Genetic in vivo engineering of human T lymphocytes in mouse models

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Receptor targeting of vector particles is a key technology to enable cell type–specific in vivo gene delivery. For example, T cells in humanized mouse models can be modified by lentiviral vectors (LVs) targeted to human T-cell markers to enable them to express chimeric antigen receptors (CARs). Here, we provide detailed protocols for the generation of CD4- and CD8-targeted LVs (which takes ~9 d in total). We also describe how to humanize immunodeficient mice with hematopoietic stem cells (which takes 12–16 weeks) and precondition (over 5 d) and administer the vector stocks. Conversion of the targeted cell type is monitored by PCR and flow cytometry of blood samples. A few weeks after administration, ~10% of the targeted T-cell subtype can be expected to have converted to CAR T cells. By closely following the protocol, sufficient vector stock for the genetic manipulation of 10–15 humanized mice is obtained. We also discuss how the protocol can be easily adapted to use LVs targeted to other types of receptors and/or for delivery of other genes of interest.

Introduction

T lymphocytes are a focus of current research in basic immunology and are used in gene therapy and immunotherapy. Their genetic manipulation is a key technology with applications in both fields. Many different methods have been described, ranging from plasmid transfer by electroporation or chemical methods to the use of viral vectors1–3. Retroviral vectors and lentiviral vectors (LVs) have emerged as basic technologies that can be used for genetic manipulations in cell-based gene therapy medicinal products4. Those products consist either of hematopoietic stem cells harboring an intact copy of the defective protein for the treatment of patients suffering from genetic diseases or lymphocytes expressing recombinant proteins such as chimeric antigen receptors (CARs) to facilitate cancer immunotherapy5. Most methods used for genetic manipulation of T lymphocytes today require modification and expansion of the T lymphocytes ex vivo before infusion into the patient. This is an established clinical approach used in immunotherapy and gene therapy. For the latter, it requires each patient to be treated with an individually produced T-cell product. In addition, manipulating the T cells outside the home organism alters their phenotypes and properties6–8. An alternative strategy is to genetically manipulate T lymphocytes directly in vivo, in their physiological environment. This strategy has become possible as a consequence of the development of so-called receptor-targeted LVs discriminating between T lymphocytes and other cells at the level of cell attachment and entry9.

In this protocol, we describe how to generate vector stocks that transfer CARs to targeted T cells in vivo and administer them to appropriate mouse models. We also provide details on how to humanize and handle mice and monitor for the presence of CAR T cells. We have used this protocol to generate CD19-CAR–specific T lymphocytes in humanized mouse models10,11. These in vivo–generated CAR T cells eliminated CD19-positive tumor cells and B lymphocytes even when the CAR was delivered exclusively to CD4-positive T lymphocytes12.

Receptor targeting

The concept of receptor targeting is based on the premise that the requirement for a gene-delivering vector to use a specific receptor to enter a cell leads to cell entry selectivity and thus restricts
expression of the gene to a specific cell type. Receptor usage can be manipulated by adding targeting ligands to the vector surface that exhibit high affinity for a cell surface receptor selectively expressed on the desired target cell type. By simultaneously destroying natural receptor usage, gene delivery to non-target cells is eliminated. This approach is limited to certain viral glycoproteins because it requires extensive protein engineering. Suitable glycoproteins can be derived from paramyxoviruses, particularly measles virus (MV) and Nipah virus (NiV). In both viruses, receptor attachment and membrane fusion functions are split across two glycoproteins. This is in contrast to the glycoprotein G of vesicular stomatitis virus (VSV), which is commonly used in conventional LVs and has both functions combined. By engineering paramyxoviral glycoproteins, LVs have been generated that deliver genes selectively into distinct cell types, such as cancer cells, subtypes of neurons or endothelial cells. Making use of the distinct surface markers of T-cell subtypes, CD8 for cytotoxic T lymphocytes and CD4 for helper and regulatory T cells, enabled the generation of LVs targeted to specific T-cell subtypes. CD8-LV carries the NiV G protein fused to a human CD8-specific single-chain antibody (single-chain variable fragment (scFv)). Although the engineered NiV glycoproteins are better incorporated into vector particles, they rely on membrane proximal target receptor binding for proper cell entry. CD4-LV, which binds to the membrane distal domain of CD4 via a human CD4-specific designed ankyrin repeat protein (DARPin), is therefore based on the MV glycoproteins. CD8-LV and CD4-LV have enabled the in vivo delivery of CARs specific for the CD19 antigen present on B-cell malignancies. A single intravenous injection of CD8-LV into humanized mice was sufficient to induce the formation of CD8\(^+\) CD19-specific CAR T cells, whereas CD4\(^+\) lymphocytes remained unmodified. The in vivo-generated CAR T cells eliminated CD19\(^+\) B lymphocytes as well as tumor cells. Conversely, CD4-LV mediated the exclusive generation of CD4\(^+\) CAR T cells, which were equally active in eliminating tumor cells.

**Applications of the protocol**

The in vivo generation of CAR T cells using CD8-LV or CD4-LV is currently the most prominent application of receptor-targeted LVs. This protocol describes the generation and use of a myc-tagged second-generation CD19-specific CAR. A prior version of this protocol has been made available via Protocol Exchange. The protocol can be applied to any other type of CAR and thus is adaptable to the constant improvements in CAR T cell technology. However, the procedure for CAR T cell detection does need to be adapted if different types of immunological tag or reporter gene are used in combination with the CAR.

In addition to targeting cancer cells, another application of CAR T cell therapy is the targeting of chronically infected cells. The humanized mouse model described in the current protocol is engrafted with a spectrum of human cells susceptible to HIV, which leads to chronic viremia accompanied by HIV-induced loss of human CD4\(^+\) T cells. It is thus well established for the testing of anti-HIV gene therapies. Moreover, the model recapitulates HIV latency and generates human immune responses similar to those seen in patients with HIV. HIV therapies have predominantly been evaluated in NOD/shi-SCID, γ\(^c\)-/- (NOG)\(^{23,24}\) or NOD/SCID, γ\(^c\)-/- (NSG)\(^{25,26}\) mice. Therefore, CD4-LV or CD8-LV delivering CARs directed against HIV can also be evaluated in these humanized mice to determine whether CAR T cells are generated and whether there is subsequent elimination of HIV-infected cells.

Beyond CARs, the provided protocol can be used to package any gene of interest into CD4-LV or CD8-LV. Recombinant T-cell-receptor genes are an obvious related example, while antiviral genes, cytokines or interfering RNA are other options, with the latter being relevant for more basic research. When used ex vivo, gene delivery rates in the same range as those reported for CAR genes can be expected. In vivo, however, it must be kept in mind that CARs (and also recombinant T-cell receptors) mediate a selective advantage for transduced T cells, potentially resulting in their preferential proliferation. Accordingly, we have observed between 5- and 25-fold higher rates of transduced cells after delivery of CAR genes compared to reporter genes.

In addition, other types of receptors can be targeted if their extracellular parts are readily accessible by the vector particle. Target receptors can be expressed on other human T-cell subtypes, on completely different cell types or even on cells of other species, such as murine CD8 or CD4. In addition to the requirement for a target receptor, the coding sequence for a suitable targeting ligand, preferably an scFv or DARPin, is required. Once confirmed that the engineered glycoprotein composed of targeting ligand and NiV G protein or MV H protein is well expressed on the surface of packaging cells, the procedure provided here for the generation of CD4-LV or CD8-LV stocks can be followed.
by exchanging the G or H protein–encoding plasmids. When targeting receptors of other species, it has to be kept in mind that restriction factors may interfere with proper transduction\(^{29}\). For primary lymphocytes from non-human primates, switching to simian immunodeficiency virus–derived vectors is a straightforward solution. Both, CD4-LV and CD8-LV, are highly active on non-human primate lymphocytes\(^{17,18}\).

Alternative methods for receptor-targeted gene delivery

The other LV-based system for receptor-targeted vectors to date relies on engineered Sindbis virus (SINV) glycoproteins\(^{30}\). The engineered SINV-LVs have been targeted to various cell types including human T lymphocytes via CD4\(^{31}\) or CD3\(^{32}\). However, data demonstrating that these vectors can successfully target primary cells is scarce, and the in vivo generation of CAR T cells has not been demonstrated with this technology. SINV-LVs have often been equipped with tissue-specific promoters to achieve sufficient selectivity. Although a side-by-side comparison has not been undertaken, it seems that they do not reach the selectivity demonstrated for engineered paramyxoviral glycoproteins as illustrated, for example, by the discrimination between CD4\(^{+}\) and CD8\(^{+}\) T lymphocytes\(^{33}\). An almost absolute selectivity in distinguishing between on-target and off-target cells can be of ultimate importance when toxic or oncogenic genes are delivered. For example, for CARs, the inadvertent delivery of a CD19-CAR into the patient’s malignant cells can result in severe adverse events with a fatal outcome, as described for CAR delivery by VSV-LV\(^{34}\). Independently from selectivity, paramyxovirus glycoprotein–based LVs follow a different cell entry mode from that of other LVs. SINV-LVs and VSV-LVs require endocytosis of the target receptor for proper entry and transduction. MV and NiV pseudotyped LVs enter cells directly at the cell membrane under neutral pH\(^{35}\). Blockage of endocytosis enhances their gene delivery activity, especially when receptors with a high endocytosis rate are targeted\(^{36,37}\). Conversely, this means that receptors with absent or low endocytosis rates can be targeted.

There has been enormous progress in the development of non-viral vector systems during the past years. Nevertheless, there was some surprise when the in vivo generation of murine CAR T cells was achieved with synthetic nanoparticles, because this requires stable integration of the delivered genetic information in the T-cell genome\(^{38}\). This was achieved by a copackaged transposase and T-cell selectivity via an incorporated CD3-specific antibody. Although this is remarkable progress for the gene-delivery field, it is also obvious that such nanoparticles will require further improvement to reach the activity and selectivity of LVs. It remains to be seen if CD3-targeted nanoparticles can be engineered that deliver CARs not only to murine but also to human T cells.

