invention thus concerns a pseudotyped viral vector particle for transferring biological material into cells, wherein said vector particle comprises at least:

**[0011]** a chimeric envelope glycoprotein which comprises or consists in a fusion of the transmembrane and extracellular domain of a baboon endogenous retrovirus (BaEV) envelope glycoprotein and the cytoplasmic tail domain of a murine leukemia virus (MLV) envelope glycoprotein; or

**[0012]** a modified BaEV envelope glycoprotein wherein the cytoplasmic tail domain is devoid of the fusion inhibitory R peptide.

**[0013]** The present invention also concerns the pseudotyped viral vector particle according to the invention for use in the treatment of a hematopoietic disorder.

**[0014]** Another object of the invention concerns a medication comprising a pseudotyped viral vector particle according to the invention as active ingredient.

**[0015]** The invention also relates to the use of a pseudotyped viral vector particle according to the invention for transferring the biological material into hematopoietic cells *ex vivo*.

**[0016]** Another object of the invention concerns a method for transducing a hematopoietic cell comprising contacting the hematopoietic cell with a pseudotyped viral vector particle according to the invention under conditions to effect the transduction of the hematopoietic cell by the pseudotyped viral vector particle.

**[0017]** The invention also concerns a stable virus packaging cell line producing the pseudotyped viral vector particle as defined above.

## DETAILED DESCRIPTION OF THE INVENTION

### Pseudotyped Viral Vector Particle

**[0018]** The present invention concerns a pseudotyped viral vector particle for transferring biological material into cells, wherein said vector particle comprises at least:

**[0019]** a chimeric envelope glycoprotein which comprises or consists in a fusion of the transmembrane and extracellular domain of a baboon endogenous retrovirus (BaEV) envelope glycoprotein and the cytoplasmic tail domain of a murine leukemia virus (MLV) envelope glycoprotein; or

**[0020]** a modified BaEV envelope glycoprotein wherein the cytoplasmic tail domain is devoid of the fusion inhibitory R peptide.

**[0021]** As intended herein, the term “vector particle” denotes any particle liable to display the chimeric envelope glycoprotein or the modified BaEV envelope glycoprotein at its surface and to reversibly bind to a biological material. It is preferred that such a vector particle is a viral vector particle, in particular a retroviral vector particle. Preferably, said retroviral vector particle is selected from the group consisting of an oncoviral vector particle, including murine leukemia virus (MLV), avian leukosis virus (ALV), respiratory syncytial virus (RSV) or Mason-Pfizer monkey virus (MPMV) vector particles, a lentiviral vector particle, such as Human Immunodeficiency Virus (HIV), e.g. HIV-1 or HIV-2, Simian Immunodeficiency Virus (SIV), feline immunodeficiency virus (FIV), equine infectious anemia virus (EIAV) and

caprine arthritis encephalitis virus (CAEV) vector particles, and a spumaviral vector particle such as human foamy virus (HFV) vector particle.

**[0022]** Lentiviral vector particles are well-known to the skilled person and are notably described in Naldini et al. (2000) *Adv. Virus. Res.* 55:599-609 and Negre et al. (2002) *Biochimie* 84:1161-1171. Usually, lentiviral vector particles according to the invention comprise at least the following components: (i) an envelope component, which is constituted of a phospholipidic bilayer associated to envelope proteins, wherein the envelope proteins comprise at least the above-defined chimeric or modified glycoproteins, said envelope surrounding (ii) a core component, constituted of the association of a gag protein, said core itself surrounding (iii) genome components, usually constituted of ribonucleic acids (RNA), and (iv) an enzyme component (pol). The biological material can be present within the envelope, within the core and/or within the genome components.

**[0023]** Lentiviral vector particles can be readily prepared by the skilled person, for example by following the general guidance provided by Sandrin et al. (2002) *Blood* 100:823-832. Briefly, the lentiviral vector particles may be generated by co-expressing the packaging elements (i.e. the core and enzyme components), the genome component and the envelope component in a so-called producer cell, e.g. 293T human embryonic kidney cells. Typically from three to four plasmids may be employed, but the number may be greater depending upon the degree to which the lentiviral components are broken up into separate units.

**[0024]** As used herein, the term “pseudotyped viral vector” refers to a viral vector comprising foreign viral envelope glycoproteins. Typically, the viral vectors according to the invention are pseudotyped with the above defined chimeric or modified glycoproteins.

**[0025]** The Baboon endogenous retrovirus or BaEV is a type C retrovirus present in multiple proviral copies in the DNA of baboons. The BaEV envelope glycoprotein is notably described in Benveniste et al. (1974) *Nature* 248:17-20 and Todaro et al. (1974) *Cell* 2:55-61.

**[0026]** In the context of the invention, the term “BaEV envelope glycoprotein” refers to the wild-type form of the BaEV envelope glycoprotein or to a mutant of said wild-type BaEV envelope glycoprotein which is at least 80%, preferably at least 85%, still preferably at least 90%, more preferably at least 95%, still more preferably at least 99% identical to said wild-type BaEV envelope glycoprotein, provided that said mutant glycoprotein retains the capacity of the wild-type glycoprotein of binding to and fusing with hematopoietic cells membrane.

**[0027]** Typically, the wild-type BaEV envelope glycoprotein is encoded by the nucleic acid sequence SEQ ID NO: 1. Preferably, it consists of the sequence SEQ ID NO: 2. As known from the skilled person, the BaEV envelope glycoprotein is constituted by a cytoplasmic tail domain, a transmembrane domain and an extracellular domain. The regions corresponding to the cytoplasmic tail domain, the transmembrane domain and extracellular domain in the envelope glycoprotein sequence can be easily determined by the skilled person. Typically, the cytoplasmic tail domain is located between amino acids 530 to 564 of the wild-type BaEV envelope glycoprotein. Preferably, the wild-type cytoplasmic tail domain of the BaEV envelope glycoprotein comprises or consists in the amino acid sequence SEQ ID NO: 3. Typically, the transmembrane domain is located between

amino acids 507 to 529 of the wild-type BaEV envelope glycoprotein. Preferably, the wild-type transmembrane domain of the BaEV envelope glycoprotein comprises or consists in the amino acid sequence SEQ ID NO: 4. Typically, the extracellular domain is located between amino acids 1 to 506 of the wild-type BaEV envelope glycoprotein. Preferably, the wild-type extracellular domain of the BaEV envelope glycoprotein comprises or consists in the amino acid sequence SEQ ID NO: 5.

**[0028]** In a particular embodiment of the invention, the cytoplasmic tail domain of the BaEV envelope glycoprotein is devoid of the fusion inhibitory R peptide.

**[0029]** In the context of the invention, the expression “fusion inhibitory R peptide” refers to the C-terminal portion of the cytoplasmic tail domain of the envelope glycoprotein which harbours a tyrosine endocytosis signal—YXXL—and which is cleaved by viral protease during virion maturation, thus enhancing membrane fusion of the envelope glycoprotein. The fusion inhibitory R peptide of the BaEV envelope glycoprotein is typically located between amino acids 547 and 564 of the wild-type BaEV envelope glycoprotein. Preferably, the fusion inhibitory R peptide of the BaEV envelope glycoprotein comprises or consists in the amino acid sequence SEQ ID NO: 6.

**[0030]** Therefore, in a particularly preferred embodiment, the modified BaEV envelope glycoprotein wherein the cytoplasmic tail domain is devoid of the fusion inhibitory R peptide comprises or consists in the amino acid sequence SEQ ID NO: 7. Such a modified BaEV envelope glycoprotein is called herein after “BaEVRLess”.

**[0031]** In another particular embodiment, the cytoplasmic tail domain of the BaEV envelope glycoprotein is replaced by the cytoplasmic tail domain of a murine leukemia virus (MLV) envelope glycoprotein.

**[0032]** The Murine Leukemia Virus envelope glycoprotein is notably described in Ott et al. (1990) *J. Virol.* 64:757-766. The Murine Leukemia Virus envelope glycoprotein is preferably that of strain 4070A.

**[0033]** In the context of the invention, the term “MLV envelope glycoprotein” refers to the wild-type form of the MLV envelope glycoprotein or to a mutant of said wild-type MLV envelope glycoprotein which is at least 80%, preferably at least 85%, still preferably at least 90%, more preferably at least 95%, still more preferably at least 99% identical to said wild-type MLV envelope glycoprotein, provided that said mutant glycoprotein retains the capacity of the wild-type envelope glycoprotein of interacting with viral core proteins, in particular with lentiviral core proteins.

**[0034]** The region corresponding to the cytoplasmic tail domain in the envelope glycoprotein sequence can be easily determined by the skilled person. Typically, the cytoplasmic tail domain of the MLV envelope glycoprotein is located between amino acids 622 and 654 of the wild-type MLV envelope glycoprotein. Preferably, the wild-type cytoplasmic tail domain of the MLV envelope glycoprotein comprises or consists in the amino acid sequence SEQ ID NO: 8.

**[0035]** Therefore, in a particularly preferred embodiment, the chimeric envelope glycoprotein which comprises or consists in a fusion of the transmembrane and extracellular domain of a BaEV envelope glycoprotein and the cytoplasmic tail domain of a MLV envelope glycoprotein comprises or consists in the amino acid sequence SEQ ID NO: 9. Such a chimeric envelope glycoprotein is called herein after “BaEV/TR”.

**[0036]** The inventors demonstrated that BaEV/TR and BaEVRLess glycoproteins were incorporated at a higher level on the lentiviral surface than the wild-type BaEV glycoprotein.

**[0037]** The inventors further demonstrated that the co-display of specific cytokines on the viral vector particles enabled enhancing the targeted transduction of cells of interest.

**[0038]** Therefore, in a particular embodiment, the viral vector particle according to the invention may further display, preferably at its surface, at least one cytokine selected from the group consisting of stem cell factor (SCF), thrombopoietin (TPO), IL-2, IL-15 and IL-7.

**[0039]** Preferably, said at least one cytokine is selected from the group consisting of human SCF, human TPO, human IL-2, human IL-15 and human IL-7.

**[0040]** In the context of the invention, the cytokine may be a wild-type cytokine or any mutant of said wild-type cytokine which is at least 80%, preferably at least 85%, still preferably at least 90%, more preferably at least 95%, still more preferably at least 99% identical to said wild-type cytokine, provided that said mutant cytokine presents essentially the same properties as the wild-type cytokine from which it derives.

**[0041]** The wild-type SCF cytokine preferably comprises or consists in the sequence SEQ ID NO: 10. The wild-type TPO preferably comprises or consists in the sequence SEQ ID NO: 11. The wild-type IL-2 preferably comprises or consists in the sequence SEQ ID NO: 12. The wild-type IL-7 preferably comprises or consists in the sequence SEQ ID NO: 13. The wild-type IL-15 preferably comprises or consists in the sequence SEQ ID NO: 14.

**[0042]** The percentage of identity may be calculated by performing a pairwise global alignment based on the Needleman-Wunsch alignment algorithm to find the optimum alignment (including gaps) of two sequences along their entire length, for instance using Needle, and using the BLOSUM62 matrix with a gap opening penalty of 10 and a gap extension penalty of 0.5.

**[0043]** In a particularly preferred embodiment, the pseudotyped viral vector particle according to the invention is obtainable or obtained by the method of production described in the section “Method for producing a pseudotyped viral vector particle” herein below.

#### Biological Material

**[0044]** The pseudotyped viral vector particles of the invention are of particular interest for transferring biological materials into cells, in particular into hematopoietic cells.

**[0045]** Accordingly, in a preferred embodiment, the pseudotyped viral vector particles as defined above further comprise a biological material.

**[0046]** As intended herein the expression “biological material” relates to one or more compounds liable to alter the structure and/or the function of a cell. Within the context of the present invention, it is preferred that the biological material is one or more nucleic acids, which in the case of lentiviral vector particles may be comprised within the genome of the vector particle. The genome typically comprises the one or more nucleic acids, preferably linked to genetic elements necessary for their expression in the target cell, such as promoters and terminators, flanked by cis-acting elements necessary for the inclusion of the genome in the core element, its reverse transcription into deoxyribonucleic acid (DNA), the import of the retrotranscribed genome into the nucleus of the

target cell and the integration of the retrotranscribed genome within the genome of the target cell.

**[0047]** Examples of nucleic acids of interest include globin genes, hematopoietic growth factors, which include erythropoietin (EPO), the interleukins (especially Interleukin-1, Interleukin-2, Interleukin-3, Interleukin-6, Interleukin-12, etc.) and the colony-stimulating factors (such as granulocyte colony-stimulating factor, granulocyte/macrophage colony-stimulating factor, or stem-cell colony-stimulating factor), the platelet-specific integrin  $\alpha\text{IIb}\beta_3$ , multidrug resistance genes, the gp91 or gp 47 genes which are defective in patients with chronic granulomatous disease (CGD), antiviral genes rendering cells resistant to infections with pathogens such as human immunodeficiency virus, genes coding for blood coagulation factors VIII or IX which are mutated in hemophilia's, ligands involved in T cell-mediated immune responses such as T cell antigen receptors, B cell antigen receptors (immunoglobulins), the interleukin receptor common  $\gamma$  chain, a combination of both T and B cell antigen receptors alone and/or in combination with single chain antibodies (ScFv), IL2, IL12, TNF, gamma interferon, CTLA4, B7 and the like, genes expressed in tumor cells such as Melana, MAGE genes (such as MAGE-1, MAGE-3), P198, P1A, gp100 etc.

**[0048]** As intended herein "transferring" relates to the capacity of the vector particle to initially deliver the biological material to the membrane or the cytoplasm of the target cell, upon being bound to the target cell. After delivery, the biological material can be translocated to other compartment of the cell.

**[0049]** As intended herein the recipient cells for the biological material to be transferred, or target cells, relate to any cell liable to be bound by the above-defined vector particle. Where the vector particle is a lentiviral vector particle, the target cell relates to any cell liable to be transduced by the vector particle. These cells are preferably hematopoietic cells, in particular human hematopoietic cells.

#### Hematopoietic Cells

**[0050]** As used herein, the term "hematopoietic cell" refers generally to blood cells, both from the myeloid and the lymphoid lineage. In particular, the term "hematopoietic cell" includes both undifferentiated or poorly differentiated cells such as hematopoietic stem cells and progenitor cells, and differentiated cells such as T lymphocytes, B lymphocytes or dendritic cells. Preferably, the hematopoietic cell is selected from the group consisting of hematopoietic stem cells, CD34<sup>+</sup> progenitor cells, in particular peripheral blood CD34<sup>+</sup> cells, very early progenitor CD34<sup>+</sup> cells, B-cell CD19<sup>+</sup> progenitors, myeloid progenitor CD13<sup>+</sup> cells, T lymphocytes, B lymphocytes, monocytes, dendritic cells, cancer B cells in particular B-cell chronic lymphocytic leukemia (BOLL) cells and marginal zone lymphoma (MZL) B cells, and thymocytes.

**[0051]** As known from the skilled person, every hematopoietic cell is produced from bone marrow hematopoietic stem cells.

**[0052]** As used herein, the term "hematopoietic stem cell" or "HSC" refers to cells able to replenish all blood cell types and to self-renew. Hematopoietic stem cells may be in particular defined as cells that keep the levels of myeloid, T and B cells at robustly detectable levels (typically more than 1% of peripheral blood cells) for 16 weeks when injected into the

circulation of a recipient mouse with a depleted hematopoietic system (Schroeder (2010) *Cell Stem Cell* 6:203-207).

**[0053]** As used herein, the term "CD34<sup>+</sup> progenitor cell" refers to a heterogeneous cell population that includes a sub-population of HSCs, pluripotent stem cells and cells in the early stages of lineage commitment. CD34<sup>+</sup> progenitor cells continuously migrate to and from the bone marrow in normal adult animals. They can differentiate to produce all hematopoietic cell lineages found in the circulation. The expression "peripheral blood CD34<sup>+</sup> cell" refers more particularly to CD34<sup>+</sup> cells present in the blood.

**[0054]** The inventors prestimulated human CD34<sup>+</sup> early progenitor cells with a strong cytokine cocktail (SCF, TPO and Flt3-L) and transduced them BaEV/TR and BaEVRLess pseudotyped lentiviral vector particles. They demonstrated that the pseudotyped lentiviral vector particles could efficiently transduce human CD34<sup>+</sup> early progenitor cells, in particular at more than 70% in presence of retronectin.

**[0055]** The inventors further demonstrated that the high human CD34<sup>+</sup> cell transduction obtained with BaEV/TR and BaEVRLess pseudotyped lentiviral vector particles was due to stable transduction of short term progenitors. Indeed, there was no significant difference between the proportion of BaEV/TR and BaEVRLess lentiviral vector particles transduced CD34<sup>+</sup> cells after a SCF/TPO prestimulation and the clonogenic progenitors derived from them. In contrast, for lentiviral vector particles pseudotyped with VSV-G, the proportion of transduced clonogenic progenitors was significantly lower than the rate of transduced CD34<sup>+</sup> cells they were derived from.

**[0056]** As used herein, the term "very early progenitor CD34<sup>+</sup> cell" refers to a subgroup of CD34<sup>+</sup> progenitor cells which is enriched in HSCs.

**[0057]** As used herein, the term "B-cell CD19<sup>+</sup> progenitor" refers to a population of B-lineage cells that express cell surface CD10, CD34 and CD19.

**[0058]** As used herein, the term "myeloid progenitor CD13<sup>+</sup> cell" refers to a population of myeloid lineage cells that express cell surface CD34 and CD13 and optionally CD33.

