Chapter 8

Lentiviral Vector Gene Transfer into Human T Cells

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Summary

Efficient gene transfer into T lymphocytes may allow the treatment of several genetic dysfunctions of the hematopoietic system, such as severe combined immunodeficiency, and the development of novel therapeutic strategies for diseases such as cancers and acquired diseases such as AIDS. Lentiviral vectors can transduce many types of nonproliferating cells, with the exception of some particular quiescent cell types such as resting T cells. Completion of reverse transcription, nuclear import, and subsequent integration of the lentivirus genome do not occur in these cells unless they are activated *via* the T-cell receptor (TCR) and/or by cytokines inducing resting T cells to enter in G_{1b} phase of the cell cycle. In T-cell-based gene therapy trials performed to date, cells have been preactivated via their cognate antigen receptor (TCR). However, TCR stimulation shifts the T cells from naïve to memory phenotype and leads to skewing of the T-cell population. Since, especially the naïve T cells will provide a long-lasting immune reconstitution to patients these are the cells that need to be transduced for effective gene therapy. Now it is clear that use of the survival cytokines, IL-2 or IL-7, allows an efficient lentiviral vector gene transfer and could preserve a functional T-cell repertoire while maintaining an appropriate proportion of naïve and memory T cells. In this protocol we give details on lentiviral transduction of T cells using TCR-stimulation or rIL-7 prestimulation. In addition, we describe the use of a new generation of lentiviral vectors displaying T-cell-activating ligands at their surface for targeted T-cell gene transfer.

Key words: IL-7, Lentiviral vector, Gene therapy, TCR, Human T-cell.

1. Introduction

1.1. T-Cell Gene Therapy

1.1.1. Human T Cells as Targets for Gene Therapy

One of the major advantages of using peripheral blood T cells is that they are easily 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 a leukemia was not observed in T-cell-based gene therapies *(1–4)*. Recently, it was also shown that retroviral vector

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integration deregulates gene expression in T cells to some extent. But this had no consequences on the function and biology of the transplanted T cells *(5)*. 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, so at least a long-term correction can be envisaged by T-cell gene therapy. Moreover, gene transfer of T cells improved enormously in the last years thanks to the engineering of new gene transfer vehicles, the lentiviral vectors (LVs). These new vehicles enable to obtain a highly efficient transduction of T cells without changing their phenotype or their functional characteristics (*see* **Subheading 1.2.3**).

1.1.2. T Cell Gene Therapy Trials

Efficient gene transfer into T lymphocytes may allow the treatment of several genetic dysfunctions of the hematopoietic system, such as severe combined immunodeficiency *(1, 2)*, and the development of novel therapeutic strategies for diseases such as cancers and for acquired diseases such as acquired immunodeficiency syndrome (AIDS) *(6)*.

Indeed, gene therapy has proven over the past years to be a solution for several inherited diseases such as severe combined immunodeficiency (SCID) *(7, 8)*, adenosine deaminase deficiency (ADA), *(9)* and hemophilia *(10)*. All these have been evaluated in clinical trials with success. Very recently a gene therapy trial of chronic granulomatous disease (CGD) resulted in successful treatment of the myeloid compartment *(11)*. These trials, however, were all stem cell-based gene therapy trials. Here we give some examples of T-cell gene therapy applications.

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. Indeed, in an allogeneic BM transplantation normal ADA-expressing T lymphocytes have a selective advantage in SCID patients and develop a protective immune system of donor-derived T lymphocytes *(12–14)*. Aiuti et al. showed immune reconstitution in ADA-SCID patients after T-cell gene therapy *(15)*. 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. Recently, also T cells of Wiskott–Aldrich Syndrome (WAS) patients were functionally corrected by transduction with lentiviral vector encoding WAS protein *(16)*.

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). Allo-BMT is widely used as a curative approach to many hematologic malignancies *(17, 18)*. Treatment with allogeneic T cells, either as a part of an allo-BMT or as an infusion of isolated allogeneic lymphocytes, offers the possibility of cure for patients with chronic myelogenous leukemia (CML) *(19)*. The specificity of this effect, called graft vs. leukemia effect (GVL), is not fully understood. Frequently, but not always, GVL is associated with graft vs. host disease (GVHD). The latter is a very severe side effect of allo-BMT, 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 herpes simplex virus thymidine kinase (HSV-tk) 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 HSV-tk-transduced T cells. This has already proven to be effective in the treatment of GVHD in clinical trials *(20–22)*.