Alternative mouse models for in vivo CAR T cell generation

The humanized mouse with reconstituted human hematopoietic and immune cells is a powerful tool for investigation of human biological systems and for translational research\(^{39,40}\). Humanized mice enable direct access to the dynamics of the human immune-hematopoietic system. Two strains of immunodeficient NOD/SCID mice homozygous for targeted mutations at the Il2rg locus are available: the NOD/SCID/IL2r\(\gamma\)cnull (NOG) strain\(^{23,24}\) and the NOD/SCID/IL2r\(\gamma\)null (NSG) strain\(^{25,26}\). Compared to NOD/SCID mice, transplantation of human CD34\(^{+}\) cells, also called hematopoietic stem and progenitor cells (HSPCs), into NSG or NOG recipient mice robustly improved the hematopoiesis and immunity in the context of gene and cancer therapy, including CAR T cell therapy\(^{42,43}\).

We describe humanization of the NSG model because it is the most widely distributed model used in Europe and beyond. Therefore, the procedure for humanization described here will be widely applicable beyond the in vivo evaluation of targeted vectors. Humanized mice are also commercially available. However, to obtain a homogenous cohort of humanized mice, we highly recommend that they be generated in house. It is important to note that HSPC–humanized NSG mice are unable to develop a functional human innate immune system and thus do not support human myeloid, natural killer cell, erythroid and macrophage lineage development\(^{44,45}\).

Two different mouse strains can be used as alternatives. The first is c-Kit receptor-mutant mice (c-Kit mutant mice) on the NSG background, a strain that supports unprecedented levels of human
engraftment, including myelo-erythroid differentiation. The second strain is MI(S)TRG mice, which are immunodeficient rag2−/− IL2rG−/− mice in which the human genes encoding M-CSF, IL-3, GM-CSF and TPO were knocked into their respective mouse loci. In addition, these mice are transgenic for human SIRPα, which allows mouse phagocytes to tolerate human engrafted cells. However, neither of these mouse models expresses human HLA molecules on thymic epithelial cells. Therefore, human T cells developing in CD34−-humanized NSG mice lack the ability to recognize antigens in a human HLA-restricted manner. However, when engrafted with CD34+ cells and a functional autologous human thymus, education of T cells on human HLA was achieved (BLT-mice). The BLT mouse model, although it has major advantages, is much more cumbersome to generate, and human fetal liver and thymic tissue is not easily accessible by many research groups.

Limitations of this protocol
Although selectivity for target cells is excellent for receptor-targeted LVs including CD4-LV and CD8-LV, the amount of particles able to transfer genes in vector stocks often lags substantially behind that of VSV-LV. To compensate for that, it is of utmost importance that the complete production process is performed under the optimal conditions provided in this protocol for the production of CD8-LV or CD4-LV. Notably, the expected yield of particle numbers does not differ between targeted and non-targeted LVs. We therefore assume that the functional activity and stability of the engineered glycoproteins is reduced. Functional titers could indeed be improved by switching from MV glycoproteins to those of NiV, which are three to four times more efficiently incorporated into the lentiviral particle. Likewise, DARPins instead of scFvs improved titers because of the former’s higher stability.

In this context, it is important to mention that functional titers provided in transducing units (t.u.) per volume very much depend on the particular experimental conditions and the cell type used. Yet, they are required to confirm the activity of a vector stock as a general quality check. They are, in our experience, rarely predictive for the in vivo performance of vector stocks, especially when comparing targeted and non-targeted vectors. Here, receptor-targeted LVs usually outperform VSV-LV, which attaches to multiple cell types in vivo via the LDL receptor, resulting in a completely different biodistribution. However, also ex vivo, CD4-LV and CD8-LV can reach or even outperform VSV-LV when applied in the presence of transduction enhancers and/or on minimally activated T lymphocytes that express low levels of the VSV receptor.

Often, a single surface marker is not sufficient to define a cell type of choice. Many subtypes of T lymphocytes exist, and they can be distinguished through combinations of particular surface markers. Therefore, receptor-targeted LVs that require more than one cell surface marker are desirable. However, such vectors have not yet been described. The display of two different targeting ligands on the particle surface expands rather than restricts the tropism of that vector, because each target receptor can be used separately. Notably, in vivo selectivity is influenced by many more parameters than just receptor expression. First, cells with a high target receptor density will be preferentially transduced over those with a lower density. Second, local administration routes directly into the tissue of choice (e.g., lymphoid tissues for CD4-LV and CD8-LV) can be an option to prevent encounters of the vector particles with the unwanted cell type. Third, promotor choice, miRNA target sequences and restriction factors expressed in particular cell types can prevent gene expression in some target receptor-positive cell types. An example of CD4-positive cells that are not transduced by CD4-LV are monocytes, which are resistant to LVs that lack vpx, the virion-associated protein encoded by most simian immunodeficiency virus strains and HIV-2. These issues should be carefully considered when designing novel types of receptor-targeted LVs.

Experimental design
The process we describe here to achieve in vivo CAR T cell generation can be split into four parts (Fig. 1). The generation of CD8-LV or CD4-LV vector stocks is the crucial first stage to achieve sufficient in vivo gene delivery rates (Fig. 1a; Steps 1–18). The second part, which runs in parallel to the vector production part, is the generation of homogenously humanized mice (Fig. 1b, phase 1; Steps 33–74). Part 3 includes preconditioning of the mice and vector administration (Fig. 1b, phase 2; Steps 75–78). In the last part, mice are monitored for 1–8 weeks for the production of CAR T cells (Fig. 1b, phase 3; Steps 79–86).
Production and quality control of vector stocks
The generation of CD8-LV and CD4-LV stocks differs from that required for conventional VSV-LVs because they carry paramyxoviral glycoproteins. An additional plasmid is required for the transfection step, and particle concentration requires a more sensitive approach, achieved by sucrose cushion centrifugation to reduce shear forces. We recommend retaining a small aliquot of packaging cell supernatant before concentration for assessment of vector particle activity to control the concentration step. In addition, depending on the stability of the displayed scFv, syncytia formation in

Fig. 1 | Overview of procedure. a, Receptor-targeted LVs are produced by transfection of Lenti-X 293T cells with four different plasmids: the attachment plasmid encoding the MV or NiV glycoprotein fused to the targeting ligand, the plasmid encoding the compatible membrane fusion protein, the packaging plasmid and the transfer vector plasmid encoding the gene of interest. After 2–3 d, LVs are harvested and concentrated by centrifugation over a sucrose cushion. b, Timeline and experimental overview for the generation of humanized mice, LV administration and CAR T cell monitoring. For phase 1, NSG mice of 3–5 weeks of age are humanized after preconditioning and injection of prestimulated CD34⁺ cells. Follow-up of humanization includes sampling blood every 3–4 weeks upon reconstitution. A humanization level of ≥40% determines the start of phase 2, where mice are treated with two s.c. injections of IL7 (day −4 and day −1) followed by LV injection after one additional day. Mice are monitored weekly for CAR T cells by flow cytometry and human cytokines in blood for ≤8 weeks (phase 3).
the packaging cells may occur, which makes the timing of particle harvest crucial. Overall, it is recommended to follow the instructions for vector stock production as precisely as possible. The quality of the starting components, especially the plasmids and the packaging cells, is of ultimate importance. As a positive control, VSV-LV stocks should be generated in parallel or in a test run and result in $\geq 5 \times 10^{11}$ particles/ml with $10^8$–$10^9$ t.u./ml. Using an identical transfer vector plasmid for generation of the VSV-LV control stock is fundamental, because the encoded gene influences the delivery activity of the vector stock.

Functionality of the LVs has to be confirmed by incubation of target receptor–positive cell lines with different concentrations of the LV stock to determine the transducing units (t.u./ml) by staining of transgene-positive cells via flow cytometer. For quantitative comparisons necessary for in vivo applications, however, the particle number of the LV stock appears to be more important. This can be measured either by nanoparticle tracking analysis (NTA), which provides very precise particle numbers and the size of the particles, or via p24 ELISA. When producing new types of targeted LVs, we recommend using GFP as a transgene for initial testing of proper gene transfer activity. In addition, selectivity can best be assessed with GFP by comparing gene transfer into target receptor–positive and -negative cells. The amount of GFP in the latter should be similar to that seen for background activity. Expression and incorporation of the glycoproteins should be verified by western blot analysis of vector stocks. As a final quality control stage, CAR-encoding LVs should be tested for functionality on primary cells such as peripheral blood mononuclear cells (PBMCs) to check that CAR T cells develop and kill target cells (e.g., CD19$^+$ cells in the case of CD19-CAR T cells) in vitro.

**Generation of humanized mice**

Immunodeficient NSG mice lack murine B, T, natural killer and functional myeloid cells and are readily engrafted by CD34$^+$ cells, subsequently resulting in the development of a human blood system. To obtain a robust and homogeneous engraftment, NSG mice are preconditioned with a sublethal dose of busulfan, which reduces the number of residual murine progenitors and creates space in the bone marrow that can be used for the engraftment of human progenitor cells. Human CD34$^+$ cells are injected into the blood stream of NSG mice to humanize them. The quality of the CD34$^+$ cells, the age of the mice, sterile housing and experimental conditions are all crucial to obtain an efficient engraftment, as explained in this procedure.

The final part of this stage of the protocol includes a quality control check of the extent of NSG humanization over time. This is required to identify the optimal time point for vector particle injection. To increase transduction levels in vivo, we describe pretreatment with IL7, which slightly activates the T cells and makes them more permissive to the vector that is subsequently injected. However, if an agonistic CD3-targeted LV is used, IL7 pretreatment can be omitted.

As a complementation to full humanization with blood stem cells, NSG mice can also be engrafted with human PBMCs before LV administration (Fig. 2). This mouse model is less expensive and easier to set up. We therefore use it for an initial analysis of the in vivo performance of newly generated vector types. Moreover, tumor cells can be administered to this mouse model to provide a target for the in vivo–generated CAR T cells. Because of the xenoreactive setting, however, T lymphocytes in this model are more activated than those of fully humanized mice. PBMC-transplanted mice therefore cannot replace, but only complement, fully humanized mice.
Detection of in vivo–generated CAR T cells

Monitoring of the mice after vector administration, detection of CAR T cells and evaluation of target cell–specific transduction in blood can be performed at different time points, and tissues are analyzed at experimental endpoint conditions. Although protocols for CAR T cell detection and analysis in mouse models have been published, it is important to emphasize that the kinetics of CAR T cell development after in vivo gene transfer differ substantially from that of transplanted ex vivo–generated CAR T cells. Ex vivo–generated CAR T cells are expanded in culture and then transplanted into mouse models in high numbers, which results in an instant high concentration of CAR T cells in vivo. Timing and detection methods therefore have been adapted in this protocol to the kinetics of in vivo CAR T cell generation.