**[0059]** The inventors demonstrated that BaEV/TR and BaEVRLess pseudotyped lentiviral vector particles were able to transduce at high efficiency hematopoietic progenitors capable of repopulating immunodeficient mice. They evaluated the long-term reconstitution capacity of these pseudotyped lentiviral vector particles transduced human CD34<sup>+</sup> cells in the Rag2<sup>-/-</sup>  $\gamma$ <sup>-/-</sup> Balbc immunodeficient mice model and the NOD/SCID/ $\gamma$ c<sup>-/-</sup> (NSG) mice model. They demonstrated that very early progenitor CD34<sup>+</sup> cells, as well as B-cell CD19<sup>+</sup> progenitors and myeloid CD13<sup>+</sup> cells were transduced to the same extent by BaEV/TR and BaEVRLess pseudotyped lentiviral vector particles of the invention at low infectious vector particle doses. Moreover, BaEV/TR-LVs and BaEVRLess-LVs transduced hCD34<sup>+</sup> cells resulted in high reconstitution levels in the bone marrow and the highest transduction levels of SCID repopulating cells (up to 90% CD45+GFP+ cells) in the bone marrow. They also demonstrated that these high transduction levels were sustained in the primary recipient mice in all hematopoietic tissues (bone marrow, thymus and spleen). Equivalent high level transduction was detected for immature progenitors (hCD34<sup>+</sup> cells), lymphoid cells (CD19<sup>+</sup>) and myeloid cells (CD13<sup>+</sup> and CD14<sup>+</sup>) in the bone marrow. On the contrary, lentiviral vector particles pseudotyped with VSV-G transduced early CD34<sup>+</sup>

progenitors, CD13+ myeloid progenitors and monocytes to much lower levels. For lentiviral vector particles pseudotyped with RD114/TR, also low transduction of all the different bone marrow cell lineages was detected. An identical picture was found for T- and B-cells and monocytes in the spleen. RD114/TR-LVs demonstrated variable transduction levels of SCID repopulating cells (ranging from 3.2-76% of GFP+ CD45+ cells).

**[0060]** The inventors also showed that in the spleen and bone marrow the percentage of GFP+ hCD45+ cells was maintained or increased after secondary engraftment of the hCD34+ cells isolated from primary recipients in immunodeficient mice. Moreover, for both BaEV/TR- and BaEVRLess-LVs, high levels of GFP+ hCD34+ early progenitor, lymphoid (CD19+) and myeloid (CD13+) cells were detected in the bone marrow of these secondary recipient mice and equivalent percentages of GFP+ cells were detected in these different lineages. The inventors thus showed that the secondary reconstituting SCID repopulating cells were capable of multilineage differentiation and that true human HSC were genetically modified to high levels by BaEV/TR- and BaEVRLess-LVs.

**[0061]** The inventors also showed that BaEV/TR and BaEVRLess pseudotyped lentiviral vector particles allowed efficient transduction of human T cells, in particular of IL-7 prestimulated human T cells. These pseudotyped lentiviral vector particles were superior over lentiviral vector particles pseudotyped with VSV-G for the same vector doses in the presence of retronectin. Importantly, the BaEV/TR and BaEVRLess pseudotyped lentiviral vector particles allowed an efficient transduction of both naïve and memory IL-7 prestimulated T cells. Indeed, they allowed transduction of IL-7 stimulated T-cells without compromising their naïve phenotype. Of high interest, they also permitted close to 100% T cell gene transfer upon T-cell receptor (TCR) stimulation at an MOI of 10.

**[0062]** Therefore, in a particular embodiment, the targeted hematopoietic cell is a T cell, preferably a naïve or a memory T cell.

**[0063]** The BaEV/TR and BaEVRLess pseudotyped lentiviral vector particles also permitted highly efficient transduction of 20-30% of resting B cells and thus outperformed by far the lentiviral vector particles pseudotyped with VSV-G or RD114/TR which enabled no transduction or very low transduction of resting human B cells. Furthermore, upon B cell receptor (BCR) stimulation, BaEV/TR and BaEVRLess pseudotyped lentiviral vector particles transduced up to 70% of the B cells while lentiviral vector particles pseudotyped with VSV-G transduction efficiency did not exceed 5%. Of importance, as well memory as naïve B cells were efficiently transduced by BaEV/TR and BaEVRLess pseudotyped lentiviral vector particles.

**[0064]** Therefore, in a particular embodiment, the targeted hematopoietic cell is a B cell, in particular a resting B cell, preferably a naïve or a memory B cell. It may also be a cancer B cell, in particular a B-cell chronic lymphocytic leukemia (BOLL) cell or a marginal zone lymphoma (MZL) B cell.

**[0065]** Because of the potential value of thymocyte gene transfer for immune modulation, the inventors evaluated the performance of BaEVgp-LVs compared to the other LV pseudotypes for the transduction of different thymocyte subpopulations. Upon isolation of thymocytes from a human thymus, they transduced them in the presence of the survival cytokine IL-7 with VSV-G-, RD114/TR-, BaEV/TR-, and

BaEVRLess-LVs encoding for the GFP reporter at an MOI of 10. The inventors showed that RD114/TR-LV, BaEV/TR-LVs and BaEVRLess-LVs allow preferential transduction of the thymocytes in the early stages of development (double negative and immature CD4 single positive cells) while they still transduce the more mature to a lower level. As already detected for adult human T cells, BaEVgp LVs transduced all different thymocyte subpopulations more efficiently than the RD114/TR-LVs. Moreover, transduction with the different lentiviral vector pseudotypes had no effect on the distribution of different thymocyte subpopulations. The inventors thus demonstrated that BaEVgp-LVs are excellent tools for transduction of thymocytes and that they are especially superior over VSV-G- and RD114/TR-LVs for the transduction of the immature double negative (DN) and immature CD4 single positive cells (ISP) thymocyte subpopulations.

**[0066]** Therefore, in a particular embodiment, the targeted hematopoietic cell is a thymocyte.

**[0067]** Finally, the inventors prestimulated freshly isolated total CB T cells with the T cell survival cytokine IL-7 to conserve the T cell naïve phenotype, and subsequently transduced them with the different vectors: VSV-G-, RD114/TR-, BaEV/TR-, and BaEVRLess-LVs encoding the GFP reporter at an MOI of 10 or 20, or at an MOI of 50 for VSV-G-LVs. They showed that BaEV/TR- and BaEVRLess-LVs present a transduction efficiency highly superior (40-50% transduction) at an MOI=10 as compared to VSV-G or RD114/TR-LVs. Importantly, BaEV/TR- and BaEVRLess-LVs reached easily 65% transduction when the vector dose was 2-fold increased (MOI=20). In all cases the naïve recent thymocyte emigrants (CD69L+ CD45RA+ CD31+) were transduced to the same extent as the more mature naïve CB T cells (CD69L+ CD45RA+ CD31-). Moreover, transduction had no effect on the distribution of these two naïve CB T cell populations.

**[0068]** Therefore, in a particular embodiment, the targeted hematopoietic cell is a naïve cell. As used herein, the term "naïve cell" refers to a cell that has not yet undergone cell activation in response to a stimulus. In particular, a "naïve cell" may be a cell that has not yet been exposed to an antigen.

**[0069]** Since a strong stimulation with a cytokine cocktail induces HSC differentiation and their loss of self renewal capacity, it is of particular interest to prestimulate the cells as less as possible, to avoid their differentiation as much as possible and to keep the more their "stem-cell features".

**[0070]** The inventors applied the pseudotyped viral vectors of the invention at a fixed MOI (number of infectious particles per cell) of 10 and different cytokine prestimulation protocols on human CD34+ early progenitor cells: 1) no stimulation; 2) stimulation with a SCF or TPO; 3) stimulation with SCF and TPO; 4) stimulation with SCF, TPO and Flt3-L. Important, the strongest cytokine prestimulation cocktail used was lower than prestimulation cocktails currently used in clinical setting with the objective to limit cell differentiation. The inventors demonstrated that BaEV/TR and BaEVRLess lentiviral vector particles were able to transduce 10-20% of unstimulated human CD34+ early progenitor cells, whereas lentiviral vectors pseudotyped with VSV-G or RD114/TR (as described in international application WO2009/013324) were unable to transduce unstimulated quiescent human CD34+ early progenitor cells efficiently at the same vector doses. Furthermore a single TPO stimulation was sufficient for a transduction of human CD34+ early progenitor cells of up to 60% for BaEV/TR and BaEVRLess pseudotyped lentiviral vectors, while

lentiviral vectors pseudotyped with VSV-G or RD114/TR only transduced 8 to 20% of human CD34<sup>+</sup> early progenitor cells under these conditions. SCF single prestimulation permitted up to 30-50% of transduction of human CD34<sup>+</sup> early progenitor cells with BaEV/TR and BaEVRLess pseudotyped lentiviral vectors, whereas lentiviral vectors pseudotyped with VSV-G or RD114/TR only reached transduction levels of 10% Finally, a combination of TPO and SCF or of TPO, SCF and Flt3-L prestimulation with a single BaEV/TR or BaEVRLess pseudotyped lentiviral vector incubation resulted in up to 75% transduction of human CD34<sup>+</sup> early progenitor cells, while lentiviral vectors pseudotyped with RD114/TR only achieved 40% of transduction.

**[0071]** Accordingly, in a preferred embodiment, the targeted hematopoietic cell is not prestimulated with at least one cytokine. In particular it is not prestimulated with SCF, TPO and/or Flt3-L.

**[0072]** In another preferred embodiment, the targeted hematopoietic cell is only prestimulated with SCF or TPO. In still another preferred embodiment, the targeted hematopoietic cell is only prestimulated with SCF and TPO.

**[0073]** As used herein, the term “prestimulation” or “pre-stimulated” means that the hematopoietic cell is contacted with at least one cytokine at a concentration or for a period suitable to induce a specific activation of the hematopoietic cell.

**[0074]** As used herein, the term “fms-like tyrosine kinase receptor-3 ligand”, “Flt3 ligand” or “Flt3-L” refers to a cytokine that increases the number of immune cells by activating the hematopoietic progenitors. Flt3-L is preferably human Flt3-L and more preferably comprises or consists in the sequence SEQ ID NO: 15.

#### Method for Producing a Pseudotyped Viral Vector Particle

**[0075]** The present invention also relates to a method for producing a pseudotyped viral vector particle comprising:

**[0076]** a) transfecting a cell with:

**[0077]** (i) at least one first nucleic acid sequence comprising a packaging competent retroviral derived genome;

**[0078]** (ii) at least one second nucleic acid sequence comprising a cDNA encoding core proteins from said retrovirus, and

**[0079]** (iii) at least one third nucleic acid sequence comprising a cDNA encoding:

**[0080]** a chimeric envelope glycoprotein which comprises or consists in a fusion of the transmembrane and extracellular domain of a baboon endogenous retrovirus (BaEV) envelope glycoprotein and the cytoplasmic tail domain of a murine leukemia virus (MLV) envelope glycoprotein, as defined above; or

**[0081]** a modified BaEV envelope glycoprotein wherein the cytoplasmic tail domain is devoid of the fusion inhibitory R peptide, as defined above;

**[0082]** to yield a producer cell;

**[0083]** b) maintaining the producer cell in culture for sufficient time to allow expression of the cDNAs to produce the encoded proteins; and

**[0084]** c) allowing the encoded proteins to form viral vector particles of the invention.

**[0085]** By “retrovirus” is meant a virus whose genome consists of a RNA molecule and that comprises a reverse-transcriptase, i.e. a member of the Retroviridae family. Ret-

roviruses are divided into Oncovirus, Lentivirus and Spumavirus. Preferably said retrovirus is an oncovirus, e.g. MLV, ALV, RSV, or MPMV, a lentivirus, e.g. HIV-1, HIV-2, SIV, EIAV, or CAEV, or a spumavirus such as HFV. Genomes of these retroviruses are readily available in databanks. More preferably said retrovirus is a lentivirus, in particular HIV-1, HIV-2 or SIV.

**[0086]** In the context of the invention “a nucleic sequence comprising a packaging competent retrovirus-derived genome” is intended for a sequence that comprises the retroviral nucleic acid sequences known as “cis-acting” sequences. These include the Long Terminal Repeats (LTRs) for the control of transcription and integration, the psi sequence necessary for encapsidation, and the Primer Binding site (PBS) and polypurine track (PPT) sequences necessary for reverse transcription of the retroviral genome. Advantageously, said nucleic acid sequence comprising a packaging competent retrovirus-derived genome further comprises a transgene.

**[0087]** Said retroviral genome may be replication-defective or replication-competent, in the absence of any trans-complementing function. A replication-competent genome would further comprise the gag, pol, and env retroviral genes. In a replication-defective genome, the viral genes gag, pol, and env are deleted. However, assembly of the viral vector particles of the invention may be achieved by providing in trans another plasmid that encodes gag, pol and/or env glycoprotein but that is defective for the “cis” sequences. Their expression allows the encapsidation of the transgene, excluding the genes necessary for the multiplication of the viral genome and for the formation of complete viral particles.

**[0088]** The “core protein from a retrovirus” refers to proteins encoded by the gag and pol genes. The gag gene encodes a polyprotein which is further processed by the retroviral protease into structural proteins that comprise the core. The pol gene encodes the retroviral protease, reverse-transcriptase, and integrase.

**[0089]** For the purpose of transfection, said first, second and third nucleic acid sequences may be carried on a same vector, or on two or three separated vectors. Generally, one plasmid encodes the core retroviral component of the viral vector particle. The origin of the gag and pol genes gives its name to the viral vector particle. For instance the expression “HIV-1-derived vector particle” usually indicates that the gag and pol genes of the vector particle are those of HIV-1.

**[0090]** The term “transfection” means the introduction of a foreign nucleic acid (DNA, cDNA or RNA) into a cell so that the host cell will express the introduced gene or sequence to produce a desired substance, typically a protein coded by the introduced gene or sequence. The introduced gene may include regulatory or control sequences, such as start, stop, promoter, signal, secretion, or other sequences used by a cell’s genetic machinery. A host cell that receives and expresses introduced DNA or RNA has been “transfected”.

**[0091]** For the production of vector particles, one may employ any cell that is compatible with the expression of lentiviral Gag and Pol genes, or any cell that can be engineered to support such expression. For example, producer cells such as 293T cells and insect cells (in particular for HIV derived vectors), TE 671 and HT1080 cells (in particular for MLV derived vectors) may be used.



### Therapeutical Applications

**[0092]** The present invention also relates to a medicament comprising a pseudotyped viral vector particle as defined above as active ingredient, in particular a pseudotyped viral vector particle as defined above further comprising a biological material which is preferably one or more nucleic acids.

**[0093]** It also relates to a pharmaceutical composition comprising a pseudotyped viral vector particle as defined above, in particular a pseudotyped viral vector particle as defined above further comprising a biological material which is preferably one or more nucleic acids, and a pharmaceutically acceptable carrier.

**[0094]** The present invention also concerns a method for treating a subject in need thereof comprising administering a therapeutically effective amount of a pseudotyped viral vector particle as defined above, in particular a pseudotyped viral vector particle as defined above further comprising a biological material which is preferably one or more nucleic acids, to the subject in need thereof.

**[0095]** In the context of the present invention, a “subject” denotes a human or non-human mammal, such as a rodent (rat, mouse, rabbit), a primate (chimpanzee), a feline (cat), a canine (dog). Preferably, the subject is human.

**[0096]** As used herein, the term “pharmaceutically acceptable carrier” refers to a carrier that may be administered to a patient, together with a viral vector of this invention, and does not destroy the pharmacological activity thereof and is non-toxic when administered in doses sufficient to deliver a pharmaceutically effective amount of the viral vector.

**[0097]** Pharmaceutically acceptable carriers and vehicles that may be used in the pharmaceutical compositions of this invention include, but are not limited to, ion exchangers, alumina, aluminium stearate, lecithin, self-emulsifying drug delivery systems (SEDDS) such as d- $\alpha$ -tocopherol polyethyleneglycol 1000 succinate, surfactants used in pharmaceutical dosage forms such as Tweens or other similar polymeric delivery matrices, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts or electrolytes, such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, polyethylene glycol, sodium carboxymethylcellulose, polyacrylates, waxes, polyethylene-polyoxypropylene-block polymers, polyethylene glycol and wool fat. Cyclodextrins such as  $\alpha$ -,  $\beta$ -, and  $\gamma$ -cyclodextrin, or chemically modified derivatives such as hydroxyalkylcyclodextrins, including 2- and 3-hydroxypropyl- $\beta$ -cyclodextrins, or other solubilized derivatives may also be advantageously used to enhance delivery of compositions according to the invention.

**[0098]** Any suitable method of administration known from one skilled in the art may be used. In particular, the pseudotyped viral vector particle according to the invention may be administered for example by the oral route or by the parenteral route (in particular by intravenous injection and intra-femur (bone marrow cavity) injection). When the parenteral route is selected, the pseudotyped viral vector particles may be in the form of injectable solutions and suspensions, conditioned in ampoules or flasks. The forms for parenteral delivery are conventionally obtained by mixing the pseudotyped viral vector particles according to the invention with buffers, stabilizers, preservatives, solubilizing agents,

isotonic agents and slurring agents. According to known techniques, these mixtures can then be sterilized and conditioned in the form of intravenous injections. One of skill in the art may use organic phosphate salts-based buffers as buffer. Examples of slurring agents include methylcellulose, acacia and sodium carboxymethylcellulose. Examples of stabilizers include sodium sulphite and sodium metasilphite, and examples of preservatives include sodium p-hydroxybenzoate, sorbic acid, cresol and chlorocresol.

**[0099]** A “therapeutically effective amount” refers to a quantity of a viral vector that confers a therapeutic effect on the treated subject. The therapeutic effect may be objective (i.e., measurable by some test or marker) or subjective (i.e., subject gives an indication of or feels an effect). As known from the skilled person, effective doses will vary depending on route of administration, the size and/or weight of the subject, as well as the possibility of co-usage with other agents.