Also for acquired diseases such as AIDS that are in demand of novel therapies, T-cell gene therapy might be an important option. While the treatment of human immunodeficiency virus (HIV)-infected people has been greatly improved by the development of highly active antiretroviral therapy (HAART) several problems remain. Limitations of HAART such as appearance of drug-resistant HIV variants and toxicity show that patients need novel additional immune therapies *(23, 24)*. Here, anti-HIV T-cell gene therapy is 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.

The first clinical HIV gene therapy trials used genes that inhibit HIV RNA and protein production (e.g., Transdominant rev and tat) and lead to low antiviral activity in the patients. Indeed, genes that inhibit production of viral RNA and protein, but allow the provirus to integrate are expected to mediate selection of cells containing a suppressed HIV provirus *(25, 26)*.

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 *(27, 28)*. A clinical phase I trial including patients with chronic HIV infection was performed using this strategy, which improved immune function in four out of five patients *(29)*. Recently, an antiviral gene was developed that encodes for a membrane-anchored peptide, which inhibits HIV entry on the level of virus-cell fusion with great efficacy *(30)*. Efficacy was also shown against several primary isolates in primary lymphocytes from different donors *(31)*. These kinds of genes that inhibit prior to integration of

the provirus are expected to lead to an accumulation of noninfected, gene-protected T cells. These kinds of therapies offer a solution for patients who do not respond any more to antiretroviral therapy.

In summary, it becomes clear that T-cell based gene therapy offers a valuable alternative for treating patients.

1.2. Lentiviral Vectors for T Cell Gene Transfer

1.2.1. MLV Retroviral Vectors Vs. Lentiviral Vectors

Vectors derived from retroviruses are probably among the most suitable tools to achieve a long-term gene transfer since they allow stable integration of a transgene and its propagation in daughter cells. To date, vectors derived from gamma-retroviruses such as murine leukemia viruses (MLVs) have been widely used for gene transfer into human T cells *(32)*. Perhaps one of the most important drawbacks associated with the use of such vectors is their inability to transduce nonproliferating target cells. Indeed, following internalization of the vector into the target cells' cytoplasm and reverse transcription, transport of the preintegration complex to the nucleus requires the breakdown of the nuclear membranes during mitosis *(33, 34)*. This provides a barrier to the use of MLV-based vectors in the many gene therapy protocols for which target cells such as T cells are quiescent or for which induction of cell proliferation is to be avoided. Lentiviral vectors, derived from HIV-1, have shown promise in the transduction of several resting cell types such as retinal cells, pancreatic islets, cells of the central nervous system, or progenitor and differentiated hematopoietic cells *(35)*. For these reasons, lentiviral vectors should be preferred gene delivery vehicles over vectors derived from gamma-retroviruses such as MLVs that cannot transduce nonproliferating target cells *(34)*. Thus, lentiviral vectors may provide a valuable alternative to overcome this problem owing to the lentivirus mechanism that allows mitosis-independent nuclear import of the preintegration complex and infection of nonproliferating cells *(36–38)*.

1.2.2. Restrictions of Lentiviral-Mediated Gene Transfer in Human T Cells

Several studies have now established the capacity of these HIV-1-derived vectors to transduce various types of nonproliferating cells both in vitro and in vivo *(35)*. However, some cell types that are important gene therapy targets are refractory to gene transfer with lentiviral vectors. This includes, in particular, early progenitor hematopoietic stem cells in G_0 *(39)*, monocytes *(40, 41)* and resting T lymphocytes *(42)*. That the parental virus, HIV-1, can enter into resting T lymphocytes but does not replicate *(43–47)*, has been attributed to multiple post-entry blocks. This includes in particular, (a) defects in initiation and completion of the reverse-transcription process *(43, 46–48)*, (b) lack of ATP-dependent nuclear import *(49)*, and (c) lack of integration of the proviral genome. Low levels of nucleotides in the resting cells do not entirely explain the restricted HIV-1 replication

since artificially raising intracellular nucleotide pools increased reverse-transcription products but not the level of productive infection *(50)*.