The most direct detection of CAR T cells is flow cytometry using fluorescently labeled antibodies recognizing the CAR. In addition, PCR can be used to detect the integrated vector sequence in the genome of T lymphocytes. The detection of the in vivo–generated CAR T cells can be challenging when the signal-to-noise ratio obtained by flow cytometry is low. It is then crucial to have sufficient numbers of control mice available. Such mice injected just with PBS or control particles are essential to set the gating for the identification of CAR-expressing T lymphocytes.

Materials

Biological materials

Plasmids

See Fig. 3 for details. All plasmids described here are available either from commercial suppliers (e.g., Addgene) or from the authors upon signing material transfer agreements (MTAs) ▲ CRITICAL All plasmids should be adjusted to a concentration of ~1 µg/µl to use similar volumes in each production. We recommend plasmid production by a commercial supplier with certified quality. The absence of RNA and endotoxin ≤100 endotoxin units (EUs)/mg DNA are most important. Alternatively, plasmids can be prepared using Macherey Nagel Maxiprep kits (Nucleo Bond Xtra Midi 100, cat. no. 740410.100) or an equivalent kit.

• Transgene plasmid: pS-CD19.CAR-W ▲ CRITICAL This plasmid contains a myc-tag for detection. Other tags, like NGFR, can be used as well. Alternatively, any other transgene plasmid packagable by HIV-derived LVs can be applied. When setting up vectors targeted to other receptors, we recommend using GFP to follow gene transfer activity and target cell selectivity.

• Second-generation HIV packaging plasmid: pCMV-dR8.91 ▲ CRITICAL Third-generation packaging plasmids can be used as well. In this case, plasmid ratios have to be adapted (see Step 4).

• Envelope plasmids used for CD8-LV production: plasmid encoding NiV envelope glycoprotein G fused to a CD8-specific scFv (pCAGGS-NiV-GcA34-aCD8opt) and NiV envelope fusion protein F–encoding plasmid (pCAGGS-NiV-FcA22)

• Envelope plasmids used for CD4-LV production: plasmid encoding MV envelope glycoprotein H fused to CD4-specific DARPin 29.2 (pCG-Hmut-CD4.DARPin29.2) and MV envelope fusion protein F–encoding plasmid (pCG-FcA30)

Cells

• Lenti-X 293T human embryonic kidney cells (Takara Bio, cat. no. 632180; RRID: CVCL_4401) ▲ CRITICAL These cells are specifically generated for production of LV particles and result in about twofold higher yields than HEK-293T/17 cells (RRID: CVCL_1926) in our hands.

• CD4– and/or CD8-positive cells for quantification of gene transfer activity of LVs (see Steps 20–23) (e.g., Molt 4.8 cells (NIH-ARP, cat. no. 175-146; RRID: CVCL_F827), A301 cells (CD4-positive T-cell line; NIH-ARP, cat. no. 166-382; RRID: CVCL_6244) and J758 (CD8-positive Jurkat cells; cells can be provided upon signing an MTA)) and primary human lymphocytes (Steps 24–32) derived from buffy coats of healthy donors regularly checked for the absence of human pathogens and having provided informed consent ▲ CRITICAL Any cell line expressing the target receptor of the used LV (e.g., CD4 and CD8) at a sufficient level can be used for titration. This should be checked via flow cytometry before first use (see Fig. 4a as an example) ! CAUTION The cell lines used in your research should be regularly checked to ensure that they are authentic and not infected with mycoplasma.

• Human CD34+ cells (alternatively called HSPCs) isolated from cord blood obtained from the ‘Etablissement Français du Sang’ (Besançon, France) upon informed consent. See Reagent setup for details on how to isolate these cells.
**Reagents**

**LV production and analysis**

- DMEM high glucose without L-glutamine with sodium pyruvate (Biowest, cat. no. L0106-500)
- RPMI 1640 without L-glutamine (Biowest, cat. no. L0501-500)
- FBS, heat-inactivated (56 °C, 30 min) (e.g., Sigma, cat. no. F7524) **CRITICAL** We test new lots experimentally after adaptation of the cells for ≥2 weeks in medium supplemented with FBS by monitoring cell growth and morphology as well as efficiency of vector production.
**Fig. 4 | Characterization of target cells and vector stocks.**

*a*, Surface expression of target receptors CD4 and CD8 as determined on Molt4.8, A301 and CD8-positive Jurkat (J76S8ab) cells by flow cytometry. Unstained cells (us) served as a control. *b*, Representative nanoparticle tracking analysis of a CD8-LV stock encoding CD19CAR. The NanoSight N5300 (Malvern Panalytical) and Nanosight NTA software version 3.3 were used to determine size distribution and calculate particle number (3 × 10^{12} particles/ml) from the area under the curve in three replicas. Samples were diluted 1:3,000 in PBS before tracking. *c*, Gene transfer activity of a CD8-LV stock encoding the CD19-CAR. Serial dilutions of the vector stock were incubated with Molt4.8 cells. CAR expression was measured by the expression of the myc-tag after 4 d via flow cytometry. Side scatter area (SSC-A) is displayed on a linear scale, whereas the axis scale for the CAR (PE) is logarithmic. *d*, Variability in gene transfer activities of various batches of VSV-LV (n = 13), CD8-LV (n = 19) and CD4-LV (n = 9). Each vector stock encoded the CD19-CAR. VSV-LVs were concentrated 180-fold, whereas targeted LVs were concentrated 300-fold. Values are displayed as t.u./ml. CD4-LV was incubated with Molt4.8 cells or A301 cells, CD8-LV with Molt4.8 cells or J76S8ab cells and VSV-LV with one of these cell lines above. Data from several experiments are displayed as means ± s.d. See individual titers for each LV in the corresponding source data.

- L-Glutamine solution (Sigma, cat. no. G7513-100 ml)
- PBS without Mg^{2+}/Ca^{2+} (Sigma, cat. no. 17-512F)
- 0.25% (wt/vol) trypsin in PBS without Mg^{2+}/Ca^{2+}/1 mM EDTA (in house)! **CAUTION** Trypsin is a hazardous chemical. Avoid contact with skin, eyes and airways.
• Polyethylenimine (PEI) (branched, molecular weight: 25,000 Da; Sigma-Aldrich, cat. no. 408727-100ML)  **CAUTION**  This is a hazardous chemical. Avoid contact with skin, eyes and airways and do not swallow.

• Sucrose BioUltra (Sigma, cat. no. 84097)

• BSA (Sigma, cat. no. A3294)

• CD3 antibody, clone: OKT3 (Miltenyi Biotec, cat. no. 130-093-387; RRID: AB_1036144)

• CD28 antibody, clone: 15E8 (Miltenyi Biotec, cat. no. 130-093-375; RRID: AB_1036134)

• Human IL-2, premium grade (Miltenyi Biotec, cat. no. 130-097-745). IL7/IL15 can be used alternatively

• Myc-Tag (9B11) mouse mAb, PE (phycoerythrin) conjugated (Cell Signaling, cat. no. 3739; RRID: AB_10889248)  **CRITICAL**  The antibody used for transgene detection must be adjusted according to the used transgene.

• CD34 MicroBead kit, human (Miltenyi, cat. no. 130-046-702)

• Lymphoprep (Stem Cell Technologies, cat. no. 07801)

• MS columns (Miltenyi, cat. no. 130-042-201)

• 10-ml syringe (Terumo, cat. no. SS-10ES1)

• Busulflex (busulfan) (Selleckchem, cat. no. S1692)  **CAUTION**  This is a hazardous chemical. Avoid contact with skin, eyes and airways and do not swallow.

• Penicillin–streptomycin (100 ml; Life Technologies, cat. no. 15140-122)  **CAUTION**  This is a hazardous chemical. Avoid contact with skin and eyes.

• Trypan blue (100 ml; Life Technologies, cat. no. 15250-061)  **CAUTION**  This is a hazardous and possibly carcinogenic chemical. Avoid contact with skin and eyes.

• Stem Cell Growth medium (CellGenix, cat. no. 20802-0500)

• Human TPO (Miltenyi Biotec, cat. no. 130-095-745)

• Human SCF (Miltenyi Biotec, cat. no. 130-096-692)

• Human Flt3-Ligand (Miltenyi Biotec, cat. no. 130-096-474)

• IL7 (Miltenyi Biotec, cat. no. 130-095-367)

• Ketamin Imalgene 1000 (Alcyon, cat. no. 6827812)

• Xylasin Rompun 2% (Alcyon, cat. no. 6835444)

• Isoflurin Isofluran Isovet 1000 mg/g (Osalia, cat. no. 240731)  **CAUTION**  This is a hazardous anesthetic agent. Avoid contact with skin and eyes.

• Tetracaine 1% (Alcyon, cat. no. 6711601)  **CAUTION**  This is a hazardous chemical. Avoid contact with skin, eyes and airways and do not swallow.

• 70% ethanol (VWR Chemicals, cat. no. 20821.330)

• Citrate phosphate dextrose (CPD) (Sigma, cat. no. C3821-50 ML)

• Red Blood Cell (RBC) lysis buffer (Miltenyi, cat. no. 130-094-183)

• Lymphoprep: human lymphocyte separation medium (EuroBio, cat. no. CMSMSL010-01)

• Formalin 10%, Q Path, buffered (VWR, cat. no. FOR0060AF59001)  **CAUTION**  This is a hazardous and possibly carcinogenic chemical. Avoid contact with skin and eyes.