**[0100]** Where the pseudotyped viral vector particle is used as a medicament and is administered to a subject in a therapeutic method, administration through the intravenous route or by the medullar route, in particular the femur or humerus medullar route, is preferred. For intravenous administration a unit dose from about  $5 \cdot 10^8$  to about  $10^9$  pseudotyped viral vector particles as defined above can be used, whereas for medullar administration a unit dose from about  $10^8$  to about  $5 \cdot 10^8$  pseudotyped viral vector particles as defined above can be used.

**[0101]** In a particular embodiment, the pseudotyped viral vector particle of the invention is for use in the treatment of a hematopoietic disorder or an autoimmune disease.

**[0102]** The present invention also relates to the use of a pseudotyped viral vector particle as defined above for the manufacture of medicament intended for the treatment of a hematopoietic disorder or an autoimmune disease. It also relates to a method for treating a hematopoietic disorder or an autoimmune disease, in a subject comprising administering a therapeutically efficient amount of a pseudotyped viral vector particle of the invention in a subject in need thereof.

**[0103]** As used herein, the expression “hematopoietic disorder” refers to a blood disease, in particular a disease involving hematopoietic cells. Preferably, said hematopoietic disorder is a monogenic hematopoietic disease.

**[0104]** As used herein, the expression “monogenic hematopoietic disease” refers to a genetic hematopoietic disease, which is due to the mutation of a single gene.

**[0105]** Examples of hematopoietic disorders include myelodysplasia, aplastic anemia, Fanconi anemia, paroxysmal nocturnal hemoglobinuria, Sickle cell disease, Diamond Blackfan anemia, Schachman Diamond disorder, Kostmann’s syndrome, chronic granulomatous disease, adrenoleukodystrophy, leukocyte adhesion deficiency, hemophilia, thalassemia, beta-thalassemia, leukaemia such as acute lymphocytic leukemia (ALL), acute myelogenous (myeloid) leukemia (AML), adult lymphoblastic leukaemia, chronic lymphocytic leukemia (CLL), B-cell chronic lymphocytic leukemia (B-CLL), chronic myeloid leukemia (CML), juvenile chronic myelogenous leukemia (JMML), and severe combined immunodeficiency disease (SCID), X-linked severe combined immunodeficiency, Wiskott-Aldrich syndrome (WAS), adenosine-deaminase (ADA) deficiency, chronic granulomatous disease, Chediak-Higashi syndrome, Hodgkin’s lymphoma, non-Hodgkin’s lymphoma (NHL) and AIDS.

**[0106]** Preferably, the hematopoietic disorder is selected from the group consisting of Fanconi anemia, hemophilia, beta-thalassemia, Wiskott-Aldrich syndrome, X-linked severe combined immunodeficiency, adenosine-deaminase deficiency, chronic granulomatous disease and adrenoleukodystrophy.

**[0107]** As used herein, the expression “autoimmune disease” refers to a disease due to an overactive immune response of the body against substances and tissues normally present in the body. Accordingly, by specifically targeting immune (hematopoietic) cells involved in this overactive immune response, the vector particles of the invention could be useful tools in the treatment of autoimmune diseases.

**[0108]** Autoimmune diseases include in particular acute disseminated encephalomyelitis, acute hemorrhagic leukoencephalitis, Addison’s disease, Agammaglobulinemia, Alopecia areata, amyotrophic lateral sclerosis, ankylosing spondylitis, antiphospholipid syndrome, antisynthetase syndrome, atopic allergy, autoimmune aplastic anemia, autoimmune cardiomyopathy, autoimmune enteropathy, autoimmune hemolytic anemia, autoimmune hepatitis, autoimmune inner ear disease, autoimmune lymphoproliferative syndrome, autoimmune peripheral neuropathy, autoimmune pancreatitis, autoimmune polyendocrine syndrome, autoimmune progesterone dermatitis, autoimmune thrombocytopenic purpura, autoimmune urticaria, autoimmune uveitis, Balo disease, Balo concentric sclerosis, Behcet’s syndrome, Berger’s disease, Bickerstaff’s encephalitis, Blau syndrome, bullous pemphigoid, cancer, Castleman’s disease, celiac disease, chronic inflammatory demyelinating polyneuropathy, chronic recurrent multifocal osteomyelitis, Churg-Strauss syndrome, cicatricial pemphigoid, Cogan syndrome, cold agglutinin disease, complement component 2 deficiency, cranial arteritis, CREST syndrome, Crohn’s disease, Cushing’s syndrome, cutaneous leukocytoclastic angitis, Deigo’s disease, Dercum’s disease, dermatitis herpetiformis, dermatomyositis, diabetes mellitus type 1, diffuse cutaneous systemic sclerosis, Dressler’s syndrome, discoid lupus erythematosus, eczema, enthesitis-related arthritis, eosinophilic fasciitis, eosinophilic gastroenteritis, epidermolysis bullosa acquisita, erythema nodosum, essential mixed cryoglobulinemia, Evan’s syndrome, firodysplasia ossificans progressiva, fibrosing aveolitis, gastritis, gastrointestinal pemphigoid, giant cell arteritis, glomerulonephritis, goodpasture’s syndrome, Grave’s disease, Guillain-Barré syndrome (GBS), Hashimoto’s encephalitis, Hashimoto’s thyroiditis, hemolytic anaemia, Henoch-Schonlein purpura, herpes gestationis, hypogammaglobulinemia, idiopathic inflammatory demyelinating disease, idiopathic pulmonary fibrosis, idiopathic thrombocytopenic purpura, IgA nephropathy, inclusion body myositis, inflammatory demyelinating polyneuropathy, interstitial cystitis, juvenile idiopathic arthritis, juvenile rheumatoid arthritis, Kawasaki’s disease, Lambert-Eaton myasthenic syndrome, leukocytoclastic vasculitis, lichen planus, lichen sclerosus, linear IgA disease (LAD), Lou Gehrig’s disease, lupoid hepatitis, lupus erythematosus, Majeed syndrome, Ménière’s disease, microscopic polyangiitis, Miller-Fisher syndrome, mixed connective tissue disease, morphea, Mucha-Habermann disease, multiple sclerosis, myasthenia gravis, myositis, neuropylitis optica, neuromyotonia, ocular cicatricial pemphigoid, opsoclonus myoclonus syndrome, ord thyroiditis, palindromic rheumatism, paraneoplastic cerebellar degeneration, paroxysmal nocturnal hemoglobinuria (PNH), Pary Romberg syndrome, Par-

sonnage-Turner syndrome, pars planitis, pemphigus, pemphigus vulgaris, pernicious anemia, perivenous encephalomyelitis, POEMS syndrome, polyarteritis nodosa, polymyalgia rheumatica, polymyositis, primary biliary cirrhosis, primary sclerosing cholangitis, progressive inflammatory neuropathy, psoriasis, psoriatic arthritis, pyoderma gangrenosum, pure red cell aplasia, Rasmussen’s encephalitis, Raynaud phenomenon, relapsing polychondritis, Reiter’s syndrome, restless leg syndrome, retroperitoneal fibrosis, rheumatoid arthritis, rheumatoid fever, sarcoidosis, Schmidt syndrome, Schnitzler syndrome, scleritis, scleroderma, Sjögren’s syndrome, spondylarthropathy, Still’s disease, stiff person syndrome, subacute bacterial endocarditis, Susac’s syndrome, Sweet’s syndrome, Sydenham chorea, sympathetic ophthalmia, Takayasu’s arteritis, temporal arteritis, Tolosa-Hunt syndrome, transverse myelitis, ulcerative colitis, undifferentiated connective tissue disease, undifferentiated spondylarthropathy, vasculitis, vitiligo and Wegener’s granulomatosis.

**[0109]** Preferably, the autoimmune disease is selected from cancer, diabetes, hemophilia, uveitis and encephalomyelitis.

**[0110]** As used herein, the term “cancer” encompasses any type of cancer but preferably includes cancers arising from hematopoietic cells, including leukemia, in particular B-CLL, CML or T cell based leukemia such as ALL, as well as melanoma.

#### Transducing Methods

**[0111]** The present invention also relates to the use of a pseudotyped viral vector particle as defined above, for transferring a biological material as defined in the section “Biological material” above, into hematopoietic cells as defined in the section “Hematopoietic cells” above, *ex vivo*.

**[0112]** Another object of the invention concerns a method for transducing a hematopoietic cell as defined in the section “Hematopoietic cells” above, comprising contacting the hematopoietic cell with a pseudotyped viral vector particle as defined above under conditions to effect the transduction of the hematopoietic cell by the pseudotyped viral vector particle.

**[0113]** As intended herein “transferring” or “transducing” relates to the capacity of the viral vector particle to initially deliver the biological material to the membrane or the cytoplasm of the target cell, upon being bound to the target cell. After delivery, the biological material can be translocated to other compartment of the cell.

**[0114]** Conditions to effect the transduction of the targeted cells are well-known from the skilled person and include typically incubating the cells to be transduced, preferably cultured in flasks, plates or dishes coated with retronectin, and optionally prestimulated with cytokine cocktails, with the pseudotyped viral vector particles of the invention, preferably at an MOI of 1, 5, 10 or 100, preferably in serum-free medium.

#### Packaging Cell Line

**[0115]** The invention also concerns a stable virus packaging cell line producing the pseudotyped viral vector particle as defined above, preferable producing the pseudotyped viral vector particle comprises at least a chimeric envelope glycoprotein which comprises or consists in a fusion of the trans-

membrane and extracellular domain of a BaEV envelope glycoprotein and the cytoplasmic tail domain of a MLV envelope glycoprotein.

**[0116]** Indeed, the inventors demonstrated that the BaEV/TR glycoprotein was not cytotoxic to the 293T producer cells.

**[0117]** In the context of the invention, a stable virus packaging cell line refers to a cell line which stably expresses the different components of the pseudotyped viral vector particle of the invention. Typically, the nucleic acids encoding the different components of the pseudotyped viral vector particle of the invention are integrated in the genome of the cell line.

**[0118]** In particular such stable virus packaging cell line may be any cell that is compatible with the expression of lentiviral Gag and Pol genes, or any cell that can be engineered to support such expression. For example, they can be 293T cells, and insect cells (in particular for HIV derived vectors), TE 671 and HT1080 cells (in particular for MLV derived vectors) may be used.

**[0119]** The present invention will be further illustrated by the following figures and example.

#### BRIEF DESCRIPTION OF THE FIGURES

**[0120]** FIG. 1 shows schematic representation of the BaEV wild-type glycoprotein, the RD114/TR and BaEV/TR chimeric glycoproteins and the BaEVRLess glycoprotein. The cytoplasmic domain of RD114 and BaEV wild-type glycoprotein was exchanged for the one of the MLV-A glycoprotein resulting in the chimeric RD114/TR and BaEV/TR glycoproteins respectively. The R peptide of the cytoplasmic tail of BaEV wild-type glycoprotein was deleted resulting in a BaEVRLess mutant glycoprotein.

**[0121]** FIG. 2 shows histograms representing the titers of the different pseudotyped lentiviral vector particles (VSV-G (1), RD114/TR (2), BaEVwt (3), BaEV/TR (4) and BaEVRLess (5) pseudotyped) coding for the GFP marker gene obtained by infection of HEK293T cells with serial dilutions of concentrated vector preparations. The percentage of GFP cells was determined by cytometry 3 days after infection. Infectious titers were calculated as GFP infectious units (IU)/ml. Averages are showed (n=7) except for BaEVwt (n=3).

**[0122]** FIG. 3 shows histograms representing the relative percentage of transduction of HEK293T cells by VSV-G (1), RD114/TR (2), BaEV/TR (3) or BaEVRLess (4) pseudotyped lentiviral vector particles which were incubated at 37° C. for 1 hour in heat inactivated human sera (white bars; the serum was heat-inactivated at 56° C. before incubation with the vectors to inactivate the complement where fresh serum contains active complement) or in fresh human sera (black bars) relative to the transduction of the same virions incubated in FCS. The values of vector incubations with sera of 3 different donors (A, B and C) are shown.

**[0123]** FIG. 4 shows histograms representing the percentage of transduction of unstimulated (white bars), SCF prestimulated (dashed bars), TPO prestimulated (vertical hatched bars), SCF+TPO prestimulated (horizontal hatched bars) or SCF+TPO+Flt3-L prestimulated (black bars) human CD34<sup>+</sup> cells by VSV-G (1), RD114/TR (2), BaEV/TR (3) or BaEVRLess (4) pseudotyped lentiviral vector particles at MOI of 10. Analysis for GFP expressing cells was performed 6 days after transduction.

**[0124]** FIG. 5 shows graphs representing the percentage of transduction of SCF/TPO prestimulated CD34<sup>+</sup> cells by VSV-G (x), RD114/TR (●), BaEV/TR (▲) or BaEVRLess (■) pseudotyped lentiviral vector particles at MOIs of 1, 5,

10, 20 (and 100 for VSV-G pseudotyped lentiviral vector particles). Analysis of GFP expressing cells was performed 6 days after transduction by FACS.

**[0125]** FIG. 6 shows graphs representing the percentage of transduction of SCF+TPO+Flt3-L prestimulated CD34<sup>+</sup> cells by VSV-G (x), RD114/TR (●), BaEV/TR (▲) or BaEVRLess (■) pseudotyped lentiviral vector particles at MOIs of 1, 5, 10, 20 (and 100 for VSV-G pseudotyped lentiviral vector particles). Analysis of GFP expressing cells was performed 6 days after transduction by FACS.

**[0126]** FIG. 7 shows histograms representing the percentage of erythrocyte, granulocyte and dendritic colony forming cells (CFCs) expressing GFP after transduction of SCF+TPO prestimulated (white bars) or SCF+TPO+Flt3-L prestimulated (black bars) cord blood-derived CD34<sup>+</sup> cells by VSV-G (1), RD114/TR (2), BaEV/TR (3) or BaEVRLess (4) pseudotyped lentiviral vector particles at MOI of 100 for VSV-G and 10 for other pseudotypes. 3 days after transduction the cells were seeded in a medium supplied with cytokines to induce myeloid differentiation. On day 14 of culture erythrocyte, granulocyte and dendritic colony forming cells (CFCs) were scored for GFP expression by fluorescence microscopy after seeding.

**[0127]** FIG. 8 shows histograms representing the percentage of erythrocyte, granulocyte and dendritic colony forming cells (CFCs) expressing GFP (black bars) and of CD34<sup>+</sup> cells expressing GFP (white bars) after transduction of SCF+TPO prestimulated cord blood-derived CD34<sup>+</sup> cells by VSV-G (1), RD114/TR (2), BaEV/TR (3) or BaEVRLess (4) pseudotyped lentiviral vector particles at MOI of 100 for VSV-G and 10 for other pseudotypes. 3 days after transduction the cells were seeded in a medium supplied with cytokines to induce myeloid differentiation. The percentage of GFP<sup>+</sup> CD34<sup>+</sup> cells was determined by FACS analysis 6 days after transduction. The percentage of GFP CFCs was determined by FACS analysis 17 days after transduction. Asterisk indicates that the proportion of GFP<sup>+</sup> CD34<sup>+</sup> cells differs statistically from the proportion of GFP colonies transduced cells (Student's T test: \*: p<0.05, NS: not significant).

**[0128]** FIG. 9 shows histograms representing the percentage of CD45<sup>+</sup> human cells (white bars), CD34<sup>+</sup> CD19<sup>-</sup> immature progenitor cells (black bars), CD34<sup>+</sup> CD19<sup>+</sup> B progenitors (dashed bars), CD20<sup>+</sup> B cells (vertical hatched bars), CD13<sup>+</sup> myeloid progenitors (horizontal hatched bars), and CD14<sup>+</sup> granulocytes and monocytes (diagonal hatched bars) in bone marrow expressing GFP after transduction of SCF+TPO prestimulated CD34<sup>+</sup> cells for 48 hours with VSV-G (1), RD114/TR (2), BaEV/TR (3) and BaEVRLess (4) pseudotyped lentiviral vector particles at MOIs indicated into brackets. Upon irradiation of newborn Rag2<sup>-/-</sup>;  $\gamma$ c<sup>-/-</sup> Balbc mice, they were injected intrahepatically with 2·10<sup>5</sup> transduced human CD34<sup>+</sup> cells. After 12 weeks of reconstitution, the mice were analyzed for human cell engraftment (CD45) in the bone marrow and the percentages of transduction (GFP<sup>+</sup> cells) of different hematopoietic cell were analyzed by FACS as indicated.

**[0129]** FIG. 10 shows histograms representing the percentage of CD45<sup>+</sup> human cells (white bars), CD34<sup>+</sup> CD10<sup>-</sup> CD19<sup>-</sup> very immature progenitor cells (black bars), CD34<sup>+</sup> CD10<sup>+</sup> CD19<sup>-</sup> B pre/pro B cells (dashed bars), CD19<sup>+</sup> CD20<sup>-</sup> preB cells (vertical hatched bars), CD34<sup>+</sup> CD10<sup>+</sup> CD19<sup>+</sup> immature B cells (horizontal hatched bars), CD20<sup>+</sup> mature B cells (diagonal hatched bars) and CD3<sup>+</sup> T cells (cross-hatched bars) in spleen expressing GFP after transduc-

tion of SCF/TPO prestimulated CD34<sup>+</sup> cells for 48 hours with VSV-G, RD114/TR, BaEV/TR and BaEVRLess pseudotyped lentiviral vector particles at MOIs indicated into brackets. Upon irradiation of newborn Rag2<sup>-/-</sup>;  $\gamma$ c<sup>-/-</sup> Balbc mice, they were injected intrahepatically with 2·10<sup>5</sup> transduced human CD34<sup>+</sup> cells. After 12 weeks of reconstitution, the mice were analyzed for human cell engraftment (CD45) in the spleen and the percentages of transduction (GFP<sup>+</sup> cells) of different hematopoietic cell were analysed by FACS as indicated.