Inclusion of the HIV-1 central polypurine track (cPPT) in lentiviral vectors has resulted in enhanced transduction of human progenitor stem cells and T cells. However, the improved lentiviral vectors that include the cPPT sequence still fail to transduce nonactivated T lymphocytes *(42)* most likely because the primary block in initiation and/or completion of reverse transcription could not be alleviated with the novel vectors. Thus still activation of these cells, causing G_0-to-G_{1b} transition of the cell cycle, is required to relieve the blocks in gene delivery *(42–48)*. It is now reported that inducing the resting T cells to enter into the G_{1b} phase of the cell cycle by stimulation through the T-cell receptor and CD28 costimulation receptor, using anti-CD3 plus anti-CD28 antibodies, was sufficient to render the cells susceptible to HIV-1 infection and replication *(48)*. Alternatively, exposing T cells to cytokines that do not trigger cell division could render them permissive to transduction with HIV-1-vectors *(51)*. These findings suggest that partial activation of resting T cells is sufficient for gene transfer by HIV-1-derived vectors and that DNA synthesis or mitosis of these cells is not necessary.

1.2.3. Conservation of T Cell Phenotype After Lentiviral Transduction

The population of mature adult T cells can be divided into two different subsets, namely, memory and naïve T cells. Naïve T cells are especially important as gene therapy target cells since they maintain the capacity to respond to novel antigens. It is also of 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_{1b} via stimulation through the T-cell receptor allows efficient transduction of adult naïve T cells by HIV-1-based vectors and wt HIV-1 *(48, 52)*. However, TCR stimulation of T cells alters their half life and immune competence and often results in an inversion of physiologic CD4/CD8 ratio, enrichment in activated memory cells associated with loss of naïve T-cell subsets and a skewed TCR repertoire *(53–56*; **Fig. 1**). Up to now T-cell gene therapy trials are based on TCR-mediated stimulation of T cells.

However, transduction of naïve T cells is a pre-requisite for any T-cell-mediated gene therapy trial aimed at providing long-lasting immune reconstitution to patients. Therefore protocols were developed that allowed efficient LV transduction of T cells in the absence of TCR triggering. It was shown that IL-7, a master regulator of T-cell survival and homeostatic proliferation *(57–59)*, and also IL-2 and IL-15 promoted long-term survival in vitro of memory and naïve T lymphocytes. Thus it is of utmost interest to note that exposure of adult T cells to cytokines such as IL-2, IL-15, and IL-7 renders them permissive to lentiviral

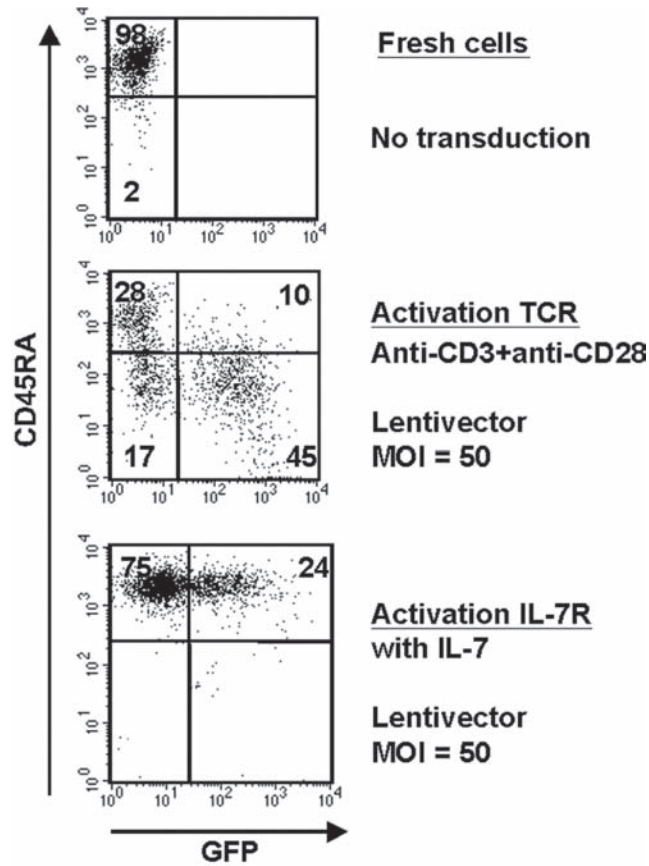

Fig. 1. Conservation of naïve phenotype after transduction of T cells with lentiviral vectors upon IL-7 prestimulation. Freshly isolated naïve adult CD4+ T cells are shown in (**a**). These naïve cells were transduced with VSV-G pseudotyped lentiviral vectors using MOIs of 10–20 after 24 h prestimulation with anti-CD3 and anti-CD28 antibody (1 μg/mL) (**b**) or after 6 days of rIL-7 (10 ng/mL) stimulation (**c**). The percentage of GFP+ naïve T cells (CD45RA+) was determined 6 days after transduction by FACS analysis.

transduction in the absence of TCR activation *(51, 56, 60–62)*. These cytokine-treated T cells move out of G_0 into the G_{1b} phase of the cell cycle, the phase in which T cells are susceptible to LV transduction, but did not start to proliferate *(56, 60–62)*.