• Antibodies for flow cytometry: antibodies to determine humanization level (panel 1; Step 63) are listed in Table 1

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**Table 1 | Antibodies to determine humanization level (Panel 1)**

| Target          | Fluoro-chrome | Clone* | Catalog no. | Company          | RRID          |
|-----------------|---------------|--------|-------------|------------------|---------------|
| Anti-human CD45 | Vio Green     | 581    | 130-113-124 | Miltenyi Biotec  | AB_2725952   |
| Anti-murine CD45| Pacific Blue  | 30-F11 | 103126      | BioLegend        | AB_493535    |
| Anti-human CD3  | APC           | BW264/56/56 | 130-113-125 | Miltenyi Biotec  | AB_2725953   |
| Anti-human CD19 | PE-Cy7        | LT19   | 560728      | BD Biosciences   | AB_1727438   |
| Anti-human CD14 | PE            | REA599 | 130-110-519 | Miltenyi Biotec  | AB_2655051   |

*aOf each antibody clone, 1.5 µl is used in 50 µl of FACS staining buffer.
**Table 2 | Antibodies for detection and characterization of in vivo-generated CAR T cells (Panel 2)**

| Target                        | Fluoro-chrome | Clone* | Catalog no. | Company         | RRID    |
|-------------------------------|---------------|--------|-------------|-----------------|---------|
| Anti-human CD45               | BV510         | 2D1    | 368526      | BioLegend       | AB_2687377 |
| Anti-human CD3                | PerCP         | BW264/56 | 130-113-131 | Miltenyi Biotec | AB_2725959 |
| Anti-human CD4                | PE-CF594      | RPA-T4 | 562281      | BD Biosciences  | AB_1115497 |
| Anti-human CD8                | FITC          | BW135/80 | 130-113-157 | Miltenyi Biotec | AB_2725985 |
| Myc-Tag                       | PE            | 9B11   | 3739        | Cell Signaling  | AB_10889248 |
| Anti-human CD19               | Alexa         | HiB19  | 56-0199-42  | Thermo Fisher   | AB_2043819 |
| Anti-human CD45RA             | BV421         | Hi100  | 304130      | BioLegend       | AB_10965547 |
| Anti-human CD62L              | BV605         | DREG-56 | 304833      | BioLegend       | AB_2562129 |
| Anti-human PD-1               | PE-Vio770     | PD1.3.1.3 | 130-117-698 | Miltenyi Biotec | AB_2660116 |
| Anti-human Lag-3              | Alexa 647     | T47-530 | 565716      | BD Biosciences  | AB_2744328 |
| Mouse isotype control         | BV510         | MOPC-21 | 400171      | BioLegend       | AB_2714004 |
| Mouse isotype control         | PerCP         | S43.10 | 130-099-190 | Miltenyi Biotec | AB_2661739 |
| Mouse isotype control         | PE-CF594      | X40    | 562292      | BD Biosciences  | AB_11207243 |
| Mouse isotype control         | FITC          | S43.10 | 130-113-271 | Miltenyi Biotec | AB_2733685 |
| Mouse isotype control         | PE            | S43.10 | 130-113-272 | Miltenyi Biotec | AB_2733883 |
| Mouse isotype control         | Alexa         | P3.6.2.8.1 | 56-4714-80  | Thermo Fisher   | AB_494014 |
| Mouse isotype control         | BV421         | MPC-11 | 400342      | BioLegend       | AB_10898160 |
| Mouse isotype control         | BV605         | MOPC-21 | 400161      | BioLegend       | AB_11125373 |
| Mouse isotype control         | PE-Vio770     | IS6-11E5.11 | 130-098-562 | Miltenyi Biotec | AB_2661759 |
| Mouse isotype control         | Alexa 647     | J606   | 560803      | BD Biosciences  | AB_2034029 |

*Of each antibody clone, 1 µl is used for staining.

**In vivo administration of LVs and analysis**

- For enrichment of CD4⁺ cells: CD4 MicroBeads, human (Miltenyi, cat. no. 130-045-101); other commonly used kits for CD4⁺ cell enrichment can be used alternatively
- For enrichment of CD8⁺ cells: CD8 MicroBeads, human (Miltenyi, cat. no. 130-045-201); other commonly used kits for CD8⁺ cell enrichment can be used alternatively
- 2% (wt/vol) BSA in PBS without Mg²⁺/Ca²⁺, sterile-filtered with a 0.2-µm filter
- Antibodies for flow cytometry: use 1 µl of each antibody per 10⁵–10⁶ cells. Antibodies to determine humanization level (panel 1; Step 63) are listed in Table 1. Antibodies for detection and characterization of in vivo-generated CAR T cells (panel 2; Step 86A) are listed in Table 2
- Fixable viability dye eFluor 780 (eBioscience, cat. no. 65-0865-14)
- Ultracomp eBeads (Life Technologies, cat. no. 01-2222-42)
- Blocking solution: mouse FcBlock CD16/CD32 (BD Biosciences; cat. no. 553142); human FcBlock (Miltenyi Biotec, cat. no. 130-059-901). Add 1 µl of each per one million cells
- Anti-FITC Microbeads (Miltenyi Biotec, cat. no.130-045-901) or anti-APC Microbeads (Miltenyi Biotec, cat. no. 130-090-855)
- DNase and RNase-free water (Sigma, cat. no. W3500)
- Tris-EDTA Buffer (Sigma, cat. no. 93283)

- Primers and probes for qPCR (Eurogentec): sequences of albumin-specific primers and probe—forward primer: 5’-CAGCTTTCGAGAAGGACGAC-3’; reverse primer: 5’-CTTGAATTCGAGTTCGCTATATTG-3’; probe: 5’-6FAM-ACTGAGGAGTATTTACTGACTGTTGCT-TBHQ1-3’; sequences of woodchuck posttranscriptional regulatory element (WPRE)-specific primers and probe—forward primer: 5’-CGCGAGTCGAGGATTCCCTCTGTTG-3’; reverse primer: 5’-GGACGATGATTCCCGACA-3’; probe: 5’-Cy5-CGCCGCCTGCCTTGCCCGCT-BHQ2-3’
- Standard plasmid for qPCR containing the sequences of WPRE and albumin separated such that individual PCR products can be amplified. The standard plasmid is available from the authors upon signing an MTA
- TaqMan-based LightCycler 480 Probes Master (Roche, cat. no. 0470749001)
Equipment

- Cell culture dishes with gripping ring, diameter: 15 cm (VWR, cat. no. TPPA93150)
- Nunc 96-well flat-bottom microplate (Thermo Fisher Scientific, cat. no. 167008)
- Corning 96-well polystyrene conical-bottom microwell plates (Corning Costar, cat. no. 3894)
- 24-well culture plate (Corning Costar, cat. no. 3526)
- T175 tissue culture flasks (Greiner Bio-One, cat. no. 660175)
- Filters for cell debris removal: Nalgene Rapid-Flow sterile disposable bottle top filters with 0.45-µm surfactant-free cellulose acetate (SFCA) membrane, 75-mm diameter, 45-mm neck (Thermo Fisher Scientific, cat. no. 291-4545)
- Filters for sterile filtration: Nalgene Rapid-Flow sterile disposable filter units with cellulose nitrate (CN) membrane, 0.2-µm pore size (Thermo Fisher Scientific, cat. no. 450-0020)
- Nalgene Rapid-Flow sterile filter storage bottle, polystyrene with PE storage cap, 45-mm neck, 500 ml (Thermo Fisher Scientific, cat. no. 455-0500)
- Nunc 250-ml wide-mouth conical centrifuge tube (Thermo Fisher Scientific, cat. no. 376814)
- 50-ml polypropylene tubes, conical bottom (Greiner Bio-One, cat. no.227261)
- Micronic tubes and Micronic Roborack-96
- Heraeus Multifuge X3R (Thermo Fisher Scientific, cat. no. 10325804) with Thermo Scientific Rotor TX-750 (Thermo Fisher Scientific, cat. no. 7500 3607) and Thermo Scientific Sorvall Legend T/RT centrifuge buckets for 4-place swinging bucket rotors (Thermo Fisher Scientific, cat. no. 75006441)
- Electronic pipette for resuspension of LV pellet: Eppendorf Xplorer, single-channel, variable, 15–300 µl, orange (Eppendorf, cat. no. 4861000031). Alternatively, resuspension of the LV pellet can be done with a conventional mechanical or other electronic pipette
- 1.5-ml microcentrifuge tubes, DNA LoBind (Eppendorf, cat. no. 525-0130) ▲CRITICAL DNA low-binding tubes are used for long-term storage of LVs.
- NanoSight NS300 equipped with a green laser module (Malvern Panalytical) with Nanosight NTA software version 3.3 for data analysis !CAUTION The Nanosight is an open system. Measurement of LVs must be performed under biosafety level 2 conditions, and you should wear safety protection or place the instrument in a safety cabinet. This instrument is equipped with a laser module, which must be handled with care to avoid exposure to hazardous voltages or class 3B laser radiation. Particle numbers of LV stocks can alternatively or also be measured via p24 ELISA (p24-ELISA kit: HIV Type 1 p24 Antigen ELISA 2.0 (96 determinations) (ZeptoMetrix, cat. no. 0801002). Read out via an ELISA reader of choice. !CAUTION This p24 ELISA contains inactivated viral antigen from HIV-1. According to the supplier, it should be handled as if capable of transmitting infectious agents.
- MACS Quant Analyzer 10 flow cytometer (Miltenyi Biotec) with MACSQuantify 2.6 software for data collection !CAUTION This instrument is equipped with three lasers. Do not remove the protective housing, to avoid hazardous laser radiation.
- BD LSR Fortessa (BD Biosciences) with FACS Diva 8.0.3 software for data collection !CAUTION This instrument is equipped with lasers. To prevent injury, do not modify the optics cover and laser shielding in any way.
- FlowJo version 10.0.8 (BD Biosciences) and FCS Express version 6 (DeNovo Software) for analysis of FACS data
- GraphPad Prism 8 for data visualization
- NanoDrop 2000c spectrophotometer
- LightCycler 480 Instrument II (Roche, cat. no. 05015278001) !CAUTION This is an electromechanical instrument with potential danger for the user of an electric shock or physical injury if the instrument is not used according to the operator’s manual.
- LightCycler 480 software version 1.5.1
- FrameStar 96-well semi-skirted PCR plate (Roche, cat. no. 4ti-0951) + qPCR seal (Brooks Life Sciences, cat. no. 4ti-0560)
- Tube holder for FACS tubes
- AutoMACS Pro Separator (Miltenyi)
- Magnetic device (MiniMACS Separator; Miltenyi, cat. no. 130-042-102)
- Balance (for weighing mice)
- Apparatus for anesthesia (e.g., from Temsega)
- Micropipettes and tips
- Pasteur pipettes for blood collection from mice
- FACS tubes, Falcon (Corning, cat. no. 352052)
• Table centrifuge for serum collection from blood
• Sterile forceps, scissors and scalpels for dissection of mice
• Laminar flow PSM2 for handling of immunodeficient mice
• Cell count chamber
• Non-woven sterile swabs, 10 × 10 cm (LCH Medical Products, cat. no. SN30-1005)
• Sterile paper towels
• 30-gauge syringe, 0.3 ml (Terumo, cat. no. 324826)
• 29-gauge syringe, 0.5 ml (Terumo, cat. no. 320926)
• 25-gauge (or 23-gauge) needle (Terumo, cat. no. 8AN2516R1)
• 10-ml syringe (Terumo, cat. no. SS-10ES1)
• 40-μm nylon cell strainer (Falcon, cat. no. 352340)
• 50-ml conical tube (Falcon, cat. no. 352070)
• 5-ml syringe plunger (Terumo, cat. no. SS-05SE1)
• Sharp sterile scissors