**[0130]** FIG. 11 shows histograms representing the relative percentage of transduction of macaques bone marrow CD34<sup>+</sup> cells by VSV-G (1), RD114/TR (2), BaEV/TR (3) or BaEVRLess (4) pseudotyped SIV vector particles which were incubated at 37° C. for 1 hour in heat inactivated macaque sera (white bars; the serum was heat-inactivated at 56° C. before incubation with the vectors to inactivate the complement where fresh serum contains active complement) or in fresh macaque sera (black bars) relative to the transduction of the same virions incubated in FCS. The values of vector incubations with sera of 2 different macaques (A and B) are shown.

**[0131]** FIG. 12 shows histograms representing the percentage of SCF+Flt3-L+IL-3+IL-6 prestimulated cynomolgus macaque bone marrow CD34<sup>+</sup> cells expressing GFP after transduction, in presence of retronectin, by VSV-G (1), BaEV/TR (2) or BaEVRLess (3) pseudotyped SIV vector particles at MOIs indicated into brackets.

**[0132]** FIG. 13 shows histograms representing the percentage of SCF+Flt3-L+IL-3+IL-6 prestimulated rhesus macaque bone marrow CD34<sup>+</sup> cells expressing GFP after transduction, in presence of retronectin, by VSV-G (1), BaEV/TR (2) or BaEVRLess (3) pseudotyped SIV vector particles at MOIs indicated into brackets.

**[0133]** FIG. 14 shows histograms representing the percentage of IL-7 prestimulated T cells expressing GFP after transduction, in presence of retronectin (black bars) or not (white bars), by VSV-G (1), RD114/TR (2), BaEV/TR (3) or BaEVRLess (4) pseudotyped lentiviral vector particles at MOIs of 10.

**[0134]** FIG. 15 shows histograms representing the percentage of IL-7 prestimulated memory T cells (white bars) and naïve T cells (black bars) expressing GFP after transduction by VSV-G (1), RD114/TR (2), BaEV/TR (3) or BaEVRLess (4) pseudotyped lentiviral vector particles at MOIs indicated into brackets.

**[0135]** FIG. 16 shows histograms representing the percentage of TCR prestimulated T cells expressing GFP after transduction, in presence of retronectin (black bars) or not (white bars), by VSV-G (1), RD114/TR (2), BaEV/TR (3) or BaEVRLess (4) pseudotyped lentiviral vector particles at MOIs of 10.

**[0136]** FIG. 17 shows histograms representing the percentage of resting B cells expressing GFP after transduction, in presence of retronectin (black bars) or not (white bars), by VSV-G (1), RD114/TR (2), BaEV/TR (3) or BaEVRLess (4) pseudotyped lentiviral vector particles at MOIs indicated into brackets.

**[0137]** FIG. 18 shows histograms representing the percentage of memory resting B cells (white bars) and naïve resting B cells (black bars) expressing GFP after transduction by VSV-G (1), RD114/TR (2), BaEV/TR (3) or BaEVRLess (4) pseudotyped lentiviral vector particles at MOIs indicated into brackets.

**[0138]** FIG. 19 shows histograms representing the percentage of BCR prestimulated memory resting B cells (white bars) and naïve resting B cells (black bars) expressing GFP after transduction by VSV-G (1), RD114/TR (2), BaEV/TR (3) or BaEVRLess (4) pseudotyped lentiviral vector particles at MOIs indicated into brackets.

**[0139]** FIG. 20 shows histograms representing the percentage of MZL B cells expressing GFP after transduction, in presence of retronectin (black bars) or not (white bars), by VSV-G (1), RD114/TR (2), BaEV/TR (3) or BaEVRLess (4) pseudotyped lentiviral vector particles at MOIs indicated into brackets.

**[0140]** FIG. 21A shows graphs representing the percentage of GFP+ hCD45+ cells detected by FACS after 1<sup>st</sup> and 2<sup>nd</sup> engraftments of mice. Cord blood hCD34+ cells were pre-stimulated with a cytokine cocktail (TPO+SCF+Flk-3L) for 16 h and transduced with BaEV/TR-LVs and BaEVRLess-LVs at an MOI=10 for 36 h. Subsequently the cells (2×10<sup>5</sup>) were injected into the liver of irradiated newborn NOD/SCID  $\gamma$ c<sup>-/-</sup> mice (NSG mice). Upon reconstitution for 8-9 weeks, the different hematopoietic tissues (bone marrow, spleen and thymus) of these primary mice engraftments (1<sup>st</sup>) were analysed for human cell engraftment by anti-hCD45 staining. The percentage of GFP+ hCD45+ cells detected by FACS is indicated for each vector type and each hematopoietic tissue. Subsequently, the hCD34+ cells were isolated from the bone marrow of each of these primary engrafted mice, and 1-2×10<sup>5</sup> isolated hCD34+ cells were injected in one or two mice following the same procedure as for the primary engraftments. 8-9 weeks upon engraftment, these secondary (2<sup>nd</sup>) engrafted mice were analysed for transduced human cell engraftment (hCD45+ GFP+) in the bone marrow, spleen and thymus. The primary and corresponding secondary engraftments are indicated by the same symbol. FIG. 21B shows graphs representing the detection of GFP+ human B-cells (CD19+), human immature progenitor cells (CD34+) and myeloid progenitor cells (CD13+) by FACS in a bone marrow sample of a representative secondary recipient NSG mice for BaEV/TR-LV transduction.

**[0141]** FIG. 22A shows the schematic presentation of thymocyte differentiation. HSC, hematopoietic stem cell; DN, double negative; ISP, immature CD4 single positive cells; DP, double positive; SP4, single positive CD4+; SP8, single positive CD8+; TCR, T cell receptor. FIG. 22B shows histograms representing the percentage of freshly isolated thymocytes expressing GFP after transduction in the presence of IL-7 (20 ng/ml) with VSV-G-, RD114/TR-, BaEV/TR-, and BaEVRLess-LVs encoding the GFP reporter at an MOI of 10. Upon multiple staining for CD3, CD4 and CD8 the transduction in the different thymocyte subpopulations were determined by FACS. FIG. 22C shows histograms representing the distribution of the different thymocyte subpopulations upon incubation of the total thymocytes with the different vectors as compared to untransduced thymocytes.

**[0142]** FIG. 23A shows histograms representing the percentage of freshly isolated total CB T cells expressing GFP after prestimulation with IL-7 for 48 h and subsequent transduction with the different vectors: VSV-G-, RD114/TR-, BaEV/TR-, and BaEVRLess-LVs encoding the GFP reporter at an MOI of 10 or 20, or VSV-G-LVs at an MOI of 50. At day 3 of transduction cells were stained for human surface marker: CD45RA, CD62L, CD31 and the transduction efficiencies in the recent thymocyte emigrants (RA+/62L+/31+) and more mature naïve CB T cells (RA+/62L+/31-) were

determined by FACS analysis. FIG. 23B shows histograms representing the distribution of the different naive CB T cell subpopulations upon incubation of CB T cells with the different vectors as compared to untransduced T cells.

#### EXAMPLE

**[0143]** The following example demonstrates the advantageous properties of BaEV/TR- and BaEVRLess pseudotyped lentiviral vector particles for use as gene transfer vehicle as compared to previous pseudotyped lentiviral vectors.

#### Materials and Methods

##### Production of Lentiviral Vectors and Titering

**[0144]** Self-inactivating HIV-1-derived vectors were generated by transient transfection of HEK 293T cells, a human embryo kidney cell line (American Type Culture Collection, Rockville, Md., CRL-1573).  $2.6 \times 10^6$  HEK 293T cells were seeded onto 10-cm<sup>2</sup> tissue culture dishes 24 h before transfection. 293T cells were grown in Dulbecco modified Eagle medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum (FCS). Cells were transfected by Ca-phosphate precipitation with 8.6  $\mu$ g of the Gag-Pol packaging construct 8.91 and the GFP-encoding HIV-1 derived SIN transfer vector pHIV-SFFV-GFP-SIN and either 2.5  $\mu$ g of pMD.G, encoding the VSV-G glycoprotein (GP), or 7  $\mu$ g of pHCMV-RD114/TR, pHCMV-BaEVWT, pHCMV-BaEV/TR or pHCMV-BaEVRLess. At 16 h post-transfection, the DMEM medium was replaced by serum-free medium (Opti-MEM, Invitrogen) and 36 h after transfection, vectors were harvested, filtrated through 0.45  $\mu$ m pore-sized membrane and concentrated using an ultra-filtration concentration system (Vivaspin, Satorius; 2 h at 3000 g (4° C.)) or by low-speed concentration by centrifugation of the viral supernatant at 3000 g at 4° C. overnight or by ultracentrifugation (4° C., 25000 rpm, 2 h). Subsequently, vectors were stored at -80° C. To determine transduction efficiency and infectious titers of HIV vectors, serial dilutions of vector preparations were added to 293T cells and percentage of GFP cells was determined by flow cytometry 3 days after transduction. The infectious titers are expressed as infectious vector particles per milliliter (Infectious unit IU/mL). Multiplicities of infection (MOIs) were determined on proliferating 293T cells and are indicated in all transduction experiments.

##### Stability of Pseudotyped Vectors in Human or Macaque Serum.

**[0145]** Infectious pseudotyped HIV or SIV-vector particles (150000 GFP infectious units in 50  $\mu$ l suspension buffer) were mixed with 50  $\mu$ l heat inactivated or fresh human sera. As a reference, virions were incubated at 37° C. for 1 h and then used to transduce HEK293T target cells. The relative percentage of transduction of human sera-incubated virions to the transduction of the same virions incubated in FCS was calculated.

##### Sample Collection and Isolation of Human CD34+ Cells

**[0146]** Umbilical cord blood (CB) samples from full-term pregnancies were collected in sterile tubes containing anticoagulant (SIGMA) after informed consent according to the Helsinki declaration. Low-density PBMC cells were separated by centrifugation over a Lymphoprep cushion (Axis shield). CD34+ stem/progenitor cells were purified from the

mononuclear cell fraction with a CD34 magnetic isolation kit (Miltenyi MACS, Miltenyi Biotec) according to the manufacturer's instructions. Purity of the selected CD34+ fraction was assessed by FACS analysis with an APC-conjugated anti-CD34 antibody (BD Pharmingen) and exceeded 95% for all experiments. Subsequently, CD34+ cells were frozen in FCS (Fetal calf serum, Lonza), 10% DMSO and stored at -80° C.

##### T Cell and B Cell Isolation and Lentiviral Transduction

**[0147]** Adult peripheral blood samples, obtained from healthy adult donors after informed consent, were collected in acid citrate dextrose (ACD) containing tubes. CD3+ T-cells and CD19+ B cells were purified by negative selection using the Rosette tetrameric complex system (StemSep Technologies, Vancouver, Canada). Purity of isolated B- and T-cells was monitored using anti-hCD19APC and anti-hCD3APC antibodies, respectively, and was analyzed by fluorescence-activated cell sorting (FACScalibur; BD). T and B lymphocytes were cultured in RPMI 1640 medium (GibcoBRL Invitrogen, Auckland, New Zealand) supplemented with 10% FCS and penicillin/streptomycin. T cells were prestimulated with anti-CD3+anti-CD28 antibodies (1  $\mu$ g/ml) in presence of hIL-2 (1 ng/ml) or were pre-stimulated for 3 days with rIL-7 as described in Verhoeyen et al. (2003) *Blood* 101:2167-2174 (10 ng/ml; BDBiosciences, Le Pont de Claix, France). B cells were immediately seeded for transduction or pre-stimulated with *Staphylococcus Aureus* Cowan (SAC; 0.001%; Calbiochem, San Diego, Calif.)+IL-2 (1 ng/ml; SIGMA) as described in Levy et al. (2010) *Blood* 116:498-500. Briefly, 1E5 cells were seeded in 48-well-plates and concentrated vector was added at indicated doses. The percentage of GFP+ cells was determined by FACS 72 h post-transduction. Transduced T-cell cultures were continued in RPMI supplemented with rhIL-7, replenished every 3 days and harvested for FACS analyses, qPCR and Alu-PCR. B-cells were transferred to MS5 cell monolayer in RPMI supplemented with 10% AB serum, 5% FCS, 50 ng/ml rhSCF, 10 ng/ml rhIL-15 and 5 ng/ml rhIL-2 and medium was refreshed every 4 days.

##### Isolation of Macaque Bone Marrow CD34+-Cells

**[0148]** Adult male cynomolgus macaques (*Macaca fascicularis*) 3-4.5 kg were imported from Mauritius and housed in single cages within level 2 biosafety animal facilities (CEA, Fontenay-aux Roses), in accordance with national guidelines. All experimental procedures were conducted in accordance with European guidelines for animal care. Studies were conducted under protocols approved by the regional committee for animal experimentation. All procedures and blood samplings were performed after animals had been anesthetized with ketamine 10 mg/kg (Imalgène 1000).

**[0149]** Bone marrow mononuclear cells were obtained from the humeri and iliac crest by aspiration and isolated by standard Ficoll density-gradient centrifugation. Cells were washed twice in phosphate buffered saline (PBS) and resuspended in PBS/1% FCS. The fraction was then enriched in CD34+ cells by positive immunomagnetic selection (clone 561; Dynabeads M-450 CD34; Dynal) accordingly to the manufacturer's instructions. Following immunoselection, the CD34+ cell purified fraction was stained using an anti-CD34 monoclonal antibody (clone 563, BD-Pharmingen). Purity of the CD34+ selected cells ranged from 85-95%.

#### Transduction of Human and Macaque HSCs.

**[0150]** Human and macaque CD34<sup>+</sup> cells were incubated overnight in 24-well plates in serum-free medium (CellGro, CellGenix) supplemented with antibiotics (PENSTREP, Invitrogen) and with human recombinant cytokines (as indicated): stem cell factor (rSCF, 100 ng/ml), Thrombopoietin (TPO, 20 ng/ml) and Flt3 ligand (Flt3-L, 100 ng/ml) (Preprotech) for human CB CD34<sup>+</sup> cells. For cynomolgus macaque bone marrow CD34<sup>+</sup> cells, human recombinant interleukin (IL-6) (20 ng/ml) and interleukin (IL-3) (20 ng/ml) (Preprotech) were added to the previous cocktail. 5·10<sup>4</sup> prestimulated CD34<sup>+</sup> cells were transduced in 48-well plates with concentrated lentiviral vector supernatant at MOIs of 1, 5, 10, 20 or 100 (as indicated) in serum-free medium (CellGro, CellGenix). Where indicated, transductions were performed on retronectin coated plates (recombinant human fibronectin fragment CH-296) (Takara). On day 3 after transduction, cells were replenished with cytokines. After 3 and 6 days of transduction, percentage of GFP<sup>+</sup> cells was determined by flow cytometry.

#### Clonogenic Assay of CD34<sup>+</sup> Cells

**[0151]** CD34<sup>+</sup> cells were transduced as described herein above, washed in PBS (Invitrogen) and plated at a density of 1000 cells/ml in complete methylcellulose medium (1% methylcellulose, 15% FBS, 1% BSA, bovine pancreatic insulin [1 µg/ml], iron-saturated transferrin [200 mg/ml], 10<sup>-4</sup> M 2-mercaptoethanol, 2 mM L-glutamine, SCF [50 ng/ml], IL-3 [10 ng/ml], IL-6 [10 ng/ml], erythropoietin [3 U/ml]) (Stem Cell technologies). GFP<sup>+</sup> colonies were scored by light and fluorescence microscopy 14 days after seeding.

#### Western Blot Analysis

**[0152]** BaEVwt, BaEV/TR and BaEVRLess pseudotyped LVs were purified over a sucrose-cushion by ultracentrifugation (2 h, 25000 rpm, 4° C.) and were frozen after resuspension in PBS. The upper part of the membrane was stained with antibodies against the surface domain of BaEV glycoprotein, the lower part of the membrane was stained with antibodies against the p24 HIV-1 capsid to assess equivalent loading of purified vectors. The positions of the BaEV mutant glycoproteins and the HIV capsid were indicated.

#### Animals

**[0153]** BALB/c Rag2<sup>-/-</sup>, γc<sup>-/-</sup> immunodeficient mice were obtained from Dr Mamoro Ito, and Taconic (CIEA, Kawasaki, Japan). All mice used were housed in the animal facility of the Ecole Normale Supérieure de Lyon, France (PBES). The animals were kept the whole time of the experiment under sterile conditions and all experiments were performed in accordance with the guidelines of the Institutional animals care upon approval of protocols by the local ethical committee.

#### Conditioning and Reconstitution of Balb-c Rag2<sup>-/-</sup>, γc<sup>-/-</sup> Mice

**[0154]** The 2- to 4-day-old newborn BALB/c Rag2<sup>-/-</sup>, γc<sup>-/-</sup> mice were subjected to a sublethal irradiation of 2×1.5Gy. For evaluation of reconstitution/differentiation capacity of transduced hCB-CD34<sup>+</sup> cells, the latter cells were transduced at an MOI of 10 with BaEV/TR-, BaEVRLess-, RD114/TR-, VSV-G-LVs and 48 h after transduction, 2.10<sup>5</sup> cells were injected intra-hepatically into newborn mice. At 10-12 weeks of

human cell engraftment, peripheral blood was taken from the facial vein to check the reconstitution efficiency by analyzing the percentage of human engraftment (hCD45<sup>+</sup> cells) by FACS.