Nevertheless, it is clear that only use of IL-2 and IL-7, but not IL-15, for lentiviral T-cell transduction could preserve a functional T-cell repertoire and maintained an appropriate proportion of naïve and memory CD4+ and CD8+ T cells with low expression of activation markers. Moreover, functional analysis of immune response to cytomegalovirus showed that IL-2- and IL-7-mediated transduced T cells were highly immunocompetent *(60)*.

Recently it was shown that (recombinant interleukin-7) rIL-7 promotes the extended survival of both naïve and memory CD4+ T cells, whereas cell-cycle progression of these two subsets is distinct and limited. In the continued presence of biologically active cytokine, IL-7-stimulated memory cells enter and exit from the cell cycle much earlier than naïve T cells *(63)*. This is in agreement with the fact that naïve adult T cells need at least 6 days of IL-7 stimulation vs. only 3 days for memory cells to allow efficient LV transduction (**Fig. 2**).

In conclusion, LV transduction of IL-2- or IL-7-stimulated cells overcomes the limitation of TCR-mediated LV gene transfer and may improve the efficacy of T-cell based gene therapy.

1.2.4. Targeting Gene Transfer to T Cells by Specific Vector-mediated Target Cell Activation

This targeting strategy consists of an interaction of a ligand displayed on the surface of the vector, which upon binding to its specific receptor will induce signaling and stimulation of the target cells. As a consequence of the specific stimulation, gene transfer into the target cell, in this case the T-cell, is significantly

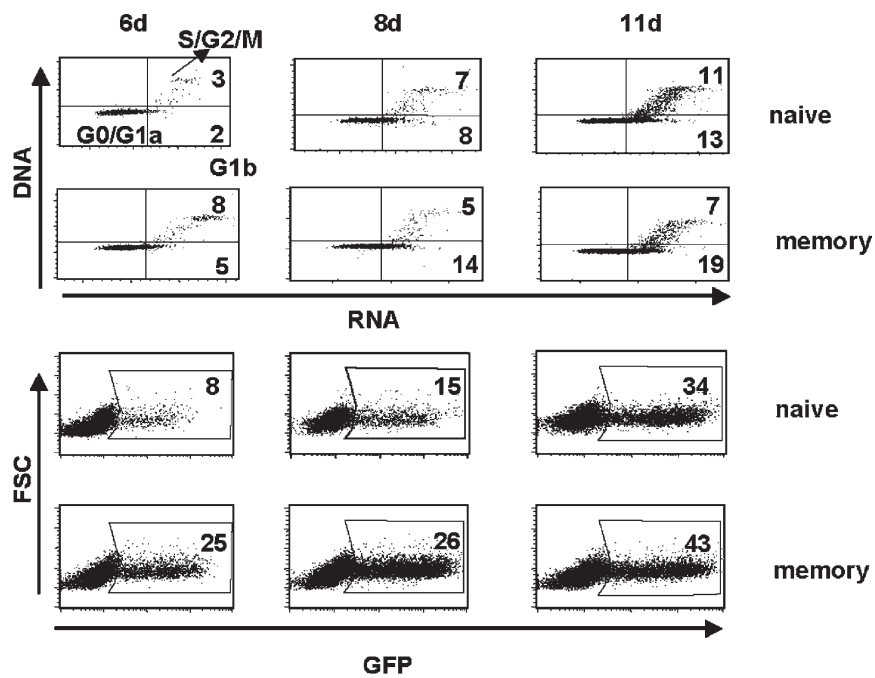

Fig. 2. Cell-cycle entry and permissiveness of IL-7 stimulated naïve and memory T cells to HIV-1 vector transduction. Naïve and memory CD4+ T cells were cultured in 10 ng/mL IL-7. Following 6, 8, 11 days of culture, cells were analyzed for cell-cycle progression by PY/7AAD staining. The percentages of cells in the G_{1b} (*lower right quadrant*) and $S/G_2/M$ (*upper right quadrant*) are indicated. Naïve and memory T cells from the same donor were infected with an HIV-1-based vector expressing enhanced green fluorescence protein (EGFP) and pseudotyped with the pantropic VSV-G envelope following 6, 8, 11 days of culture in rIL-7. Infections were performed at an MOI of 20, and infected cells were detected by monitoring EGFP expression. The percentages of EGFP+ infected cells are indicated.