Reagent setup

Medium for cultivation of Lenti-X 293T cells
This medium is DMEM supplemented with 10% (vol/vol) FBS and 2 mM L-glutamine (store at 4 °C for ~1 month).

Medium for medium exchange before transfection
This medium is DMEM supplemented with 15% (vol/vol) FBS and 2 mM L-glutamine (store at 4 °C for ~1 month). Note that the medium contains 15% (vol/vol) FBS to compensate for dilution with the transfection mixture (containing DMEM without further additives).

Cultivation of Lenti-X 293T cells
Cultivate Lenti-X 293T cells in DMEM containing 10% (vol/vol) FBS and 2 mM L-glutamine. For passaging, detach with 0.25% (wt/vol) trypsin (PBS without Mg2+/Ca2+/1 mM EDTA). Cells should be passaged twice a week at a 1:8–1:10 ratio. To have enough cells for the production, expand your cells to ~18 T175 flasks 4 d before seeding. Check the condition of the cells before seeding. ▲CRITICAL We strongly recommend cultivation of cells without antibiotics to avoid hidden contamination. LV production is also possible with HEK-293T/17 cells (American Type Culture Collection CRL-11268), but yields will be two-fold lower. ▲CRITICAL Cells should not be passaged >20 times.

T-cell medium (TCM) for cultivation of PBMCs
RPMI supplemented with 10% (vol/vol) FBS, 2 mM L-glutamine, 25 mM HEPES and 0.4% (vol/vol) penicillin–streptomycin (store at 4 °C for ~1 month).

PEI solution
Prepare PEI solution in several dilutions to achieve the desired concentration. First, prepare a 25% (wt/wt) solution by weighing 5 g of PEI (molecular weight: 25,000 Da); add a triple amount of water and dissolve by mixing. As a next step, prepare a 100 mM (referring to free phosphate residues) stock solution by mixing 0.71 g of the 25% (wt/wt) PEI solution with 39.5 g of water. Finally, prepare the 18 mM (0.92 µg/µl) ready-to-use solution by mixing 9.25 g of the 100 mM PEI stock solution with 35 ml of water, adjust to pH 7 with 1 N HCl (should be ~350 µl) and fill up to 50 g with water. Sterilize by filtration (0.2 µm). (Prepare 5-ml aliquots and store them at −20 °C for ≤2 years).

Sucrose solution for concentration of LV stocks
Prepare 20% (wt/vol) sucrose in PBS without Mg2+/Ca2+ and sterile-filter through a Nalgene 0.2-μm filter (can be stored at 4 °C for years). ▲CRITICAL Regularly check for contamination.

Freezing medium
The freezing medium is FBS and 10% (vol/vol) dimethyl sulfoxide (prepare fresh).

FACS wash buffer
This buffer is PBS without Mg2+/Ca2+ with 2% (vol/vol) sterile-filtered FBS + 0.1% (wt/vol) NaN3 (store at 4 °C for ~6 months).
FACS fixation buffer
This buffer is PBS without Mg$^{2+}$/Ca$^{2+}$ with 1% (vol/vol) paraformaldehyde (store at 4 °C for ~2 months; protect from light).

Complete stem cell growth medium
This medium is stem cell growth medium supplemented with SCF (100 ng/ml), TPO (30 ng/ml) and FLT3 (100 ng/ml). Stocks or cytokines should be stored at −20 °C. ▲CRITICAL The complete CellGro medium should be prepared shortly before using it.

Blocking solution for FACS staining of in vivo samples (panel 2)
Add 1 µl each of mouse and human FcBlock per sample. 2 µl of FcBlock + 48 µl of FACS wash buffer = 50 µl/sample (further referred to as 'Tube 1').

Antibody mixture for FACS staining of in vivo samples (panel 2)
Add 1 µl of each anti-human antibody listed in Table 1 to 40 µl of FACS wash buffer per sample. 1 µl × 10 different antibodies + 40 µl of FACS wash buffer = 50 µl/sample (further referred to as 'Tube 2'). ▲CRITICAL The volume of each mAb may vary depending on the antibody clone, fluorochrome-conjugate and its concentration. Titration of all mAbs is recommended, especially when using other fluorochromes.

Isotype controls for FACS staining of in vivo samples (panel 2): we include 1 µl each of anti-human CD45, CD3, CD4 and CD8 in our isotype control vial and add 1 µl of isotype controls for the rest of the channels, namely PE, Alexa Fluor 700, BV421, BV605, PE-Vio770 and Alexa 647, to 40 µl of FACS wash buffer per sample. 4× anti-human + 6× isotype controls + 40 µl of FACS wash buffer = 50 µl/sample (further referred to as 'Tube 3'). ▲CRITICAL Isotypes are used to determine the background signal from nonspecific binding of the particular isotype of a given antibody to the cells. They are used at the same concentration as the corresponding antibody of interest.

Viability dye for FACS staining of in vivo samples (panel 2)
Use a 1:10,000 dilution of Fixable Viability dye eFluor 780 (further referred to as 'Tube 4').

Plasmid-based PCR standard for albumin and WPRE
Prepare a series of 1:10 dilutions from 1 × 10^8–1 × 10^9 molecules/µl in DNase- and RNase-free water. This results in 1 × 10^7–1 × 10^8 molecules/well when using 10 µl for qPCR. ▲CRITICAL The standard plasmid can be diluted in advance to a concentration of 1 × 10^9 molecules/µl, divided into aliquots and stored at −20 °C in DNA low-binding tubes for several years. Lower dilutions should be prepared fresh and stored at 4 °C until usage. The standard plasmid must be handled separately from the actual samples to avoid cross-contamination.

Isolation of human CD34$^+$ cells from cord blood (CD34$^+$ cells are also commercially available (ABCellBio, Lymphobank and Merck/Sigma-Aldrich) and provided cryopreserved (−150 °C in cryotubes.) ▼CAUTION Human tissues should be handled using biosafety level 2–recommended protocols and should be collected and used in accordance with all institutional and governmental ethics guidelines. ▲CRITICAL We use cord blood collected in citrate phosphate dextrose–coated bags obtained from the 'Etablissement Français du Sang' (Besançon, France) upon informed consent.
1 Dilute cord blood 1:1 with PBS and gently layer 35 ml of this diluted product on top of 15 ml of Lymphoprep in a 50-ml tube without mixing the blood with Lymphoprep. Ficoll-Paque can be used as an alternative for Lymphoprep without any need for changes in this protocol, because both have a density of 1.077 g/ml.
2 Centrifuge the cells at 850g for 20 min at 20 °C without brake and collect the layer containing the mononuclear cells situated on top of the Lymphoprep layer.
3 Wash the collected mononuclear cell interface in 30 ml of PBS buffer (PBS/2% (vol/vol) FBS) at 850g and 8–10 °C for 10 min.
4 Resuspend the cells with 300 µl of PBS buffer for up to 10^8 total cells. First, add FcR blocking reagent for 5 min, then add anti-hCD34$^+$ microbeads (CD34 MicroBead kit, human) according to the manufacturer’s instructions and incubate for 30 min while shaking at 4 °C.
5 Wash the cells to remove the unbound anti-hCD34$^+$ microbeads in 10 ml of PBS buffer at 300g and 8–10 °C for 10 min.
6 Resuspend the cells at a maximum concentration $1 \times 10^8$ cells in 500 µl of PBS buffer.
7 Proceed to magnetic separation with the AutoMACS Pro Separator using the Posseld2 program adapted for CD34$^+$ positive separation according to the manufacturer’s instructions. **CRITICAL** If no AutoMACS Pro Separator is available, one can instead proceed with Steps 8–11 using manual magnetic separation.
8 Preincubate the MACS separation column with PBS/2% (vol/vol) FBS and let labeled cells pass through this first column placed on a magnetic device.
9 Wash the column once with 2 ml of PBS buffer.
10 Remove the column from the magnetic device to flush out CD34$^+$ cells with 1 ml of PBS buffer using a 5-ml syringe.
11 Repeat Steps 9 and 10 once. **CRITICAL** The purity of CD34$^+$ cells is usually 90–95% using either the manual procedure or the AutoMACS Pro Separator.

**PAUSE POINT** CD34$^+$ cells can be frozen in freezing medium at $-150 \degree C$ for ~1 year.

**Procedure**

### Production of CD4-LV and CD8-LV

#### Seeding of Lenti-X 293T cells  ● **Timing** Day 1, early afternoon

**CRITICAL** To have enough cells for production, expand your cells to ~18 T175 flasks 4 d before starting the procedure. It is also important to check the condition of the cells before seeding.