#### FACS Analysis

**[0155]** The mice were sacrificed at 10-12 weeks of human cell engraftment in agreement with bioethical procedures and all hematopoietic tissues were harvested. Subsequently the cells from bone marrow, spleen, thymus and peripheral blood were extracted. For the detection of LV transduction of the engrafted cells, flow cytometry analysis was performed using APC-coupled anti-hCD45 antibody for the detection of total human cell engraftment in the bone marrow. APC-coupled antibodies (BD Pharmingen) were used for the detection of hCD19 (B cells), hCD34 (early progenitor cells) and hCD13 (myeloid progenitors).

#### Results

##### The Mutant BaEV/TR Glycoprotein and BaEVRLess Glycoprotein can Pseudotype HIV-1 Vector Efficiently

**[0156]** The BaEV/TR chimeric glycoprotein was engineered by the inventors by exchange of its cytoplasmic domain for the one of the MLV-A glycoprotein (FIG. 1). A second mutant was engineered by the deletion of the fusion inhibitory R peptide from the carboxy-terminal cytosolic domain resulting in BaEVRLess glycoprotein (FIG. 1). The inventors compared these different mutant BaEV glycoproteins in their ability to pseudotype lentiviral vectors derived from HIV-1 to VSV-G and RD114/TR glycoproteins.

**[0157]** RD114/TR, BaEV/TR, BaEVRLess and VSV-G pseudotyped HIV vectors were generated by transient transfection in HEK293T cells together with the gagpol packaging and HIV vector encoding for GFP. The vector supernatants were concentrated by low speed or ultracentrifugation (100-fold). Infections assays on HEK293T cells indicated that titers higher than 1·10<sup>7</sup> IU/ml were obtained for BaEV/TR and BaEVRLess pseudotyped LVs resulting in a 40-fold titer increase as compared to BaEVwt-LVs. RD114/TR and VSV-G pseudotyped vectors gave titers of 6·10<sup>7</sup> IU/ml and 1·10<sup>9</sup> IU/ml (FIG. 2). The immunoblot confirmed a lower incorporation of the BaEVwt glycoprotein on the lentiviral vector surface as compared to BaEV/TR and BaEVRLess glycoproteins at equal amount of HIV-1 capsid. These data were in agreement with the increase in titers obtained for BaEV/TR and BaEVRLess pseudotyped LVs as compared to BaEVwt-LVs.

**[0158]** Infectious titers of BaEV mutant glycoproteins pseudotyped LVs (BaEV/TR and BaEVRLess) were similar to the ones of RD114/TR-LVs and were therefore further considered for transduction of human CD34<sup>+</sup> early progenitor cells and T and B cells.

##### Stability of BaEV Mutant Pseudotyped Vectors and Resistance to Human Serum Inactivation

**[0159]** In view of clinical in vivo application, BaEV mutant pseudotyped LVs must resist to the human complement system. This stability was determined by comparing infectious titers of LVs incubated in human sera 1 hour at 56° C. Heat is reported to destroy human complement, so heat inactivated human sera were used as control. Residual infectious titers obtained were reported to the infectivity of LVs incubated

with fetal calf serum (100%) (FIG. 3). As expected the VSV-G pseudotyped LVs were inactivated by the 3 different human sera. BaEV/TR and BaEVRLess pseudotyped LVs remained stable in presence of complement containing sera since no reduction in transduction efficiency was detected upon incubation in the presence of human complement for the three different donors. Thus, the inventors concluded that BaEV/TR and BaEVRLess LVs were resistant to the human complement system to the same extent as RD114/TR-LVs. The stability of BaEV mutant pseudotyped LVs in human serum makes them better candidates than VSV-G LV pseudotypes for in vivo applications.

**BaEV Pseudotyped LVs are Highly Superior Over VSV-G- and RD114/TR-LVs for the Transduction of Human CD34<sup>+</sup> Cells**

**[0160]** Both BaEV/TR and BaEVRLess pseudotyped LVs bind to hASCT1 and hASCT2 on the cell membrane of hCD34<sup>+</sup> cells while the RD114/TR LVs only bind to hASCT2. It was described previously that RD114/TR-LVs can only transduce hCD34<sup>+</sup> cells efficiently in the presence of retronectin. Indeed, retronectin is a fragment of fibronectin that is reported to allow binding of both cells and vectors and therefore increases transduction of hCD34<sup>+</sup> cells. To evaluate the role of retronectin in BaEV pseudotyped LVs transduction, the inventors initially prestimulated hCD34<sup>+</sup> cells with a strong cytokine cocktail (rSCF+rTPO+rFlt3-L) and transduced them with VSV-G, RD114/TR, BaEV/TR, BaEVRLess pseudotyped LVs at a MOI of 10 in wells coated or not with retronectin. The inventors observed that, in the presence of retronectin, both BaEV-LVs transduced hCD34<sup>+</sup> cells at more than 70%.

**[0161]** As mentioned above, a strong stimulation with a cytokine cocktail will induce HSCs into differentiation and their loss of self renewal capacity. Indeed, the less the cells are prestimulated, the less they differentiate and the more they keep their 'stem cell' character. Moreover, the stronger the cytokine stimulation the higher the risk is that multiple vector integrations per cell will occur at high vector doses. This might increase the risk of genotoxicity in gene therapy applications. Therefore, the inventors tested if BaEV pseudotyped LVs could transduce efficiently hCD34<sup>+</sup> cells after a low cytokine prestimulation combined with low vector doses.

**[0162]** Firstly, they applied vectors at a fixed MOI (number of infectious particles per cells) of 10 and different cytokine prestimulation protocols for hCD34<sup>+</sup> cells (FIG. 4): 1) no stimulation; 2) stimulation with a single cytokine: rSCF or rTPO; 3) rSCF and rTPO; 4) rSCF+rTPO+rFlt3-L. Importantly, the strongest cytokine pre-stimulation used here was lower than stimulation cocktails currently used in clinical setting with the objective to limit cell differentiation. The inventors observed that a single TPO stimulation was sufficient for a transduction of hCD34<sup>+</sup> cells up to 60% for BaEV pseudotyped LVs, while VSV-G-LVs and RD114/TR-LVs transduced only 8-20% hCD34<sup>+</sup> cells upon TPO stimulation. SCF single prestimulation permitted up to 30-50% hCD34<sup>+</sup> cell transduction while VSV-G-LVs and RD114/TR-LVs did only reach transduction levels of 10%. A combination of TPO+SCF or TPO+SCF+FLK-3 prestimulation with a single BaEV-LV incubation resulted in up to 75% hCD34<sup>+</sup> cell transduction, while RD114/TR-LVs achieved not more than 40% of transduction. Concluding, for all the different cytokine prestimulation protocols BaEV LV pseudotypes outperformed VSV-G- and RD114/TR-LVs for hCD34<sup>+</sup> cell trans-

duction. Moreover, results obtained on unstimulated cells indicated that BaEV/TR LVs and BaEVRLess LVs were able to transduce 10-20% of unstimulated hCD34<sup>+</sup> cells in the presence of retronectin. In contrast, VSV-G- and RD114/TR LVs were unable to transduce unstimulated quiescent hCD34<sup>+</sup> cells efficiently at the same vector doses (MOI=10).

**[0163]** Secondly, the risk of insertional mutagenesis must be avoided to limit gene therapy side effects. Therefore, the inventors decreased the vector dose in order to limit multi-copy integrations. Different MOIs were tested under rSCF+rTPO and rSCF+rTPO+rFlt3-L stimulations (FIGS. 5 and 6). BaEV/TR pseudotyped LVs transduced notably better hCD34<sup>+</sup> cells at low vector doses reaching 60% (rSCF+rTPO) and 70% (rSCF+rTPO+rFlt3-L) at a MOI of 5. BaEVRLess-LVs were highly superior over the RD114/TR-LVs at a MOI of 5. VSV-G LVs, remained at low vector doses (MOI 5-10) much less efficient. Only at an MOI of 100 these VSV-G-LVs allowed efficient transduction of hCD34<sup>+</sup> cells, a vector dose currently used for clinical gene therapy trials. But the VSV-G-LV transduction rate remained far below that of BaEV-LV (VSV-G-LVs (MOI of 100)=50-60% and BaEV-LVs (MOI 20)=75-90%; FIGS. 5 and 6). Under the higher stimulation condition, the inventors noted that BaEV/TR-, BaEVRLess-LVs and RD114/TR-LVs reached a plateau at a MOI of 10 for BaEV/TR-LVs and RD114/TR LVs (75% and 50% of transduction respectively). Consequently, incubation with low vector doses of BaEV LVs inferior or equal to 10 were sufficient to transduce hCD34<sup>+</sup> cells very efficiently and to a much higher extent than RD114/TR-LVs and VSV-G LVs. In conclusion, BaEV pseudotyped LVs and particularly BaEV/TR-LV appeared to be more effective to transduce hCD34<sup>+</sup> cells than the currently available VSV-G-LV and RD114/TR-LV vectors. Clearly, the VSV-G-LV hCD34<sup>+</sup> cell transduction efficiency is dependent on the cytokines stimulation cocktail while for the BaEV pseudotyped LVs this is not the case.

**Low BaEV-L V Vector Doses Allow Transducing at a High Rate Short Term Progenitors.**

**[0164]** The inventors wanted to confirm that the high hCD34<sup>+</sup> cell transduction obtained with BaEV pseudotypes were due to stable transduction of short term progenitors. Thus, they determined the transduction efficiency of BaEV pseudotyped LVs on hematopoietic progenitors thanks to a clonogenic assay performed in a semi-solid methylcellulose medium. Cells prestimulated with rSCF+rTPO and rSCF+rTPO+rFlt3-L were transduced with RD114/TR, BaEV/TR, BaEVRLess pseudotyped vectors at a MOI of 10 and VSV-G at a MOI of 100. The inventors chose a higher MOI for VSV-G because clinical trials currently work with a MOI of 100 for this pseudotype to transduce hCD34<sup>+</sup> cells. It thus appeared more relevant to use this high vector dose for comparison. Three days after transduction, cells were cultured in a methylcellulose medium (complemented with cytokines) which allowed their differentiation into myeloid colonies. The inventors observed after 14 days erythroblastic, granulomonocytic and mixed colonies expressing GFP or not. First, they compared the number of GFP expressing colonies for different prestimulations (FIG. 7). For all vectors tested, both stimulations gave similar results. The proportion of clonogenic progenitors transduced with both BaEV mutant pseudotyped vectors at a MOI of 10 was higher than with VSV-G ones at a MOI of 100 (BaEV/TR-LV=50-70%, BaEVRLess-LV=45-70%, VSV-G-LV=25-50%). At the same

vector dose used for BaEV/TR and BaEVRLess pseudotyped LVs, only 10-35% of clonogenic progenitors were transduced with RD114/TR LV pseudotypes. There was no significant difference between the proportion of RD114/TR-, BaEV/TR-, BaEVRLess LV transduced CD34<sup>+</sup> cells after a rSCF+rTPO prestimulation and the clonogenic progenitors derived

ment of a complete human blood system since they lack T, B and NK cells.

**[0167]** After 8-10 weeks, the animals were sacrificed and the percentage of total human blood nucleated cells (CD45<sup>+</sup>) in the bone marrow was determined by FACS analysis (Table 1).

TABLE 1

Pseudotype	BaEV/TR- and BaEVRLess-LVs allow efficient transduction of SCID repopulating cells							
	Bone marrow	Bone marrow					Spleen	Thymus
	Engraftment (%)	CD45+ GFP+ (%)	CD34+ GFP+ (%)	CD19+ GFP+ (%)	CD13+ GFP+ (%)	CD14+ GFP+ (%)	CD45+ GFP+ (%)	CD45+ GFP+ (%)
<b>VSV-G</b>								
1(*)	22.1	32.0	18.3	41.0	5.7	7.7	NA	NA
2(*)	2.2	6.8	27.7	15.5	9.6	4.0	NA	NA
3(*)	57.3	5.7	17.7	12.5	22.3	19.2	NA	NA
<b>RD114/TR</b>								
1(*)	32	3.2	2.9	2.8	4	7	NA	NA
2(*)	15.3	39.0	33.8	38.6	34.2	35.0	NA	NA
3(*)	2.3	17.6	16.5	21.8	11.3	13.5	NA	NA
4(*)	34.0	27.2	40.1	27.1	24.1	18.7	NA	NA
5(°)	30.6	76.5	64.5	78.8	57.4	45.2	37.0	41.2
6(°)	25.3	12.6	13.8	10.0	11.1	4.8	20.5	15.8
<b>BaEV/TR</b>								
1(*)	23.6	36.0	41.4	39.1	37.4	56.6	NA	NA
2(*)	21.6	69.7	78.9	68.0	73.7	68.7	NA	NA
3(°)	27.6	92.0	91.7	89.7	82.5	72.7	84.2	84.3
4(°)	36.4	59.3	40.0	59.8	68.0	33.3	69.4	65.5
<b>BaEVRLess</b>								
1(*)	23.8	33.9	35.7	38.7	35.6	38.6	NA	NA
2(*)	10.0	56.2	70.2	66.1	59.3	65.5	NA	NA
4(*)	17.6	70.5	64.4	76.2	67.9	67.6	NA	NA
5(°)	64.1	55.5	51.5	56.0	56.9	59.4	52.0	76.9
6(°)	52.8	52.8	56.0	46.6	61.1	60.0	42.5	4.1
7(°)	89.5	87.5	83.8	86.7	83.1	83.9	69.4	74.8

(\*)Balbc, Rag2<sup>-/-</sup>, γc<sup>-/-</sup> mice

(°)NOD/SCID/γc<sup>-/-</sup> mice

from them indicating that hCD34<sup>+</sup> cell transduction was stable (FIG. 8). In contrast, for VSV-G LVs, the proportion of transduced clonogenic progenitors was significantly lower than the rate of transduced CD34<sup>+</sup> cells they were derived from (FIG. 8) suggesting that VSV-G pseudotyped vectors transduced more differentiated CD34<sup>+</sup> cells or were more toxic than other vectors tested.

**[0165]** These results showed that BaEV/TR- and BaEVRLess-LV pseudotypes stably transduced at a high rate short term repopulating progenitor cells.

#### BaEV Pseudotyped LVs Transduce at High Efficiency Repopulating Hematopoietic Progenitors in Immunodeficient Mice

**[0166]** To determine the transduction efficiency of both BaEV mutant pseudotyped vectors on repopulating hematopoietic progenitors, cord blood CD34<sup>+</sup> cells were prestimulated 16 h with rSCF, rTPO and FLk-3L and then transduced with RD114/TR, BaEV/TR, BaEVRLess pseudotyped vectors at a MOI of 10 or with VSV-G-pseudotyped LVs at a MOI of 100 and transplanted into sublethally irradiated immunodeficient Balbc, Rag2<sup>-/-</sup>, γc<sup>-/-</sup> new born mice or NOD/SCID/γc<sup>-/-</sup> (NSG) mice (Table 1). Both mice models allow engraft-

**[0168]** A comparable high level of engraftment with human cells was detected for all transduction conditions (up to 89%, Table 1). However, overall VSV-G-LVs allowed a much lower transduction of scid repopulating cells as compared to the other vectors. For the VSV-G-LVs, the inventors detected 32% transduced hCD45<sup>+</sup> cells in the total bone marrow of mice 1 (Table 1). In bone marrow, for VSV-G pseudotyped LV used at a MOI of 100, they observed a transduction bias since a majority of pre-B and pro B cells (CD19<sup>+</sup> CD34<sup>+</sup> cells) showed 33% of transduction but early progenitors (CD34<sup>+</sup> CD19<sup>-</sup>) and myeloid progenitors (CD13<sup>+</sup>) and monocytes (CD14<sup>+</sup>) showed only 5 to 8% of transduction at most (FIG. 9).

**[0169]** In contrast, for BaEV/TR- and BaEVRLess-LVs detailed phenotyping showed that very early progenitor cells (CD34<sup>+</sup> CD19<sup>-</sup>), as well as B-cell CD19<sup>+</sup> progenitors and myeloid progenitor CD13<sup>+</sup>-cells were transduced to the same extent since over 30% transduction for all these different cell lineages was detected (FIG. 9). Table 1 shows that BaEV/TR-LVs and BaEVRLess-LVs transduced hCD34<sup>+</sup> cells resulted in high reconstitution levels in the bone marrow and the highest transduction levels of SCID repopulating cells (up to 90% CD45+GFP+ cells) in the bone marrow. Moreover, these



high transduction levels were sustained in the primary recipient mice in all hematopoietic tissues (bone marrow, thymus and spleen Table 1). Equivalent high level transduction was detected for immature progenitors (hCD34+ cells), lymphoid cells (CD19+) and myeloid cells (CD13+ and CD14+) in the bone marrow (Table 1).

**[0170]** For RD114/TR-LVs less than 5% transduction in all the different bone marrow cell lineages was detected for mice NR1. An identical picture was found for progenitors, T and B cells and monocytes in the spleen (FIG. 10). RD114/TR-LVs demonstrated variable transduction levels of SCID repopulating cells (ranging from 3.2-76% of GFP+ CD45+ cells, Table 1).

**[0171]** Overall, these data strongly suggested that BaEV/TR- and BaEVRLess-LVs allowed transduction of very early progenitor repopulating cells, so-called hHSC, able to differentiate into all the different lineages in immunodeficient mice. In contrast, VSV-G LV pseudotypes did not result in equivalent transduction of the different hematopoietic lineages indicating that VSV-G-LVs transduced more differentiated progenitors and less true HSCs as compared to BaEV/TR- and BaEVRLess-LVs.

**[0172]** To confirm that true HSC were gene modified, secondary reconstitutions of NSG mice were performed with bone marrow hCD34 cells of primary NSG mice previously reconstituted with BaEV/TR- and BaEVRLess-LV transduced hCD34+ cells for at least 2 months.