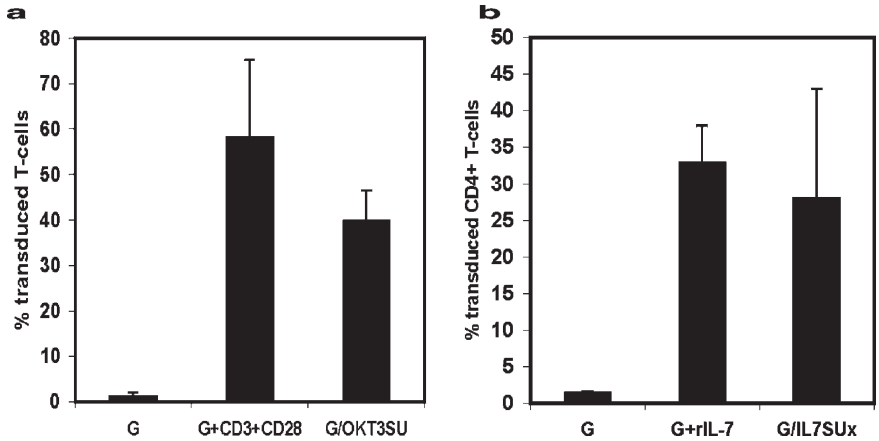

Fig. 3. Transduction of adult T cells by anti-CD3scFV- or IL-7-displaying lentiviral vectors. (**a**) PBLs were transduced with lentiviral vectors encoding for EGFP immediately upon isolation. Cells were transduced with VSV-G-pseudotyped lentiviral vectors (G) in the absence or in the presence of anti-CD3 and anti-CD28 antibodies or with anti-CD3scFv-displaying vectors (G/OK3SU). (**b**) CD4+ freshly isolated T cells were transduced with VSV-G-pseudotyped lentiviral vectors (G) in the absence or in the presence of rIL-7. Alternatively, transduction was performed with IL-7-displaying lentiviral vectors (G/IL7Sux). Multiplicities of infection of 20 were used. The % of GFP+ cells was determined on day 3 post-transduction by FACS.

enhanced. T cells are refractory to gene transfer with LVs as discussed earlier. Upgraded lentiviral vectors have therefore been engineered in order to overcome their inability to transduce nonactivated T cells. A T-cell-activating polypeptide was displayed on HIV-1 vector particles in order to target and stimulate the T cells at time of transduction. A single-chain antibody variable fragment (scFv) derived from the anti-CD3 OKT3 monoclonal antibody, which recognizes and activates the T-cell receptor, was fused to the amino-terminus of the SU subunit of the MLV envelope glycoprotein. This chimeric CD3-targeted MLV glycoprotein demonstrated reduced infectivity; thus, coexpression of an "escorting" wild-type VSV-G envelope protein was necessary to render the LV particles fully infectious. Stimulation by this surface-modified lentiviral vector was sufficient to allow efficient gene transfer in T lymphocytes, i.e., 100-fold more than the performance of unmodified lentiviral vectors in nonactivated T cells *(52*; **Fig. 3a**). However, it was demonstrated that the phenotype of the transduced naïve T cells was modified to memory cells as is the case for anti-CD3 + anti-CD28 antibody stimulation. Therefore, to transduce resting T cells while conserving their phenotype *(64, 65)*, human IL-7 gene was fused to the amino-terminus of the MLV envelope glycoprotein. IL-7 displaying LVs allowed efficient transduction of naïve neonatal CD4+ T cells as

well as memory CD4+ T cells allowing to maintain the functional characteristics of the naïve T cells *(56*; **Fig. 3b**). Importantly, a recent breakthrough has been made by engineering measles virus gp pseudotyped LVs that allow transduction of quiescent naive and memory T-cells *(66)*.