1. On the day before transfection, seed $2 \times 10^7$ cells in 18 ml of DMEM (10% (vol/vol) FBS, 2 mM L-glutamine) per 15-cm cell culture plate (growth surface: 147.8 cm$^2$) in 40 plates. This should achieve 75–90% cell confluence for transfection. We recommend adding 8 ml of medium to the culture plates, then diluting the cells to a concentration of $2 \times 10^6$ cells/ml and adding 10 ml of this cell suspension to the plates. We prefer to transfect cells in 15-cm culture plates instead of T175 flasks, although transfection of cells in T175 flasks is also possible. There is a higher risk of contamination in plates; however, they allow a quicker workflow because they are easier to handle, especially when many plates have to be processed. **CRITICAL** When handling the plates, be careful not to move your hand or material above an uncovered plate, to avoid contamination. Evenly spread the cells on the plates by carefully tilting the plate back and forth and from left to right after seeding.

#### Transfection of Lenti-X 293T cells  ● **Timing** Day 2, late afternoon

**CRITICAL** Transfection can also be performed in the morning (if cell density is >75%). In this case, medium is replaced (Step 9) 6–8 h after transfection on the same day. Harvesting of LV particles (Step 10) should still be carried out 2 d after transfection, as described. Alternative transgene plasmids can be used. When setting up LVs targeted to other receptors, we recommend using GFP for validation. Plasmid ratios may have to be adapted when using transgenes of different sizes. For third-generation packaging LVs, plasmid amounts have to be adapted as follows. Instead of using 577.8 µg of pCMV-dR8.91, use 385.0 µg of the plasmid coding for Gag/Pol (pMDLg/pRRE, addgene #12251) and 192.77 µg of the plasmid coding for Rev (pRSV-Rev, addgene #12253). The other plasmid amounts are not changed.

2. Observe cells under the microscope to check for their condition and possible contamination. The cells should be equally distributed over the plate (no cluster formation) with a confluency of ~75–90% and should not be growing three-dimensionally. **CRITICAL** The color of the medium should be orange, not red (low cell density) or turbid yellow (indicating contamination). The cells must cover ≥75% of the plate’s surface for optimal transfection. Ideally, they should be above this threshold, although they should not be too confluent because they are in this culture for a further 2 d.

**TROUBLESHOOTING**

3. Remove and discard the medium and replace the medium in each dish with 12 ml of DMEM containing 15% FBS (vol/vol) and 2 mM L-glutamine. **CRITICAL** Cells should not be kept in this low amount of medium for >1 h 30 min. This can be achieved by first changing the medium of 20 plates, then starting to prepare the DNA mixture and PEI mixture (Step 4) and performing the medium change of the remaining 20 plates during the incubation time of the transfection mixture.

4. Separately prepare the DNA mixture and PEI mixture required for transfection in conical-bottom, sterile polypropylene tubes. First, prepare DNA mixture in a 250-ml tube and then prepare PEI
mixture in another 250-ml tube. The following amounts are for transfection of 40 cell culture plates. Note that amounts can be downscaled to, for example, five 15-cm cell culture plates per vector. The total amount of plasmid DNA per plate is 35 µg with a ratio of 1:3:11.3:10.7 between targeted glycoprotein plasmid, fusion protein plasmid, transgene plasmid and packaging plasmid for CD8-LV (as for other NiV glycoprotein-based LVs) and a ratio of 1:5:16.9:16.1 for CD4-LV (as for other MV glycoprotein-based LVs). The amount of PEI per microgram of DNA is 3.68 µg.

- **DNA mixture.** Combine 92 ml of DMEM without any supplement with 606.9 µg of pS-CD19.CAR-W and 577.8 µg of pCMV-dR8.91. Add 35.9 µg of pCAGGS-NiV-GcΔ34-αCD8opt and 179.5 µg of pCAGGS-NiV-FcΔ22 for production of CD8-LV or 53.9 µg of pCG-Hmut-CD4.DARPin29.2 and 161.5 µg of pCG-FcΔ30 for production of CD4-LV.

- **PEI mix.** Combine 5.6 ml of PEI (0.92 µg/µl) with 88 ml of DMEM without supplements.

5. Thoroughly mix PEI-mixture and DNA-mixture individually by vortexing for 30 s, respectively.

6. Then, add PEI-mixture to DNA-mixture and vortex again for 30 s. Incubate for 20–30 min at room temperature (22–25 °C).

7. After the incubation period, pipette 4.6 ml of the transfection mixture from Step 6 to each plate using a 10-ml pipette. Carefully tilt the plates to disperse the medium evenly. Avoid disturbing the attached cells by slightly tilting the plate and carefully pipetting the solution slowly to its border. Distribution of the transfection mixture drop by drop is not necessary. Transfect only 10 plates at a time and leave the rest of the plates in the incubator to keep the cells in optimal culture condition.

8. Return the dishes to the tissue culture incubator with a humidified atmosphere containing 5% CO₂ at 37 °C and incubate them overnight for 16–18 h. Stack the dishes horizontally to make sure that cells are completely covered with medium at any time.

### Medium change

- **Timing** Day 3, early morning (~16 h after transfection)

- **CRITICAL** If cells were transfected in the morning, the medium is replaced 6–8 h after transfection on the same day.

9. Gently aspirate the medium from the cells and add 18 ml of DMEM (10% (vol/vol) FBS, 2 mM L-glutamine). Incubate the plates in a tissue culture incubator with a humidified atmosphere containing 5% CO₂ at 37 °C for 24 h. Note that when using T175 flasks, 20 ml of DMEM is required.

- **CRITICAL** Take only 10 plates at a time out of the incubator to keep the cells in optimal culture condition. Change the medium of only two plates at a time, to avoid cell dehydration.

### Harvesting of LV particles

- **Timing** Day 4, morning

10. To get rid of cell debris, filter the cell supernatant through a 0.45-µm bottle top filter placed on a 500-ml storage bottle. One filter is sufficient for filtration of the supernatant of 20 plates. Filter the LV-containing supernatant by connecting a pump to the filter and collect the flow-through. An additional vector harvest after another 24 h can be considered to increase vector yields. If this is required, the filtered supernatant from the first harvest can be kept at 4 °C overnight and pooled with the supernatant of the second harvest. It has to be checked for each type of LV, if a second harvest is worth the effort required, because yield depends on the level of syncytia formation in the packaging cells.

- **CRITICAL** Keep the supernatant on ice after filtration.

- **PAUSE POINT** Store at least one aliquot of the unconcentrated filtered cell culture supernatant at −80 °C (4 °C in the case of titration on the next day) to later compare titers of unconcentrated and concentrated vector stocks.

### Concentration of vector stock over sucrose cushion

- **Timing** Days 4 and 5

11. Distribute the filtered supernatant to four 250-ml conical centrifuge tubes.

12. Aspirate 14 ml of the 20% (wt/vol) sucrose solution with a 10-ml pipette. Insert the pipette to the bottom of the centrifuge tube containing the filtered supernatant and slowly underlay with 9 ml of
the sucrose solution. Stop expelling the solution when it hits the 5-ml mark on the pipette. Discard the rest of the solution and use a new pipette for the next tube.

▲ CRITICAL The extra volume in the pipette is necessary to avoid bubbles in the tube, because the 5-ml mark of a 10-ml pipette is still visible when being inserted in the centrifuge tube containing the supernatant.

▲ CRITICAL Weighing can be done outside the cell culture hood, although the tubes have to be kept closed. However, PBS has to be added under the cell culture hood to maintain sterility.

13 Balance the tubes by addition of PBS without Mg²⁺/Ca²⁺ until the weight difference is <0.1 g.

▲ CRITICAL Weighing can be done outside the cell culture hood, although the tubes have to be kept closed. However, PBS has to be added under the cell culture hood to maintain sterility.

14 Centrifuge for 24 h at 4 °C (4,500 g: acceleration: 6; deceleration: 6) in a benchtop centrifuge. You can also concentrate the vectors by ultracentrifugation (SW32 Ti Rotor) for 2 h at 100,000g, but due to the volume restrictions, we prefer slow-speed centrifugation. If you decide on ultracentrifugation, we recommend using open-top thinwall polypropylene tubes (Beckmann Coulter, 38.5 ml, 25 × 89 mm, cat. no. 326823) for centrifugation of the supernatant. These tubes have to be filled to the maximum to prevent collapsing. If necessary, fill up the supernatant to exactly 30 ml with PBS without Mg²⁺/Ca²⁺ before adding 5 ml of sucrose solution. If production is downscaled to <10 plates per vector, concentration of LVs can be performed in 50-ml conical centrifuge tubes. In this case, the supernatant should be aspirated with a 10-ml pipette and filtered with a 0.45-µm syringe filter. One filter can be sufficient for two plates by carefully detaching the pipette without clogging the filter (e.g., by keeping an ~3-ml volume in the pipette). The filtered supernatant of two culture plates is transferred in a 50-ml centrifuge tube and underlayed with 4.5 ml of 20% (wt/vol) sucrose by aspirating 7.5 ml of the sucrose solution with a 5-ml pipette and stopping to expel the solution when it hits the 3-ml mark on the pipette.

▲ CRITICAL The centrifugation time should be set for ≥24 h. Set the centrifuge on ‘Hold’ to be sure that it does not finish before you are ready to proceed.

Resuspension of concentrated LVs • Timing Day 5

15 Carefully remove the tubes from the centrifuge and discard the supernatant. Remove residual liquid by leaving the tube for 5 min upside down in a rack lined with paper towels. Wipe the tubes with fresh paper towels without touching the pellet. Change gloves after that step. A tiny, beige-brownish pellet should be visible at the bottom of the tube.

▲ CRITICAL Pour the supernatant quickly but carefully and avoid bubbles.

16 Add 600–1,000 µl of PBS without Mg²⁺/Ca²⁺ to each pellet (equals 60–100 µl/plate) without touching it and let the pellet resuspend for 30 min by placing the tubes on ice on a plate shaker.

17 Pipette up and down 80 times with an electronic pipette. Alternatively, it is possible to use a conventional mechanical pipette for resuspension of the pellet.

▲ CRITICAL Avoid foam formation during resuspension of the pellets, to prevent aerosols.

18 Pool the liquids from all resuspended pellets in one of the conical tubes and distribute aliquots of the vector stock in low-binding tubes in volumes of 15–200 µl (15–25 µl for subsequent NTA or p24 ELISA and titration; 200 µl for subsequent in vivo analyses). Store the vectors at −80 °C.