**[0173]** To improve engraftment into secondary recipients the primary NSG bone marrow derived hCD34+ cells were cultured in SCF and TPO to increase or at least maintain the homing capacity of these cells. Following 16-18 h of pre-stimulation,  $1-2 \times 10^5$  hCD34+ cells were injected into the liver of newborn NSG mice subjected to sub-lethal irradiation. At 8-9 weeks post-transplantation, the bone marrow, spleen and thymus of the secondary recipient mice were analysed for the percentage of human CD45+ GFP+ cells (FIG. 21A). In the spleen and bone marrow the percentage of GFP+ hCD45+ cells was maintained or increased in the secondary recipient mice (FIG. 21A). Moreover, for both BaEV/TR- and BaEVRLess-LVs high levels of GFP+ hCD34+ early progenitor, lymphoid (CD19+) and myeloid (CD13+) cells were detected in the bone marrow of these secondary recipient mice and equivalent percentages of GFP+ cells were detected in these different lineages (FIG. 21B). This indicated that the secondary reconstituting SCID repopulating cells were capable of multilineage differentiation and thus that true human HSC were genetically modified to high levels by BaEV/TR- and BaEVRLess-LVs.

BaEV/TR and BaEVRLess Pseudotyped SIV Vectors Transduce Efficiently Rhesus and Cynomolgus Macaque CD34+ Bone Marrow Cells.

**[0174]** Lentiviral vectors based on HIV transduce hCD34+ cells very efficiently, while they transduce the corresponding cells from old world monkeys (baboons, rhesus and cynomolgus macaques) very poorly. This is attributed to restriction factors (TRIM5a) interfering at several post-entry steps of HIV in simian cells. Thus, since the cynomolgus macaque is an important animal model, the inventors switched to a SIV (Simian Immunodeficiency Virus) based LV system, previously developed by the inventors (Negre et al. (2000) *Gene Ther.* 7:1613-1623), which was not subjected to these post-entry restrictions and allowed efficient transduction of

macaque T-cell and CD34+ cells. For all pseudotypes, they obtained similar infectious titers for SIV-derived pseudotypes as compared to HIV vectors.

**[0175]** The stability of the pseudotyped vectors in cynomolgus macaque sera was evaluated. The same quantities of pseudotyped infectious particles were mixed with fresh macaque sera and with macaque sera incubated for 1 h at 56° C. which enabled inactivating the complement system. In contrast to VSV-G LVs that were readily inactivated in fresh sera, RD114/TR-, BaEV/TR- and BaEVRLess-LV pseudotypes remained stable upon incubation with macaque sera evaluated (FIG. 11). This was confirmed for two independent macaque donors.

**[0176]** In order to evaluate the macaque CD34+ cell transduction, the inventors pre-stimulated cynomolgus macaque CD34+ cells derived from bone marrow with a strong cytokine cocktail (rSCF+rTPO+rFlt3-L+rIL-3+rIL-6) during 16 h. They transduced them with VSV-G, RD114/TR, BaEV/TR, BaEVRLess pseudotyped SIV-LVs at a MOI of 10 or 20 in culture wells coated with retronectin (FIG. 12). As reported for VSV-G-LV pseudotypes, they observed that they allow low level transduction of the macaque CD34+ cells independently of the presence of retronectin. Importantly, RD114/TR, BaEV/TR and BaEVRLess LV pseudotypes needed retronectin to transduce macaque CD34+ cells. BaEV/TR pseudotyped SIV-LVs at an MOI of 20 transduced up to 60% of cynomolgus macaque CD34+ cells while the BaEVRLess LVs showed up to 45% of transduction (FIG. 12). The transduction of rhesus macaque CD34+ cells was slightly different since BaEV/TR-LVs allowed the highest transduction levels (50%) and were superior over VSV-G- and BaEVRLess-LVs in efficiency (FIG. 13).

**[0177]** These first results indicated that BaEV/TR- and BaEVRLess-LV pseudotypes efficiently transduced cynomolgus and rhesus macaque CD34+ cells which means that it is possible to evaluate these vectors for pre-clinical testing of gene therapy vectors in these primate models, which are biologically very close to humans.

BaEV/TR- and BaEVRLess-LVs Allow Efficient Transduction of rIL-7 and T Cell Receptor Stimulated Human T Cells

**[0178]** Another essential target for gene therapy is T cells. Naive T cells are 'the' gene therapy targets of interest since these are the long-living cells that when corrected might persist over years. BaEV/TR- and BaEVRLess-LVs allowed efficient transduction of rIL-7 pre-stimulated human T cells and were superior over VSV-G-LVs for the same vector doses (MOI=10) in the presence of retronectin (% transduction, BaEV/TR-LV=25-43%, BaEVRLess=25-45%, VSV-G-LV=5-15%; FIG. 14). Surprisingly, the RD114/TR-LVs allowed much lower transduction efficiency of rIL-7 pre-stimulated T cells as compared to BaEV-LVs (RD114/TR-LV=5-15%). Moreover, the BaEV/TR- and BaEVRLess-LVs allowed an efficient high transduction level of both naive and memory IL-7 prestimulated T cells (FIG. 15). Since rIL-7 is a T cell survival cytokine regulating T cell homeostasis, no naive to memory phenotypic switch is induced as compared to a T-cell receptor (TCR) stimulation which induced a switch of naive to memory T cell phenotype. Indeed, BaEV/TR- and BaEVRLess-LVs allowed transduction of IL-7 stimulated T-cells without compromising their naive phenotype. This is highly relevant for many gene therapy applications.

**[0179]** Up to date the majority of the clinical trials uses TCR stimulated T cells. Of high interest, the BaEV/TR- and BaEVRLess-LVs permitted up to 90% T cell gene transfer in

TCR stimulated at low vector doses (MOI=10) and thus outperformed by far VSV-G- and RD114/TR-LVs for transduction of TCR prestimulated T cells (FIG. 16).

**[0180]** Overall, the novel BaEV/TR- and BaEVRLess-LVs are promising gene transfer tools for T cell gene therapy.

#### BaEV/TR- and BaEVRLess-LVs Allow Efficient Transduction of Unstimulated and BCR Stimulated Human B Cells

**[0181]** B lymphocytes are attractive targets for gene therapy of genetic diseases associated with B-cell dysfunction. In addition, long-lasting transgene expression in B-cells is of particular interest for immunotherapy by its potential to induce specific immune activation or tolerance. Moreover, efficient delivery of genes or shRNAs for gene expression knock-down into primary human B lymphocytes would allow studying gene functions in these cells. However, primary B-cells remain very poorly transducible with VSVG-pseudotyped LVs even when stimulated into proliferation by cross-linking of CD40 in the presence of various cytokines.

**[0182]** Since BaEV glycoprotein pseudotyped LVs were able to transduce T cells, the inventors evaluated them for gene transfer into primary human B cells.

**[0183]** VSV-G-LVs were unable to transduce unstimulated resting human B cells efficiently even at high vector doses (MOI=100, VSV-G-LV<5% transduction). Of interest, RD114/TR-LVs allowed only low transduction levels of up to 8% of primary resting B cells (FIG. 17). BaEV/TR-LVs and BaEVRLess-LVs permitted highly efficient transduction of 20-30% of resting B cells and thus outperformed the VSV-G-LVs and RD114/TR-LVs by far (FIG. 17, Retronectin). Upon B cell receptor (BCR) stimulation, BaEV/TR- and BaEVRLess-LVs transduced up to 70% of the B cells while VSV-G-LV transduction efficiency did not exceed 5%. RD114/TR-LVs allowed transduction of 25% of BCR stimulated primary B cells. Of importance, BaEV/TR- and BaEVRLess-LVs transduced as well memory as naive B cells efficiently in the presence of retronectin (FIGS. 18 and 19). Of interest, primary marginal zone lymphoma (MZL) B cells were efficiently transduced by BaEV/TR- and BaEVRLess-LVs (up to 75%) in the presence of retronectin (FIG. 20).

**[0184]** Thus, BaEV/TR-LVs and BaEVRLess-LVs outperformed VSV-G-LVs and RD114/TR-LVs for the transduction of human healthy and cancer B cells and might be promising tools for B cell based gene and immunotherapy

#### BaEVgp LVs Allow High-Level Transduction of Thymocytes in Early Stages of Differentiation

**[0185]** The thymus is the primary site of T cell development and plays a key role in the induction of self-tolerance. Therefore, targeting the thymus with an antigen of interest can result in the induction of tolerance. Indeed, this has been achieved by the direct injection of soluble antigen, of viral vector harboring an antigen of interest or even of entire cells (Khoury et al. (1993) *J Exp Med.* 178:559-66, Marodon et al. (2006) *Blood* 108:2972-8, Posselt et al. (1992) *Science* 256: 1321-4). Secondly, in situ correction of a genetic immunodeficiency, ZAP-70, by intra-thymus injection of a lentiviral vector expressing the correcting gene was demonstrated (Adjali et al. (2005) *J Clin Invest.* 115:2287-95).

**[0186]** Thymocyte differentiation is a highly regulated process that occurs via the interaction with the thymic stroma cells and cytokine stimulation (FIG. 22A). Hematopoietic

stem cells (CD34+ CD1a-) are migrating from the bone marrow to the thymus where they first gain CD1a expression and lack the expression of CD8 and CD4 markers (CD8-CD4- double negative (DN) stage; 2-5% of total thymocytes). These DN cells subsequently develop into immature CD4 single positive cells (ISP) and gain expression of the pre-T cell receptor (pre-TCR; FIG. 22A). The appearance of the CD8 surface marker together with CD4 and maturation of the TCR is called the double positive (DP) stage of thymocyte development (80-90% of the total thymocytes). Upon selection in the thymus DP thymocytes develop into CD8+ CD4- (SP8) and CD8-CD4+ (SP4) thymocytes (FIG. 22A).

**[0187]** Because of the potential value of thymocyte gene transfer for immune modulation, it is important to develop vectors for efficient thymocyte transduction. Therefore, the inventors evaluated the performance of BaEVgp-LVs compared to the other LV pseudotypes for the transduction of all these different thymocyte subpopulations. Upon isolation of thymocytes from a human thymus, they transduced them in the presence of the survival cytokine IL-7 with VSV-G-, RD114/TR-, BaEV/TR-, and BaEVRLess-LVs encoding for the GFP reporter at an MOI of 10. For all transduction experiments retronectin coated plates were used. Three days later we determined the transduction levels by FACS in the different thymocyte subpopulations using CD4/CD8/CD3 triple staining.

**[0188]** VSV-G-LVs do not allow efficient transduction of all these different subpopulations reaching between 10 and 20% of transduced cells (FIG. 22B). RD114/TR-LV, BaEV/TR-LVs and BaEVRLess-LVs though allow preferential transduction of the thymocytes in the early stages of development (DN and ISP) while they still transduce the more mature to a lower level (FIG. 22B). As already detected for adult human T cells, BaEVgp LVs transduced all different thymocyte subpopulations more efficiently than the RD114/TR-LVs. Moreover, transduction with the different lentiviral vector pseudotypes had no effect on the distribution of different thymocyte subpopulations (FIG. 22C). This indicates that BaEVgp-LVs are excellent tools for transduction of thymocytes and that they are especially superior over VSV-G- and RD114/TR-LVs for the transduction of the immature DN and ISP thymocyte subpopulations.

#### BaEVgp-LVs Allow High-Level Transduction of Cord Blood Recent Thymocyte Emigrants

**[0189]** It might be of high interest for T cell gene therapy or immunotherapy to genetically modify truly naive T cells, which are understood to be the cells persisting for the entire life-span of an individual. Therefore, the inventors focused on cord blood (CB) T cells since they are gaining importance in the context of allogeneic CB transplantations. The majority of CB T cells are naive and provide the advantage that they represent a reduced risk of graft-versus-host disease (GVHD), a common side effect seen in bone marrow allogeneic transplantation including allogeneic adult T cells. In contrast to adult T cells, though, CB T cells exert a reduced graft-versus-leukemia effect.

**[0190]** The majority of CB T cells (70-80%) are identified by CD31, CD62L and CD45RA positive staining which is a highly immature naive T cell population and are called recent thymocyte emigrants (RTEs). Genetic modification of these cells in the context of allogeneic transplantation to increase their graft-versus-leukemia effect without increasing GVHD would be beneficial (Mickelthwaite et al. (2010) *Blood* 115:

2695-703). Therefore, the inventors tested several lentiviral vector pseudotypes for their transduction efficiency of CB RTEs.

**[0191]** To conserve the T cell naive phenotype they pre-stimulated freshly isolated total CB T cells with the T cell survival cytokine, IL-7, and subsequently transduced them with the different vectors: VSV-G-, RD114/TR-, BaEV/TR-, and BaEVRLess-LVs encoding the GFP reporter at an MOI of 10 or 20, except for VSV-G-LVs for which a higher vector dose (MOI of 50) was applied. FIG. 23A shows that BaEV/TR-, and BaEVRLess-LVs show a transduction efficiency highly superior (40-50% transduction) at an MOI=10 as compared to VSV-G or RD114/TR-LVs (FIG. 23A). Importantly, BaEV/TR- and BaEVRLess-LVs reached easily 65% transduction when the vector dose was 2-fold increased (MOI=20; FIG. 23A). In all cases the naive recent thymocyte emigrants (CD69L+ CD45RA+ CD31+) were transduced to the same extent as the more mature naive CB T cells (CD69L+ CD45RA+ CD31-; FIG. 23A). Moreover, transduction had no effect on the distribution of these two naive CB T cell populations (FIG. 23B).

#### Discussion

**[0192]** In this example, the inventors described efficient pseudotyping of lentiviral vectors using a novel envelope glycoprotein BaEV derived from the Baboon endogenous virus. Two mutants of this glycoprotein: the BaEV/TR chimeric glycoprotein and the BaEVRLess glycoprotein allowed a better incorporation on the LV surface as compared to the BaEVwt glycoprotein. LVs pseudotyped by these envelopes could be produced at high titers ( $1 \cdot 10^7$  IU/ml). Like RD114/TR-LV pseudotypes, they were stable in human and macaque sera, which allow potential clinical perspectives for in vivo delivery of transgenes.

**[0193]** Regarding transduction of hCD34+ cells, BaEV mutant LV pseudotypes were superior to VSV-G and RD114/TR. First, they were able to transduce hCD34+ cells upon very low prestimulation protocols. Indeed, people currently use cytokine cocktails containing rSCF, rTPO, rFlt3-L and IL-3 but it was reported that the more hCD34+ cells are stimulated, the more they differentiate and they lose their long term hematopoietic reconstitution capacity. Furthermore, IL-3 was thought to notably increase the CD34+ differentiation. Here, the inventors showed that BaEV/TR- and BaEVRLess-LVs achieved high transduction rates of hCD34+ cells when they were prestimulated with rSCF, rTPO or rSCF and rTPO co-stimulation for a short time of 16 h. These mild cytokine stimulation protocols strongly reduced the risk of differentiation of HSCs present in the hCD34+ cell population. Of high importance, these results indicated that BaEV/TR-LV pseudotypes could transduce 10-20% of unstimulated hCD34+ cells while VSV-G-LVs and RD114/TR-LVs remained inefficient. Thus, since no cytokine stimulation is needed to achieve efficient hCD34+ cell transduction with BaEV/TR- or BaEVRLess-LVs they possibly allow to conserve better the stem cell character of hCD34+ cells.

**[0194]** Another important observation was that at low MOIs (5 and 10) lentiviral vector pseudotyped with BaEV/TR and BaEVRLess respectively, consistently transduced human CD34+ cells better than VSV-G LV pseudotypes at MOI of 100. The use of a lentiviral vector at a very low MOI could limit insertional mutagenesis which occurs when the proviral DNA is inserted in an oncogene of the host cells. If too many vectors integrate into the host cell genome, it could

lead to genotoxicity due to host cell gene deregulation. Thus, in the case of BaEV-LVs, it would be possible to decrease significantly the vector doses to safe levels without a significant loss of the transduction rate. In contrast, for VSV-G-LVs very high vector doses were needed to allow efficient hCD34+ cell transduction.

**[0195]** The ability of BaEV/TR- and BaEVRLess-LV pseudotypes to highly transduce short term repopulating cells and the results on reconstitution of immunodeficient mice bone marrow and spleen with gene marked cells in all the different human cell lineages are arguments that strongly suggest they actually transduce HSCs.

**[0196]** Moreover, the novel BaEV/TR- and BaEVRLess-LVs are promising gene transfer tools for T cell gene therapy since they allow high transduction of IL-7 prestimulated T cells without inducing a phenotypic switch. Additionally, if one considers the need for a life time correction, it is necessary to transduce the long-lived naive T cells which are shown here to be highly susceptible to BaEV/TR- and BaEVRLess-LV mediated gene transfer and might know therefore many applications in vivo.

**[0197]** Finally, these BaEV/TR- and BaEVRLess-LVs represent a new tool allowing high stable gene transfer of primary quiescent B cells that now makes it possible to study with ease gene function and therapeutic gene transfer in these cells. These novel vectors may allow expression of co-stimulatory molecules such as CD40L or B7 molecules in non-dividing tumor B-cells such as B-CLLs in order to elicit immune responses against gene modified and unmodified cells. They also enable introducing DNA encoding the light and heavy chains of specific antibodies (for example antibodies specifically directed against HIV or HCV) or epitopes into autologous memory B cells in order to allow immunotherapy and tolerance induction upon reinfusion of the engineered cells.