2. Materials

2.1. Buffers and Solutions

1. *2× Hepes-buffered saline (HBS) and 2 M $CaCl_2$*. Calphos Mammalian Transfection Kit (Clontech, BD Biosciences, Location San Diego, USA).
2. Phosphate-buffered saline (PBS) without calcium and magnesium, without sodium bicarbonate, sterile.
3. Trypsin-ethylenediminetetraacetric acid (EDTA) 1× Hank's balanced salt solution without calcium and magnesium, sterile.
4. Ficol-Paque Plus, sterile.
5. *Nucleic acid-staining solution (NASS)*. 0.15 M NaCl in 0.1 M phosphate-citrate buffer containing 5 mM sodium EDTA and 0.5% bovine serum albumin.
6. *Nucleic acid staining solution (NASS)*. 0.15 M NaCl in 0.1 M phosphate-citrate buffer containing 5 mM sodium EDTA and 0.5 bovine serum albumin (BSA, fraction 5, Sigma, Saint Quentin Fallavier, France pH 6.0).
7. *7AAD- (7-amino-actinomycin D-) staining buffer*. 0.03% saponin in NASS buffer containing 20 µM 7AAD.
8. Pyronine-Y (PY) stock solution 100 µM in water.

2.2. Media

1. Fetal calf serum (FCS), sterile.
2. DMEM (Dulbecco's modified Eagle medium) with 0.11 g/L sodium pyridoxine and pyridoxine. DMEM is supplemented with 10% FCS, 100 µg/L streptomycin, 100 U/mL penicillin (stored at 4°C).
3. RPMI medium is supplemented with 10% FCS, 100 µg/L streptomycin, 100 U/mL penicillin (stored at 4°C).

2.3. Nucleic Acids

1. Lentiviral vector DNA encoding for an HIV-1-derived self-inactivating vector with the internal SFFV (spleen focus foamy virus) promoter driving the reporter gene GFP.
2. Envelope glycoprotein expressing plasmids:
   (a) Fusion glycoprotein: stomatitis virus G glycoprotein (VSV-G).
   (b) *Activating and targeting* glycoproteins for T cells *(1)* OKT3-SU Env (anti-CD3scFv fused to the murine

leukemia virus envelope) and *(2)* IL-7SU Env (human interleukin-7 fused to murine leukemia virus envelope glycoprotein).

3. Virus structural protein (gagpol) expressing plasmid (pCMV8.91).

2.4 Cells and Tissue

1. 293T cells.
2. *Source of T cells.* Fresh adult or cord blood (*see* **Note 1**).

2.5. Special Equipment

Magnetic separation device (Dynal Location Biotech ASA, Oslo, Norway).

2.6. Additional Reagents

1. Rosette Sep cocktail from stem cell Technologies for separation of total T cells or CD4+ T cells (*see* **Note 2**).
2. Pan Mouse IgG Kit (Dynal Biotech ASA, Oslo, Norway) (*see* **Note 3**).
3. 24-well cell culture-coated tissue culture plates.
4. 0.45-µm filter.
5. Mouse monoclonal antibodies:
   (a) *For identification of phenotype.* Anti-hCD45RA-phycoerythrin (PE), anti-hCD45RO-PE, anti-hCD3-PE, anti-hCD4-PE, anti-hCD69-PE, anti-HLADR-PE, anti-hCD25-PE, and corresponding PE-conjugated mouse IgG controls.
   (b) *For isolation of nonactivated naïve and memory CD4+ T-cell population.* Anti-hCD69, anti-hHLADR, and anti-hCD45RA or anti-hCD45RO (BD Pharmingen, San Diego, USA).
   (c) *For stimulation via the T-cell receptor.* Anti-CD3 and anti-CD28 (BD Pharmingen, San Diego, USA).
6. *Cytokines.* Human rIL-7 (Preprotech, LocationRocky Hill, USA).

3. Methods

3.1. Production of VSV-G Pseudotyped Lentiviral Vectors and Lentivectors Displaying T-Cell-Activating polypeptides

1. *Day 0.* 2.5×10^6 293T cells are seeded the day before transfection in 10-cm plates in a final volume of 10 mL DMEM.
2. *Day 1.* Cotransfection of HIV packaging construct (8.6 µg) with the lentiviral gene transfer vector (8.6 µg) and (a) the glycoprotein VSV-G (3 µg) for VSV-G pseudotyped lentiviral vectors or (b) the glycoproteins VSV-G (1.5 µg) and OKT3SU env or (c) the glycoproteins VSV-G (1.5 µg) and IL7SUx env (1.5 µg) is performed using the Clontech calcium-phosphate transfection system.
3. *Day 2.* 15 h after transfection, the medium is replaced with 6 mL of fresh DMEM medium (*see* **Note 4**).