▲ CRITICAL Resuspension of the pellet in too little PBS can be counterproductive for efficient gene transfer. This is a particular issue for LVs containing MV envelope proteins. We recommend the addition of 100 µl of PBS per plate when starting with a new type of targeted LV.

■ PAUSE POINT LVs can be stored at −80 °C. Although formal test results are not available, you can store the LVs for several years in our experience. However, if stored long term, vectors should be tested for functionality in vitro before in vivo application. If you want to proceed directly with the titration, be sure to freeze/thaw the respective aliquot first to make it comparable with LVs used after storage (e.g., for in vivo applications).

Quantification of particle number and gene transfer activity of LVs

Determine particle number via NTA • Timing ~1 h per stock

▲ CRITICAL Particle numbers of LVs can also be measured via p24 ELISA according to the manufacturer’s protocol. In our laboratory, we often use both methods for each vector stock.

▲ CRITICAL Regularly check PBS solution for the absence of salt crystals before usage. Always sterile-filter PBS for ≥24 h before preparation of LV dilutions, to reduce air bubbles.

19 Determine particle number using your preferred method. We use NTA with Nanosight NS300 (Malvern Panalytical) according to the user’s manual, diluting LVs 1:1,000–1:10,000 in sterile-
filtered (0.2 µm) PBS without Mg²⁺/Ca²⁺ (typically 1:3,000) in a total volume of 1 ml for measurement. Ensure that the particle number per frame ranges between 20 and 50 events for analysis. Perform at least three consecutive measurements for each dilution to obtain robust results. The possibility of measuring the concentration and particle size of LV stocks simultaneously while also seeing inhomogeneity and aggregates is the advantage of NTA. Representative data measured via NTA are shown in Fig. 4b. We provide two different NTA software scripts that can be used (Supplementary Data 1; also available at figshare (doi:10.6084/m9.figshare.13221602)). We prefer to use the script for continuous flow, which is only applicable in combination with a syringe pump (Malvern Panalytical), because it is time saving and simplifies the work flow. If a syringe pump is not available, you can still perform a measurement, but you will have to use the stop flow script.

Gene transfer activity on target receptor–positive cell lines  ● Timing 3–4 d

20 To determine the gene transfer activity of the produced vector particles, seed 4 × 10⁴ CD8⁺/CD4⁺ cells in 100 µl of RPMI medium (10% (vol/vol) FBS, 2 mM L-glutamine) per well in a 96-well plate.
21 Dilute 10 µl of the vector stock in 190 µl of medium and make consecutive dilutions of 1/5.
22 Add 100 µl of the diluted vector (resulting in 5 µl of LV in the lowest dilution) to the cells to get a final volume of 200 µl.
23 Determine the percentage of transduced cells via flow cytometry 3–4 d later. Appropriate antibodies to use will depend on the corresponding transgene. For the example we describe here, an anti-myc-PE antibody was used for detection of the CAR (Fig. 4c).

▲ CRITICAL When producing other targeted vectors, the cell line for titration and the antibody used for detection of the transgene must be adjusted. Vector stocks should have ≥1 × 10⁷ t.u./ml (Fig. 4d).

? TROUBLESHOOTING

Gene transfer activity on primary cells  ● Timing 8–11 d

▲ CRITICAL It is very important to check whether the LVs are able to successfully transduce primary cells. In this section, we describe how to use human PBMCs purified from whole blood. Alternatively, buffy coats from healthy donors, purified via a Ficoll gradient (e.g., Histopaque 1077; Sigma Aldrich, cat. no. 10771) can be used, as described in the manufacturer’s protocol.

24 In preparation for the activation of 2 × 10⁶ PBMCs, coat one well of a 24-well plate with 500 µl of 1 µg/ml anti-human CD3 mAb (clone: OKT3; Miltenyi Biotec) and incubate for 2 h at 37 °C or overnight at 4 °C.
25 Remove the medium and replace with 2% (wt/vol) BSA in PBS without Mg²⁺/Ca²⁺ (sterile-filtered) for 30 min at 37 °C.
26 Wash with PBS without Mg²⁺/Ca²⁺ twice.
27 Seed 2 × 10⁶ PBMCs in 2 ml of TCM supplemented with 3 µg/ml anti-human CD28 mAb (clone: 15E8; Miltenyi Biotec) and 50 U/ml IL-2 into the plate prepared in Steps 24–26 and incubate the plate for 72 h at 37 °C, 5% CO₂ and 90% humidity.

▲ CRITICAL After 72 h, the PBMCs should be activated.
▲ CRITICAL PBMC activation can be adjusted according to the amount of cells needed. For example, you can activate 1 × 10⁷ PBMCs in one well of a 6-well plate using 1 ml of anti-human CD3 mAb (1 µg/ml) for coating, and seeding cells in a total volume of 6 ml per well.

28 Seed 0.4–1 × 10⁵ activated PBMCs per well in a 96-well plate in a volume of 50 µl in TCM + 50 U/ml IL-2.
29 Add 5 µl of vector stock diluted in 50 µl of medium per well of PBMCs. Vectofusin-1 can be used to enhance gene delivery, especially for gene transfer into suspension cells. For details, see Jamali and Kapitza et al.33.

▲ CRITICAL The amount of vector stock per well when transducing primary cells might have to be adjusted according to the gene transfer activity of the vectors, as determined on Molt 4.8 cells. We use 5 µl of LVs with titers of 1–5 × 10⁷ t.u./ml.
30 Perform spinfection at 850g (acceleration: 7; deceleration: 7) for 90 min in a preheated centrifuge (32 °C) to enhance transduction efficiency.
31 Afterward, add 100 µl of TCM to achieve a total volume of 200 µl in each well and incubate cells at 37 °C, 5% CO₂ and 90% humidity.
32 Determine the number of transduced cells after 4 and 7 d of cultivation via flow cytometry with the corresponding antibodies.

? TROUBLESHOOTING
Humanization of mice

Mice conditioning with sublethal injection of Busulfen

**Timing 1 h (for 20 mice)**

▲ **CRITICAL** Busulfen injection, as described in this section, has to be performed 30–36 h before cell injection in Step 56. Mice should be 3–5 weeks old.

**! CAUTION** This part of the protocol must be carried out by personnel trained in animal experimentation.

33 Weigh each NSG mouse and calculate the precise volume of Busulfen required for a dose of 20 mg/kg per mouse. Stock solution of Busulfen (6 mg/ml) can be prediluted twofold with sterile PBS just before injection.

34 Carefully remove the animal from the cage and restrain it gently in the head-down position.

35 Insert the needle with the bevel facing ‘up’ into the lower right quadrant of the abdomen toward the head at a 30–40° angle to the horizontal line.

36 Inject the appropriate volume of Busulfen solution intraperitoneally in a steady, fluid motion with a 29-gauge 0.5-ml syringe.

▲ **CRITICAL** Busulfen solution for injection is very viscous; therefore, slow injection is recommended.

37 Repeat Steps 33–36 until all mice have been injected. Place the young adult mice back in their box. These mice will be used in Step 51 after 30–36 h. In the meantime, if using fresh CD34⁺ cells in the next section, proceed with culture of CD34⁺ cells (as described in Reagent setup).

Cell preparation for human CD34⁺ cells before injection

**Timing 40–50 min**

▲ **CRITICAL** This procedure needs to be performed 24 h before injection into preconditioned mice in Step 56.

▲ **CRITICAL** CD34⁺ cells from three to four donors can be pooled to obtain enough cells to humanize a larger cohort of mice with homogeneous engraftment levels.

▲ **CRITICAL** It is crucial to use CD34⁺ cells with a purity that is >90% to obtain a high level of human cell engraftment in NSG mice. A contamination of T cells (>3%) might result in low human cell engraftment.

38 Warm up 30 ml of CellGro medium in a 37 °C water bath.

39 If using frozen CD34⁺ cells, take CD34⁺ cells from the liquid nitrogen tank and thaw them in the water bath at 37 °C for 1–2 min. Take cells out of the water bath when they are almost thawed. If using fresh CD34⁺ cells instead, proceed straight to Step 41.

40 Transfer CD34⁺ cells very quickly to a prewarmed 50-ml tube containing 30 ml of CellGro medium.

41 Spin the tube at 300 g for 10 min at room temperature.

42 Aspirate the supernatant and resuspend cells in 2 ml of CellGro medium.

43 Count the cells using a hemocytometer after trypan blue staining (mix 10 μl of cells with 10 μl of trypan blue and count clear cells as live cells and blue cells as dead cells).

44 Spin down the cell suspension at 300 g for 10 min at 4 °C and discard the supernatant.

45 Resuspend the cells in 1 ml of complete CellGro medium at a density of 1–10⁶ living cells/ml and seed in a 24-well plate.

46 Incubate cells at 37 °C, 5% CO₂ and 20% O₂ overnight (16–24 h).

Cell preparation for injection

**Timing 1 h**

47 In preparation for transplantation, collect cells in 1.5-ml tubes.

48 Count cells using a hemocytometer after trypan blue staining (as described in Step 43).

49 Spin down CD34⁺ cells at 300g for 10 min at room temperature and resuspend them with sterile PBS without Mg²⁺/Ca²⁺ to 0.7–2 × 10⁵ cells per 35-μl total volume for each mouse to be injected. The dose of CD34⁺ cells injected will determine the speed of NSG humanization. If one injects 2 × 10⁵ CD34⁺ cells per mouse, 40% human cell reconstitution in blood should be achieved 12–16 weeks after engraftment.

▲ **CRITICAL** Cell suspension must be prepared in 35 μl of PBS for retro-orbital injection.

Transplantation of human cells

**Timing 1 h (for 20 mice)**

▲ **CRITICAL** This part of the protocol must be carried out by personnel trained in animal experimentation.

▲ **CRITICAL** Intravenous injection can also be performed into the tail vein. This has the advantage that there is no need for anesthesia of the mice but does require an appropriate ‘contention box’ and a mouse-tail illuminator for dilatation of the tail vein.
**NATURE PROTOCOLS**

**PROTOCOL**

▲ CRITICAL It is also important to perform Steps 51–58 on control mice, but instead of CD34^+^ cells, inject a vehicle using a buffer without cells.