**[0198]** In vivo gene delivery of lentiviral vectors displaying BaEV/TR- or BaEVRLess-LVs in HSCs, B cells or T cells would make gene therapy much easier. The advantages of in vivo gene delivery are multiple. In vivo gene transfer could target all HSCs in their stem cell niche, which creates a microenvironment regulating HSC survival and maintenance. Moreover, for the majority of diseases there is a requirement of a minimal number of gene-corrected cells to be re-infused. This requisite might be a limiting factor when treating children since CD34+ isolation results in significant loss of this target population or in bone marrow failure syndromes like Fanconi anemia, in which patients already suffer from a reduced number of hCD34+ cells early on. All these concerns might be overcome by in vivo targeted gene delivery to HSCs. Identical reasoning is possible for T and especially for B cells since the latter cells go easily into apoptosis during ex vivo culture.

**[0199]** In summary, the inventors have developed a novel and apparently effective pseudotype for lentiviral vectors. BaEV/TR and BaEVRLess pseudotyped LV will probably become useful for many fundamental and clinical applications because they present a lot of advantages such as very high transduction of human primary HSCs, T cells and B cells.

Sequence Identifiers Reference Table:

-continued

[0200]

SEQ ID NO:	Feature	SEQ ID NO:	Feature
1	Nucleic acid encoding the wild-type BaEV envelope glycoprotein	7	Modified BaEV envelope glycoprotein wherein the cytoplasmic tail domain is devoid of the fusion inhibitory R peptide (BaEVRless)
2	Wild-type BaEV envelope glycoprotein	8	Cytoplasmic tail domain of the wild-type MLV envelope glycoprotein
3	Cytoplasmic tail domain of the wild-type BaEV envelope glycoprotein	9	Fusion of the transmembrane and extracellular domain of the BaEV envelope glycoprotein and the cytoplasmic tail domain of the MLV envelope glycoprotein (BaEV/TR)
4	Transmembrane domain of the wild-type BaEV envelope glycoprotein	10	Human wild-type SCF
5	Extracellular domain of the wild-type BaEV envelope glycoprotein	11	Human wild-type TPO
6	Fusion inhibitory R peptide of the wild-type BaEV envelope glycoprotein	12	Human wild-type IL-2
		13	Human wild-type IL-7
		14	Human wild-type IL-15
		15	Human wild-type Flt3-L

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 15

<210> SEQ ID NO 1

<211> LENGTH: 1692

<212> TYPE: DNA

<213> ORGANISM: Baboon endogenous virus

<400> SEQUENCE: 1

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gacgaccctc gaaaagccat agaactagta caaaagcgat atggccgacc atgcgattgc    120
agcggaggac aagtgtccga gcccccgta gacagggta gcaagtgc ttgctcaggc    180
aagacagctt acttaatgcc cgaccaaaga tggaaatgta agtcaattcc aaaagacacc    240
tccccaaagc ggccactcca agagtgcctc tgtaattctt accagtcctc agtacacagt    300
tcttggtata cctcatacca acaatgcaga tcaggcaata agacatatta tacggctact    360
ctgctaaaaa cacaaactgg gggcaccagt gatgtacaag tattaggatc caccaacaaa    420
cttatacaat ctcccgtgaa tggcataaaa gggcagtcct tttgctggag cactacagct    480
cctatccacg tctctgatgg aggaggtcca ttagacacca caagaattaa aagtgttcag    540
agaaaactgg aagaaattca taaagcccta taccctgaac ttcagatca ccctttggcc    600
atacctaagg ttagagataa cctcatggtc gatgcccaga ctttaaacat tctcaatgcc    660
acttacaact tactcctaata gtccaacacg agcctagtgg acgactgttg gctttgttta    720
aaattaggtc ccctactcc cctcgcaata cctaacttcc tattatccta cgtgactcgc    780
tctctcgata atatctcttg ttaataaatt ccccccttc tagttcaacc gatgcagttt    840
tccaattcat ctgctctt ttccccctcc tacaacagta cagaagaaat agatctaggc    900
catgttcctc tcagcaactg tacctccata accaatgtca cgggtcccat atgctctgta    960
aatggttcgg tctttctctg tggcaataac atggcataca ctatctacc cacgaaactgg    1020
acggggcttt gcgtcctagc aactctctc cccgacattg acatcattcc cggagatgaa    1080
ccggccccca tccctgctat tgatcatttt atatatagac ctaaacgggc catacagttt    1140
attcctttac tagcagggct agggatcacc gcagccttca caacaggagc tacaggccta    1200
gggtctctg tgaccaata tacaataata tctaactcagc taatttctga tgtacaaatc    1260
ttatctagca ccatacaaga tctgcaagat caagtagact cattagccga agtggttctc    1320
    
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cagaacagaa gggggctaga tctacttaca gcagaacaag gaggaatctg tttagcctg 1380
caagaaaaat gctgctttta tgtaacaag tcagggattg tgagagacaa aataaaaacc 1440
ttacaagaag aactagaag acgtagaaaa gatctagctt ccaacccact ttggactggg 1500
cttcaagggc tctccctta cctcctgccc tttcttgccc ctctacttac cctcctgctc 1560
ttactcacca ttgggccgtg catttttaac cgtctaaccg cttttattaa tgataagta 1620
aacataatac acgctatggt gctaaccxaa cagtatcagg tgctcagaac cgatgaagaa 1680
gctcaagatt ga 1692

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<210> SEQ ID NO 2
<211> LENGTH: 563
<212> TYPE: PRT
<213> ORGANISM: Baboon endogenous virus
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (1)..(506)
<223> OTHER INFORMATION: extracellular domain
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (507)..(529)
<223> OTHER INFORMATION: transmembrane domain
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (530)..(563)
<223> OTHER INFORMATION: cytoplasmic tail
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (547)..(563)
<223> OTHER INFORMATION: R peptide

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<400> SEQUENCE: 2

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Met Gly Phe Thr Thr Lys Ile Ile Phe Leu Tyr Asn Leu Val Leu Val
 1          5          10          15
Tyr Ala Gly Phe Asp Asp Pro Arg Lys Ala Ile Glu Leu Val Gln Lys
 20          25          30
Arg Tyr Gly Arg Pro Cys Asp Cys Ser Gly Gly Gln Val Ser Glu Pro
 35          40          45
Pro Ser Asp Arg Val Ser Gln Val Thr Cys Ser Gly Lys Thr Ala Tyr
 50          55          60
Leu Met Pro Asp Gln Arg Trp Lys Cys Lys Ser Ile Pro Lys Asp Thr
 65          70          75          80
Ser Pro Ser Gly Pro Leu Gln Glu Cys Pro Cys Asn Ser Tyr Gln Ser
 85          90          95
Ser Val His Ser Ser Cys Tyr Thr Ser Tyr Gln Gln Cys Arg Ser Gly
100          105          110
Asn Lys Thr Tyr Tyr Thr Ala Thr Leu Leu Lys Thr Gln Thr Gly Gly
115          120          125
Thr Ser Asp Val Gln Val Leu Gly Ser Thr Asn Lys Leu Ile Gln Ser
130          135          140
Pro Cys Asn Gly Ile Lys Gly Gln Ser Ile Cys Trp Ser Thr Thr Ala
145          150          155          160
Pro Ile His Val Ser Asp Gly Gly Gly Pro Leu Asp Thr Thr Arg Ile
165          170          175
Lys Ser Val Gln Arg Lys Leu Glu Glu Ile His Lys Ala Leu Tyr Pro
180          185          190
Glu Leu Gln Tyr His Pro Leu Ala Ile Pro Lys Val Arg Asp Asn Leu
195          200          205

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Met Val Asp Ala Gln Thr Leu Asn Ile Leu Asn Ala Thr Tyr Asn Leu
 210                               215                               220

Leu Leu Met Ser Asn Thr Ser Leu Val Asp Asp Cys Trp Leu Cys Leu
 225                               230                               235                               240

Lys Leu Gly Pro Pro Thr Pro Leu Ala Ile Pro Asn Phe Leu Leu Ser
                               245                               250                               255

Tyr Val Thr Arg Ser Ser Asp Asn Ile Ser Cys Leu Ile Ile Pro Pro
                               260                               265                               270

Leu Leu Val Gln Pro Met Gln Phe Ser Asn Ser Ser Cys Leu Phe Ser
 275                               280                               285

Pro Ser Tyr Asn Ser Thr Glu Glu Ile Asp Leu Gly His Val Ala Phe
 290                               295                               300

Ser Asn Cys Thr Ser Ile Thr Asn Val Thr Gly Pro Ile Cys Ala Val
 305                               310                               315                               320

Asn Gly Ser Val Phe Leu Cys Gly Asn Asn Met Ala Tyr Thr Tyr Leu
                               325                               330                               335

Pro Thr Asn Trp Thr Gly Leu Cys Val Leu Ala Thr Leu Leu Pro Asp
                               340                               345                               350

Ile Asp Ile Ile Pro Gly Asp Glu Pro Val Pro Ile Pro Ala Ile Asp
 355                               360                               365

His Phe Ile Tyr Arg Pro Lys Arg Ala Ile Gln Phe Ile Pro Leu Leu
 370                               375                               380

Ala Gly Leu Gly Ile Thr Ala Ala Phe Thr Thr Gly Ala Thr Gly Leu
 385                               390                               395                               400

Gly Val Ser Val Thr Gln Tyr Thr Lys Leu Ser Asn Gln Leu Ile Ser
                               405                               410                               415

Asp Val Gln Ile Leu Ser Ser Thr Ile Gln Asp Leu Gln Asp Gln Val
 420                               425                               430

Asp Ser Leu Ala Glu Val Val Leu Gln Asn Arg Arg Gly Leu Asp Leu
 435                               440                               445

Leu Thr Ala Glu Gln Gly Gly Ile Cys Leu Ala Leu Gln Glu Lys Cys
 450                               455                               460

Cys Phe Tyr Val Asn Lys Ser Gly Ile Val Arg Asp Lys Ile Lys Thr
 465                               470                               475                               480

Leu Gln Glu Glu Leu Glu Arg Arg Arg Lys Asp Leu Ala Ser Asn Pro
 485                               490                               495

Leu Trp Thr Gly Leu Gln Gly Leu Leu Pro Tyr Leu Leu Pro Phe Leu
 500                               505                               510

Gly Pro Leu Leu Thr Leu Leu Leu Leu Thr Ile Gly Pro Cys Ile
 515                               520                               525

Phe Asn Arg Leu Thr Ala Phe Ile Asn Asp Lys Leu Asn Ile Ile His
 530                               535                               540

Ala Met Val Leu Thr Gln Gln Tyr Gln Val Leu Arg Thr Asp Glu Glu
 545                               550                               555                               560

Ala Gln Asp

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&lt;210&gt; SEQ ID NO 3

&lt;211&gt; LENGTH: 34

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Baboon endogenous virus

&lt;400&gt; SEQUENCE: 3

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Asn Arg Leu Thr Ala Phe Ile Asn Asp Lys Leu Asn Ile Ile His Ala  
1 5 10 15

Met Val Leu Thr Gln Gln Tyr Gln Val Leu Arg Thr Asp Glu Glu Ala  
20 25 30

Gln Asp

<210> SEQ ID NO 4  
<211> LENGTH: 23  
<212> TYPE: PRT  
<213> ORGANISM: Baboon endogenous virus

<400> SEQUENCE: 4

Tyr Leu Leu Pro Phe Leu Gly Pro Leu Leu Thr Leu Leu Leu Leu Leu  
1 5 10 15

Thr Ile Gly Pro Cys Ile Phe  
20

<210> SEQ ID NO 5  
<211> LENGTH: 506  
<212> TYPE: PRT  
<213> ORGANISM: Baboon endogenous virus

<400> SEQUENCE: 5

Met Gly Phe Thr Thr Lys Ile Ile Phe Leu Tyr Asn Leu Val Leu Val  
1 5 10 15

Tyr Ala Gly Phe Asp Asp Pro Arg Lys Ala Ile Glu Leu Val Gln Lys  
20 25 30

Arg Tyr Gly Arg Pro Cys Asp Cys Ser Gly Gly Gln Val Ser Glu Pro  
35 40 45

Pro Ser Asp Arg Val Ser Gln Val Thr Cys Ser Gly Lys Thr Ala Tyr  
50 55 60

Leu Met Pro Asp Gln Arg Trp Lys Cys Lys Ser Ile Pro Lys Asp Thr  
65 70 75 80

Ser Pro Ser Gly Pro Leu Gln Glu Cys Pro Cys Asn Ser Tyr Gln Ser  
85 90 95

Ser Val His Ser Ser Cys Tyr Thr Ser Tyr Gln Gln Cys Arg Ser Gly  
100 105 110

Asn Lys Thr Tyr Tyr Thr Ala Thr Leu Leu Lys Thr Gln Thr Gly Gly  
115 120 125

Thr Ser Asp Val Gln Val Leu Gly Ser Thr Asn Lys Leu Ile Gln Ser  
130 135 140

Pro Cys Asn Gly Ile Lys Gly Gln Ser Ile Cys Trp Ser Thr Thr Ala  
145 150 155 160

Pro Ile His Val Ser Asp Gly Gly Gly Pro Leu Asp Thr Thr Arg Ile  
165 170 175

Lys Ser Val Gln Arg Lys Leu Glu Glu Ile His Lys Ala Leu Tyr Pro  
180 185 190

Glu Leu Gln Tyr His Pro Leu Ala Ile Pro Lys Val Arg Asp Asn Leu  
195 200 205

Met Val Asp Ala Gln Thr Leu Asn Ile Leu Asn Ala Thr Tyr Asn Leu  
210 215 220

Leu Leu Met Ser Asn Thr Ser Leu Val Asp Asp Cys Trp Leu Cys Leu  
225 230 235 240







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Asp Val Gln Ile Leu Ser Ser Thr Ile Gln Asp Leu Gln Asp Gln Val  
                   420                                  425                                  430  
 Asp Ser Leu Ala Glu Val Val Leu Gln Asn Arg Arg Gly Leu Asp Leu  
                   435                                  440                                  445  
 Leu Thr Ala Glu Gln Gly Gly Ile Cys Leu Ala Leu Gln Glu Lys Cys  
                   450                                  455                                  460  
 Cys Phe Tyr Val Asn Lys Ser Gly Ile Val Arg Asp Lys Ile Lys Thr  
                   465                                  470                                  475                                  480  
 Leu Gln Glu Glu Leu Glu Arg Arg Arg Lys Asp Leu Ala Ser Asn Pro  
                   485                                  490                                  495  
 Leu Trp Thr Gly Leu Gln Gly Leu Leu Pro Tyr Leu Leu Pro Phe Leu  
                   500                                  505                                  510  
 Gly Pro Leu Leu Thr Leu Leu Leu Leu Thr Ile Gly Pro Cys Ile  
                   515                                  520                                  525  
 Phe Asn Arg Leu Thr Ala Phe Ile Asn Asp Lys Leu Asn Ile Ile His  
                   530                                  535                                  540  
 Ala Met  
 545

<210> SEQ ID NO 8  
 <211> LENGTH: 33  
 <212> TYPE: PRT  
 <213> ORGANISM: Murine leukemia virus

<400> SEQUENCE: 8

Asn Arg Leu Val Gln Phe Val Lys Asp Arg Ile Ser Val Val Gln Ala  
 1                  5                                  10                                  15  
 Leu Val Leu Thr Gln Gln Tyr His Gln Leu Lys Pro Leu Glu Tyr Glu  
                   20                                  25                                  30  
 Pro

<210> SEQ ID NO 9  
 <211> LENGTH: 562  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Synthetic protein BaEV/TR

<400> SEQUENCE: 9

Met Gly Phe Thr Thr Lys Ile Ile Phe Leu Tyr Asn Leu Val Leu Val  
 1                  5                                  10                                  15  
 Tyr Ala Gly Phe Asp Asp Pro Arg Lys Ala Ile Glu Leu Val Gln Lys  
                   20                                  25                                  30  
 Arg Tyr Gly Arg Pro Cys Asp Cys Ser Gly Gly Gln Val Ser Glu Pro  
                   35                                  40                                  45  
 Pro Ser Asp Arg Val Ser Gln Val Thr Cys Ser Gly Lys Thr Ala Tyr  
                   50                                  55                                  60  
 Leu Met Pro Asp Gln Arg Trp Lys Cys Lys Ser Ile Pro Lys Asp Thr  
                   65                                  70                                  75                                  80  
 Ser Pro Ser Gly Pro Leu Gln Glu Cys Pro Cys Asn Ser Tyr Gln Ser  
                   85                                  90                                  95  
 Ser Val His Ser Ser Cys Tyr Thr Ser Tyr Gln Gln Cys Arg Ser Gly  
                   100                                  105                                  110  
 Asn Lys Thr Tyr Tyr Thr Ala Thr Leu Leu Lys Thr Gln Thr Gly Gly  
                   115                                  120                                  125

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Thr Ser Asp Val Gln Val Leu Gly Ser Thr Asn Lys Leu Ile Gln Ser  
 130 135 140

Pro Cys Asn Gly Ile Lys Gly Gln Ser Ile Cys Trp Ser Thr Thr Ala  
 145 150 155 160

Pro Ile His Val Ser Asp Gly Gly Gly Pro Leu Asp Thr Thr Arg Ile  
 165 170 175

Lys Ser Val Gln Arg Lys Leu Glu Glu Ile His Lys Ala Leu Tyr Pro  
 180 185 190

Glu Leu Gln Tyr His Pro Leu Ala Ile Pro Lys Val Arg Asp Asn Leu  
 195 200 205

Met Val Asp Ala Gln Thr Leu Asn Ile Leu Asn Ala Thr Tyr Asn Leu  
 210 215 220

Leu Leu Met Ser Asn Thr Ser Leu Val Asp Asp Cys Trp Leu Cys Leu  
 225 230 235 240