4. *Day 3.* 36 h after transfection, vectors are harvested, filtrated through 0.45-µm pore-sized membrane, and stored at –80°C for 2–3 months.

3.2. Immunoselection of Human T Cells

1. Add to the adult or cord blood Rossette Sep isolation cocktail for T-cell or CD4+ T-cell (25 µL/mL blood) and incubate for 20 min at room temperature while rocking.
2. Dilute blood + Rosette Sep cocktail 1: 1 with PBS and gently layer 35 mL of this diluted product on 15 mL Ficoll in a 50-mL tube.
3. Centrifuge the cells at 850 g for 30 min, 20°C without brake and collect the layer containing mononuclear cells (*see* **Note 2**).
4. Wash the collected mononuclear cell interface in PBS/2% FCS at 850 g, 20°C for 10 min.
5. If further isolation of memory and naïve T-cell subsets is wanted the T cells are resuspended at 10^8/mL PBS and incubated for 30 min at 4°C with anti-CD45RA antibody (1 µg/10^6 target cells) for isolation of memory T-cell or with anti-CD45RO antibody (1 µg/10^6 target cells) for isolation of naïve T cells (*see* **Note 5**).
6. Wash cells to remove the unbound antibody and resuspend in PBS/2% FCS.
7. Add the Pan Mouse beads to the T cells according to the manufacturer's indication (DynalBiotech ASA, Oslo, Norway) and incubate for 30 min while rocking at 4°C.
8. Place the tube into the Dynal magnetic device for 2 min and collect the unbound cells.
9. Remove the tube from the magnet and wash the beads once with PBS/2% FCS.
10. Repeat **step 8**.
11. The purity of the T-cell subsets is routinely 90–95%.

3.3. Titer Determination

1. *Day-1.* 293T cells are seeded in DMEM at a density of 2 × 10^5 cells per well in 6-well plates in a final volume of 2 mL.
2. *Day 0.* Serial dilutions of vector preparations were added to 293T cells and incubated O/N.
3. *Day 1.* Medium on the cells is replaced with 2 mL fresh DMEM and cells are incubated for 72 h.
4. *Day 3.* Cells are trypsinized and transferred in FACS tubes. The percentage of green fluorescent protein (GFP)-positive cells is determined by fluorescence-activated cell sorter (FACS) analysis.

3.4. Analysis of Transduction and Titer

1. Transduction efficiency is usually determined as the percentage of GFP-positive cells after transduction of 3×10^5 target cells with 1 mL of viral supernatant.
2. *Infectious titers.* Are provided as transducing units (TU)/mL and can be calculated by using the formula: Titer = %inf × $(3 \times 10^5/100) \times d$,; where "*d*" is the dilution factor of the viral supernatant and "%inf" is the percentage of GFP-positive cells as determined by FACS analysis using dilutions of the viral supernatant that transduce between 5 and 10% of GFP-positive cells.
3. *Multiplicities of infection (MOI).* Ratio between infectious particles and target cells that are required to optimally transduce target cells of interest, which are generally much less permissive to transduction than the cells used for titrations

3.5. Cell-Cycle Fractionation by 7AAD/Pyronin Y Staining (Fig. 2a)

1. *First DNA staining with 7AAD is performed.* T cells are resuspended at concentration of 10^6 cells/mL in 7AAD-staining buffer and cells are incubated for 30 min at room temperature (*see* **Note 6**).
2. Cells are put on ice for at least 10 min.
3. Subsequently, RNA staining is performed by adding to the T cells, resuspended in the 7AAD-staining buffer (no washing is required), PY at a final concentration of 5 µM; cells are kept on ice for 10 min and are immediately analyzed on a FACS Calibur (BD Biosciences, San Diego, USA).
4. The living T cells are gated and in this gate cells in G_0 are identified by their minimal RNA (PY) and DNA content (7AAD), whereas cells in G_{1b} are identified by low DNA content but increased RNA content, S + G_2 + M phases were defined as those with high or maximal PY staining and increased DNA staining (**Fig. 2a**).