Lys Leu Gly Pro Pro Thr Pro Leu Ala Ile Pro Asn Phe Leu Leu Ser  
 245 250 255

Tyr Val Thr Arg Ser Ser Asp Asn Ile Ser Cys Leu Ile Ile Pro Pro  
 260 265 270

Leu Leu Val Gln Pro Met Gln Phe Ser Asn Ser Ser Cys Leu Phe Ser  
 275 280 285

Pro Ser Tyr Asn Ser Thr Glu Glu Ile Asp Leu Gly His Val Ala Phe  
 290 295 300

Ser Asn Cys Thr Ser Ile Thr Asn Val Thr Gly Pro Ile Cys Ala Val  
 305 310 315 320

Asn Gly Ser Val Phe Leu Cys Gly Asn Asn Met Ala Tyr Thr Tyr Leu  
 325 330 335

Pro Thr Asn Trp Thr Gly Leu Cys Val Leu Ala Thr Leu Leu Pro Asp  
 340 345 350

Ile Asp Ile Ile Pro Gly Asp Glu Pro Val Pro Ile Pro Ala Ile Asp  
 355 360 365

His Phe Ile Tyr Arg Pro Lys Arg Ala Ile Gln Phe Ile Pro Leu Leu  
 370 375 380

Ala Gly Leu Gly Ile Thr Ala Ala Phe Thr Thr Gly Ala Thr Gly Leu  
 385 390 395 400

Gly Val Ser Val Thr Gln Tyr Thr Lys Leu Ser Asn Gln Leu Ile Ser  
 405 410 415

Asp Val Gln Ile Leu Ser Ser Thr Ile Gln Asp Leu Gln Asp Gln Val  
 420 425 430

Asp Ser Leu Ala Glu Val Val Leu Gln Asn Arg Arg Gly Leu Asp Leu  
 435 440 445

Leu Thr Ala Glu Gln Gly Gly Ile Cys Leu Ala Leu Gln Glu Lys Cys  
 450 455 460

Cys Phe Tyr Val Asn Lys Ser Gly Ile Val Arg Asp Lys Ile Lys Thr  
 465 470 475 480

Leu Gln Glu Glu Leu Glu Arg Arg Arg Lys Asp Leu Ala Ser Asn Pro  
 485 490 495

Leu Trp Thr Gly Leu Gln Gly Leu Leu Pro Tyr Leu Leu Pro Phe Leu  
 500 505 510

Gly Pro Leu Leu Thr Leu Leu Leu Leu Thr Ile Gly Pro Cys Ile  
 515 520 525

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Phe Asn Arg Leu Val Gln Phe Val Lys Asp Arg Ile Ser Val Val Gln
 530          535          540

Ala Leu Val Leu Thr Gln Gln Tyr His Gln Leu Lys Pro Leu Glu Tyr
 545          550          555          560

Glu Pro

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<210> SEQ ID NO 10
<211> LENGTH: 273
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

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<400> SEQUENCE: 10

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Met Lys Lys Thr Gln Thr Trp Ile Leu Thr Cys Ile Tyr Leu Gln Leu
 1          5          10          15

Leu Leu Phe Asn Pro Leu Val Lys Thr Glu Gly Ile Cys Arg Asn Arg
          20          25          30

Val Thr Asn Asn Val Lys Asp Val Thr Lys Leu Val Ala Asn Leu Pro
          35          40          45

Lys Asp Tyr Met Ile Thr Leu Lys Tyr Val Pro Gly Met Asp Val Leu
          50          55          60

Pro Ser His Cys Trp Ile Ser Glu Met Val Val Gln Leu Ser Asp Ser
 65          70          75          80

Leu Thr Asp Leu Leu Asp Lys Phe Ser Asn Ile Ser Glu Gly Leu Ser
          85          90          95

Asn Tyr Ser Ile Ile Asp Lys Leu Val Asn Ile Val Asp Asp Leu Val
          100          105          110

Glu Cys Val Lys Glu Asn Ser Ser Lys Asp Leu Lys Lys Ser Phe Lys
          115          120          125

Ser Pro Glu Pro Arg Leu Phe Thr Pro Glu Glu Phe Phe Arg Ile Phe
          130          135          140

Asn Arg Ser Ile Asp Ala Phe Lys Asp Phe Val Val Ala Ser Glu Thr
 145          150          155          160

Ser Asp Cys Val Val Ser Ser Thr Leu Ser Pro Glu Lys Asp Ser Arg
          165          170          175

Val Ser Val Thr Lys Pro Phe Met Leu Pro Pro Val Ala Ala Ser Ser
          180          185          190

Leu Arg Asn Asp Ser Ser Ser Ser Asn Arg Lys Ala Lys Asn Pro Pro
          195          200          205

Gly Asp Ser Ser Leu His Trp Ala Ala Met Ala Leu Pro Ala Leu Phe
          210          215          220

Ser Leu Ile Ile Gly Phe Ala Phe Gly Ala Leu Tyr Trp Lys Lys Arg
 225          230          235          240

Gln Pro Ser Leu Thr Arg Ala Val Glu Asn Ile Gln Ile Asn Glu Glu
          245          250          255

Asp Asn Glu Ile Ser Met Leu Gln Glu Lys Glu Arg Glu Phe Gln Glu
          260          265          270

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Val

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<210> SEQ ID NO 11
<211> LENGTH: 353
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

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<400> SEQUENCE: 11

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Met Glu Leu Thr Glu Leu Leu Leu Val Val Met Leu Leu Leu Thr Ala  
 1 5 10 15

Arg Leu Thr Leu Ser Ser Pro Ala Pro Pro Ala Cys Asp Leu Arg Val  
 20 25 30

Leu Ser Lys Leu Leu Arg Asp Ser His Val Leu His Ser Arg Leu Ser  
 35 40 45

Gln Cys Pro Glu Val His Pro Leu Pro Thr Pro Val Leu Leu Pro Ala  
 50 55 60

Val Asp Phe Ser Leu Gly Glu Trp Lys Thr Gln Met Glu Glu Thr Lys  
 65 70 75 80

Ala Gln Asp Ile Leu Gly Ala Val Thr Leu Leu Leu Glu Gly Val Met  
 85 90 95

Ala Ala Arg Gly Gln Leu Gly Pro Thr Cys Leu Ser Ser Leu Leu Gly  
 100 105 110

Gln Leu Ser Gly Gln Val Arg Leu Leu Leu Gly Ala Leu Gln Ser Leu  
 115 120 125

Leu Gly Thr Gln Leu Pro Pro Gln Gly Arg Thr Thr Ala His Lys Asp  
 130 135 140

Pro Asn Ala Ile Phe Leu Ser Phe Gln His Leu Leu Arg Gly Lys Val  
 145 150 155 160

Arg Phe Leu Met Leu Val Gly Gly Ser Thr Leu Cys Val Arg Arg Ala  
 165 170 175

Pro Pro Thr Thr Ala Val Pro Ser Arg Thr Ser Leu Val Leu Thr Leu  
 180 185 190

Asn Glu Leu Pro Asn Arg Thr Ser Gly Leu Leu Glu Thr Asn Phe Thr  
 195 200 205

Ala Ser Ala Arg Thr Thr Gly Ser Gly Leu Leu Lys Trp Gln Gln Gly  
 210 215 220

Phe Arg Ala Lys Ile Pro Gly Leu Leu Asn Gln Thr Ser Arg Ser Leu  
 225 230 235 240

Asp Gln Ile Pro Gly Tyr Leu Asn Arg Ile His Glu Leu Leu Asn Gly  
 245 250 255

Thr Arg Gly Leu Phe Pro Gly Pro Ser Arg Arg Thr Leu Gly Ala Pro  
 260 265 270

Asp Ile Ser Ser Gly Thr Ser Asp Thr Gly Ser Leu Pro Pro Asn Leu  
 275 280 285

Gln Pro Gly Tyr Ser Pro Ser Pro Thr His Pro Pro Thr Gly Gln Tyr  
 290 295 300

Thr Leu Phe Pro Leu Pro Pro Thr Leu Pro Thr Pro Val Val Gln Leu  
 305 310 315 320

His Pro Leu Leu Pro Asp Pro Ser Ala Pro Thr Pro Thr Pro Thr Ser  
 325 330 335

Pro Leu Leu Asn Thr Ser Tyr Thr His Ser Gln Asn Leu Ser Gln Glu  
 340 345 350

Gly

&lt;210&gt; SEQ ID NO 12

&lt;211&gt; LENGTH: 153

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Homo sapiens

&lt;400&gt; SEQUENCE: 12

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Met Tyr Arg Met Gln Leu Leu Ser Cys Ile Ala Leu Ser Leu Ala Leu  
 1 5 10 15  
 Val Thr Asn Ser Ala Pro Thr Ser Ser Ser Thr Lys Lys Thr Gln Leu  
 20 25 30  
 Gln Leu Glu His Leu Leu Leu Asp Leu Gln Met Ile Leu Asn Gly Ile  
 35 40 45  
 Asn Asn Tyr Lys Asn Pro Lys Leu Thr Arg Met Leu Thr Phe Lys Phe  
 50 55 60  
 Tyr Met Pro Lys Lys Ala Thr Glu Leu Lys His Leu Gln Cys Leu Glu  
 65 70 75 80  
 Glu Glu Leu Lys Pro Leu Glu Glu Val Leu Asn Leu Ala Gln Ser Lys  
 85 90 95  
 Asn Phe His Leu Arg Pro Arg Asp Leu Ile Ser Asn Ile Asn Val Ile  
 100 105 110  
 Val Leu Glu Leu Lys Gly Ser Glu Thr Thr Phe Met Cys Glu Tyr Ala  
 115 120 125  
 Asp Glu Thr Ala Thr Ile Val Glu Phe Leu Asn Arg Trp Ile Thr Phe  
 130 135 140  
 Cys Gln Ser Ile Ile Ser Thr Leu Thr  
 145 150

<210> SEQ ID NO 13  
 <211> LENGTH: 177  
 <212> TYPE: PRT  
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 13

Met Phe His Val Ser Phe Arg Tyr Ile Phe Gly Leu Pro Pro Leu Ile  
 1 5 10 15  
 Leu Val Leu Leu Pro Val Ala Ser Ser Asp Cys Asp Ile Glu Gly Lys  
 20 25 30  
 Asp Gly Lys Gln Tyr Glu Ser Val Leu Met Val Ser Ile Asp Gln Leu  
 35 40 45  
 Leu Asp Ser Met Lys Glu Ile Gly Ser Asn Cys Leu Asn Asn Glu Phe  
 50 55 60  
 Asn Phe Phe Lys Arg His Ile Cys Asp Ala Asn Lys Glu Gly Met Phe  
 65 70 75 80  
 Leu Phe Arg Ala Ala Arg Lys Leu Arg Gln Phe Leu Lys Met Asn Ser  
 85 90 95  
 Thr Gly Asp Phe Asp Leu His Leu Leu Lys Val Ser Glu Gly Thr Thr  
 100 105 110  
 Ile Leu Leu Asn Cys Thr Gly Gln Val Lys Gly Arg Lys Pro Ala Ala  
 115 120 125  
 Leu Gly Glu Ala Gln Pro Thr Lys Ser Leu Glu Glu Asn Lys Ser Leu  
 130 135 140  
 Lys Glu Gln Lys Lys Leu Asn Asp Leu Cys Phe Leu Lys Arg Leu Leu  
 145 150 155 160  
 Gln Glu Ile Lys Thr Cys Trp Asn Lys Ile Leu Met Gly Thr Lys Glu  
 165 170 175

His

<210> SEQ ID NO 14

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<211> LENGTH: 136
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 14

Met Asp Phe Gln Val Gln Ile Phe Ser Phe Leu Leu Ile Ser Ala Ser
1          5          10          15
Val Ile Met Ser Arg Ala Asn Trp Val Asn Val Ile Ser Asp Leu Lys
          20          25          30
Lys Ile Glu Asp Leu Ile Gln Ser Met His Ile Asp Ala Thr Leu Tyr
          35          40          45
Thr Glu Ser Asp Val His Pro Ser Cys Lys Val Thr Ala Met Lys Cys
          50          55          60
Phe Leu Leu Glu Leu Gln Val Ile Ser Leu Glu Ser Gly Asp Ala Ser
65          70          75          80
Ile His Asp Thr Val Glu Asn Leu Ile Ile Leu Ala Asn Asn Ser Leu
          85          90          95
Ser Ser Asn Gly Asn Val Thr Glu Ser Gly Cys Lys Glu Cys Glu Glu
          100          105          110
Leu Glu Glu Lys Asn Ile Lys Glu Phe Leu Gln Ser Phe Val His Ile
          115          120          125
Val Gln Met Phe Ile Asn Thr Ser
          130          135

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<210> SEQ ID NO 15
<211> LENGTH: 235
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Gln His Ser Pro Ile Ser Ser Asp Phe Ala Val Lys Ile Arg Glu Leu
          35          40          45
Ser Asp Tyr Leu Leu Gln Asp Tyr Pro Val Thr Val Ala Ser Asn Leu
          50          55          60
Gln Asp Glu Glu Leu Cys Gly Gly Leu Trp Arg Leu Val Leu Ala Gln
65          70          75          80
Arg Trp Met Glu Arg Leu Lys Thr Val Ala Gly Ser Lys Met Gln Gly
          85          90          95
Leu Leu Glu Arg Val Asn Thr Glu Ile His Phe Val Thr Lys Cys Ala
          100          105          110
Phe Gln Pro Pro Pro Ser Cys Leu Arg Phe Val Gln Thr Asn Ile Ser
          115          120          125
Arg Leu Leu Gln Glu Thr Ser Glu Gln Leu Val Ala Leu Lys Pro Trp
          130          135          140
Ile Thr Arg Gln Asn Phe Ser Arg Cys Leu Glu Leu Gln Cys Gln Pro
          145          150          155          160
Asp Ser Ser Thr Leu Pro Pro Pro Trp Ser Pro Arg Pro Leu Glu Ala
          165          170          175
Thr Ala Pro Thr Ala Pro Gln Pro Pro Leu Leu Leu Leu Leu Leu
          180          185          190

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	210					215					220				
Pro	Ser	Pro	Gln	Asp	Leu	Leu	Leu	Val	Glu	His					
225					230					235					

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**1.** A pseudotyped viral vector particle for transferring biological material into cells, wherein said vector particle comprises at least:

- a chimeric envelope glycoprotein which comprises or consists in a fusion of the transmembrane and extracellular domain of a baboon endogenous retrovirus (BaEV) envelope glycoprotein and the cytoplasmic tail domain of a murine leukemia virus (MLV) envelope glycoprotein; or
- a modified BaEV envelope glycoprotein wherein the cytoplasmic tail domain is devoid of the fusion inhibitory R peptide.

**2.** The pseudotyped viral vector particle according to claim 1, wherein the viral vector particle is a lentiviral vector particle.

**3.** The pseudotyped viral vector particle according to claim 2, wherein the lentiviral vector particle is selected from the group consisting of HIV and SIV.

**4.** The pseudotyped viral vector particle according to claim 1, wherein the biological material is one or more nucleic acids.

**5.** The pseudotyped viral vector particle according to claim 1, wherein said viral vector particle further displays at least one cytokine selected from the group consisting of stem cell factor (SCF), thrombopoietin (TPO), IL-2, IL-15 and IL-7.

**6.** The pseudotyped viral vector particle according to claim 1, wherein the pseudotyped viral vector particle is intended for transferring biological material into hematopoietic cells.

**7.** A method for treating a hematopoietic disorder or an autoimmune disease in a subject in need thereof comprising administering a therapeutically effective amount of a pseudotyped viral vector particle according to claim 1.

**8.** The method according to claim 7, wherein the hematopoietic disorder is selected from the group consisting of Fanconi anemia, hemophilia, beta-thalassemia, Wiskott-Aldrich syndrome, X-linked severe combined immunodeficiency, adenosine-deaminase deficiency, chronic granulomatous disease and adrenoleukodystrophy.

**9.** A method for producing a pseudotyped viral vector particle comprising:

- a) transfecting a cell with:
  - (i) at least one first nucleic acid sequence comprising a packaging competent retroviral derived genome;

- (ii) at least one second nucleic acid sequence comprising a cDNA encoding core proteins from said retrovirus, and

- (iii) at least one third nucleic acid sequence comprising a cDNA encoding:

- a chimeric envelope glycoprotein which comprises or consists in a fusion of the transmembrane and extracellular domain of a baboon endogenous retrovirus (BaEV) envelope glycoprotein and the cytoplasmic tail domain of a murine leukemia virus (MLV) envelope glycoprotein, as defined above; or
- a modified BaEV envelope glycoprotein wherein the cytoplasmic tail domain is devoid of the fusion inhibitory R peptide, as defined above;

to yield a producer cell;

b) maintaining the producer cell in culture for sufficient time to allow expression of the cDNAs to produce the encoded proteins; and

c) allowing the encoded proteins to form pseudotyped viral vector particles.

**10.** A medicament comprising a pseudotyped viral vector particle as defined in claim 1 as active ingredient.

**11.** (canceled)

**12.** A method for transducing a hematopoietic cell comprising contacting the hematopoietic cell with a pseudotyped viral vector particle as defined in claim 1 under conditions to effect the transduction of the hematopoietic cell by the pseudotyped viral vector particle.

**13.** The method according to claim 12, wherein the hematopoietic cell is selected from the group consisting of hematopoietic stem cells, progenitor CD34+ cells, very early progenitor CD34+ cells, B-cell CD19+ progenitors, myeloid progenitor CD13+ cells, T lymphocytes, B lymphocytes, monocytes, dendritic cells, peripheral blood CD34+ cells, cancer B cells (BCLL), marginal zone lymphoma (MZL) B cells and thymocytes.

**14.** The method according to claim 13 wherein the hematopoietic cell is not prestimulated with at least one cytokine.

**15.** A stable virus packaging cell line producing the pseudotyped viral vector particle as defined in claim 1.

\* \* \* \* \*





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