3.6. Transduction of Human T Cells

1. 1×10^6 T cells are seeded in RPMI medium 10% FCS in 24-well plates and are then prestimulated:
   (a) For TCR-mediated activation stimulation with anti-CD3 antibody (1 µg/mL) and anti-CD28 antibody (1 µg/mL) is performed during 24 h to obtain efficient lentiviral transduction (*see* **Note 7**).
   (b) In the absence of TCR activation stimulation with rhIL-7 (10 ng/mL) was performed during 3–4 days for total or memory adult T cells; naïve T cells need to be prestimulated for 6–12 days to obtain efficient lentiviral transduction (**Fig. 2b**).
   (c) No prestimulation is needed for lentiviral vector displaying T-cell-activating ligands.

2. Prestimulated T cells are transduced at an MOI of 10 overnight and transduced cells are washed and resuspended in RPMI/10% FCS supplemented with IL-2 (1 ng/mL) for the TCR-activated cells or with rhIL-7 (10 ng/mL) for the IL-7 prestimulated cells during a further 72 h before transduction efficiency is determined by flow cytometry. Alternatively, the OKT3SU/VSV-G and IL-7SUx/VSV-G codisplaying vectors are added to the freshly isolated T cells at an MOI of 10. The vector is not removed until analysis by flow cytometry at 72 h post-transduction (**Fig. 3**).

3.7. Evaluation of Transduced T Cell Phenotype by Cell Surface Marker Staining (Fig. 3)

1. The transduced T cells were divided into aliquots in PBS/2% FCS to stain for different phenotypic markers: anti-hCD45RA-PE for naïve T-cell identification, anti-hCD45RO-PE, antibodies for memory T-cell phenotype and anti-CD25-PE, anti-CD69PE, and anti-HLADR-PE to verify T-cell activation. In all cases, corresponding PE-conjugated mouse IgG controls need to be used to evaluate specific labeling. Incubation is performed at 4°C for 20 min at concentrations indicated by the manufacturer.
2. Cells are washed once with PBS/2% FCS.
3. GFP+ cells are detected for the different T-cell subsets by two-color flow cytometry analysis.

4. Notes

1. T cells from cord blood contain over 90% naïve T cells that reside in a more immature stage than adult naïve T cells which make up only one-third of the adult T-cell population. The former naïve cells are considered as recent thymic emigrants and enter much easier into cell cycle after cytokine stimulation with IL-7 as compared to adult naïve T cells.
2. The Rossette sep cocktail works through negative selection in order not to activate the T cells. This cocktail contains tetrameric antibody complexes which crosslink unwanted cells to the red blood cells. This increases the weight of the unwanted cells that will be pelleted together with erythrocytes after a Ficoll gradient. The layer of mononuclear cells that appears at the top of the Ficoll after centrifugation contains only T cells (purity of T cells is over 95%).
3. The Pan Mouse magnetic beads will allow to retrieve all unwanted cells that are bound by a cell type-specific mouse monoclonal antibody. This methodology allows to remove different cell types from the T-cell population isolated by

Rosette Sep isolation, e.g., all activated cells and memory cell can be removed in a single step through negative selection, without activating the target cells.

4. For the lentiviral vector displaying T-cell-activating polypeptides, extra Hepes is added to the DMEM medium to guarantee stability of the OKTSU and IL-7SU env gps displayed on the lentiviral vectors after freezing at –80°C.
5. If removal of activated T cells is wanted, add anti-CD69 and anti-HLADR mouse monoclonal antibody in addition to the anti-CD45RA or anti-CD45R0 antibody at 1 (1 µg/10^6 target cells). Freshly isolated T cells contain approximately 50% memory cells, 50% naïve cells of which maximum 10% express the T-cell activation markers CD69 and HLA-DR.
6. For 7AAD/PY cell-cycle staining it is very important to respect the order of staining: first the DNA is stained with 7AAD followed by staining of the RNA by PY. PY can stain as well DNA as RNA and will only stain the RNA when the DNA was previously stained with 7AAD. This allows to identify the different cell-cycle phases: G_0, G_{1b}, $S/G_2/M$ as depicted in **Fig. 2a**.
7. Alternatively, TCR stimulation can be performed by (1) precoating the culture plates with anti-CD3 antibody (1 µg/mL PBS) for 2 h at RT. The anti-CD3 antibody solution is then removed and replaced with 1 mL of T cells in RPMI/10% FCS to which anti-CD28 is added; (2) incubating T cells with beads that are coated with optimized amounts of anti-CD3 and anti-CD28 according to the manufacturer's protocol.

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