50 Take the CD34^+^ cells from Step 49 to the animal facility (experimental area).

51 Anesthetize mice. We anesthetize mice using a decontaminated induction box infused with 3.5% isoflurane-enriched air until the mouse is non-responsive and recumbent and demonstrates a slower, even respiratory pattern. Anesthesia is maintained using a nose cone releasing 1–2% isoflurane-enriched air.

▲ CRITICAL Mice should be anesthetized because the needle is being placed in the retrobulbar space.

▲ CRITICAL Be sure to perform anesthesia and retro-orbital injection under a laminar flow (PSM2, sterile conditions). Decontaminate the surface of the laminar flow and your gloves regularly with 70% (vol/vol) alcohol before touching the NSG mice.

▲ CRITICAL It is preferable to use inhalant anesthetic, because it ensures rapid induction and quick recovery times.

▲ CRITICAL Ensure that the mouse is completely under anesthesia before injecting the cells. This usually takes 2–5 min and can be checked by pressing the foot pad to make sure that the mouse has no reflexes.

52 Remove the mouse from the isoflurane chamber and place it on a sterile compress with its belly facing the surface of the laminar flow.

▲ CRITICAL Injection (Steps 54–57) takes <15 s per mouse. Mice can be maintained under anesthesia using a nose cone and 1–2% (vol/vol) isoflurane, but injection can also be performed directly after withdrawing the mouse from the induction box if personnel are trained to perform it quickly.

53 Mix the cells immediately before each injection by pipetting up and down. Load the syringe (29-gauge needles, 0.3- to 0.5-ml insulin syringes) with the cell suspension at a volume of 30 µl per mouse. The same syringe can be used for several mice.

▲ CRITICAL Air bubbles in the syringe must absolutely be avoided before injection into the bloodstream, because this will lead to cardiac arrest.

54 Protrude the mouse’s right eyeball from the eye socket by applying gentle pressure to the skin at the dorsal and ventral part of the eye.

55 Introduce the needle bevel down at an angle of ~30° into the medial canthus.

56 Inject the cell suspension slowly and smoothly and remove the needle slowly and smoothly once the injection is complete.

57 Make sure that there is little or no bleeding.

58 Place mice back into the cage for recovery. A warming device is not required, because the injection procedure takes only a very short time (<15 s). Thus, the mouse is usually ambulatory within 30–45 s.

▲ CRITICAL The NSG mice are immunodeficient and should be housed in a sterile environment according to national and institutional requirements. Mice must be strictly handled under a laminar flow, get sterile food and water and be kept in sterile cages. This is essential to ensure an efficient high-level humanization of these mice. If this is not respected, opportunistic infections of NSG mice can strongly impair CD34^+^ cell engraftment. The complete humanization process can take 12–16 weeks depending on the CD34^+^ cell donor and the number of injected cells. We recommend proceeding to the next step after 8 weeks.

**Determination of the humanization level in the peripheral blood (PB)**

▲ CRITICAL We recommend taking blood from the mice every 3 weeks from 8 weeks after injection to monitor humanization in the PB (i.e., at 8, 11 and 14 weeks after injection).

59 Apply a drop of tetracaine 5 min before sampling of blood from the eye.

▲ CRITICAL Mice can be briefly anesthetized with isoflurane (as described in Step 51) to facilitate blood sampling.

60 Perform retro-orbital blood sampling by penetrating the retro-orbital sinus in mice with a sterile hematocrit capillary tube or Pasteur pipette.

▲ CRITICAL Sterile tubes or pipettes are required to avoid peri-orbital infections and potential long-term damage to the eye. The eyelid is pulled back to protrude the eye and facilitate blood harvesting.

61 Take ~100 µl of blood per mouse in 1.5-ml sterile microcentrifuge tubes containing 20 µl of CPD (for plasma collection and genomic DNA (gDNA) analysis) and immediately pipette 50 µl in a new tube containing 20 µl of CPD and place on ice for FACS staining.

■ PAUSE POINT Samples can be kept on ice for ≤12 h before proceeding to the next step.
62 Add 50–100 µl of PBS without Mg²⁺/Ca²⁺ to the blood (1:1 ratio).
63 Transfer 50 µl of 2×-diluted blood into 5-ml FACS tubes containing 50 µl of FACS wash buffer supplemented with the antibody cocktail described in panel 1 (Table 1). Alternatively, staining and RBC lysis (Step 68) can be performed directly in a 96-well plate (conical bottom), especially if many samples (>10 mice) are processed.
64 Incubate for 30 min at 4 °C in the dark.
65 Add 500 µl of FACS wash buffer.
66 Centrifuge at 300 g for 10 min at 4 °C.
67 Discard the supernatant by inverting the tube and gently tapping it on a paper towel to remove the remaining drop of supernatant.
68 Add 700 µl of 1× RBC lysis buffer for 10 min at room temperature (dark).
69 Centrifuge at 300g for 10 min at 4 °C, discard the supernatant and add 3–4 ml of FACS wash buffer.
70 Spin the cells again at 300 g for 10 min at 4 °C and remove the supernatant.
71 Resuspend the cell pellet in 100–200 µl of FACS fixation buffer.
72 Analyze the cells via MACS Quant measurement.

CRITICAL Cells can be retained in the FACS tubes on ice for 1–2 h before MACS Quant measurement.
73 First, gate the white blood cells by granularity and size (forward scatter versus side scatter). Then, evaluate by gating for hCD45 (negative for mCD45) combined with hCD3 (total T cells), hCD19 (total B cells) or hCD14 (monocytes) using flow cytometry analysis (Fig. 5).
74 Determine the human immune reconstitution by applying the following formula:

\[
\text{Human immune reconstitution} = \frac{\% \text{ human CD45 cells}}{\% \text{ human CD45 cells} + \% \text{ murine CD45 Cells}}
\]

CRITICAL Use this calculation to identify the time point when the human cell engraftment has become a relevant fraction in blood. The percentage of human cells is more relevant than absolute cell numbers. Animals with ≥40% engraftment are suitable for use in further experiments.

TROUBLESHOOTING

In vivo CAR T cell generation: IL7 conditioning

CRITICAL Human IL7 needs to be administered to the humanized NSG mice 4 d and again 1 d before vector application.
75 Resuspend human IL7 according to the manufacturer’s protocol. The IL7 stock solution is usually at 100 µg/ml.
76 Prepare a working solution at 2 µg/ml in sterile PBS without Mg²⁺/Ca²⁺.
77 Inject 100 µl of the working solution of huIL7 (200 ng) via the subcutaneous route. To do this, restrain the animal by grasping the skin along its back and insert the needle at the base of the skin fold between your thumb and finger. Administer IL7 in a steady, fluent motion with a 29-gauge 0.5-ml syringe.

CRITICAL Inject the control group with PBS using the same route of administration.

In vivo CAR T cell generation: administration of the vector

CRITICAL A maximal volume of 100 µl can be injected into the eye. Alternatively, up to 200 µl can be injected into the tail vein.
78 Inject a single dose of 2 × 10¹¹ LV particles intravenously using the same procedure as for human CD34⁺ cell injection (Steps 51–58).

CAR-T longitudinal analysis

CRITICAL Appropriate experimental endpoints (physical appearance, behavioral changes and weight loss) should have been agreed upon and outlined in the protocol that was submitted to the local ethics committee before commencing the protocol.
**Fig. 5 | Follow-up of humanization of NSG mice.**

**a**, Gating strategy for determination of humanization levels in NSG mice. Mononucleated cells are gated in a side scatter area (SSC-A) and forward scatter area (FSC-A) plot according to their size and granularity. This population is represented in the FSC-A versus forward scatter height (FSC-H) plot to gate for single cells and exclusion of doublets. Using this gate, the hCD45+ cells (upper left quadrant) are plotted versus mCD45+ cells (lower right quadrant) to calculate the humanization (%hCD45/(hCD45+mCD45)) in the blood of the CD34+-engrafted mice. A subgate on hCD45 is presented and plotted to determine the %hCD19+ (B cells) and %hCD3+ (T cells) in the blood.

**b**, Follow-up (6–20 weeks) of hCD45 levels in the PB of the mice, showing different mice reconstituted with the same CD34+ cell donor. See individual data points for each mouse in the corresponding source data.

**c**, Follow-up (6–20 weeks) of %hB cells (%hCD19+hCD45+ B cells) and T cells (%hCD3+hCD45+ cells) in the PB, showing different mice reconstituted with the same CD34+ cell donor (week 6, n = 7; weeks 10, 14, 17 and 20, n = 9). Data were obtained in a single experiment and are displayed as means ± s.d. See individual data points for each mouse in the corresponding source data. Data depicted with ◊ indicate mice that had hCD45 levels <40% after 20 weeks and were therefore not included in **b**. Experiments were performed in accordance with the European Union guidelines upon approval of the animal experimentation protocols by the local ethics committee and the French government (authorization agreement number C2EA-15: CECCAPP, Lyon, France). K, thousand.
In vivo sampling and analyses

Euthanization and organ collection ● Timing 2 h (for 20 mice)
▲ CRITICAL At least two trained people are necessary for mouse euthanasia and organ collection to enable quick processing of organs before the cells start to die.
80 Anesthetize mice by intraperitoneal administration of 100 mg/kg ketamine/20 mg/kg xylasin.
81 Collect >500 µl of blood in a 1.5-ml tube containing 100 µl of CPD via retro-orbital blood sampling or intracardiac puncture and keep it on ice until further use.
▲ CRITICAL Put 20 µl of blood into a 1.5-ml tube and store at −80 °C for gDNA extraction and qPCR (Step 86B).
82 Perform cervical dislocation.
83 Collect mouse tissues (spleen, lymph nodes, liver and others) in FACS wash buffer and immediately place on ice.
■ PAUSE POINT Organs can be stored at 4 °C overnight before cell isolation for the analysis by flow cytometry (Step 86A). The following step (Step 84) needs to be performed immediately.
84 For histology and immunohistochemistry, fix the tissue in 4% (vol/vol) formalin (for 24 h) and wash in 70% (vol/vol) ethanol.
■ PAUSE POINT Keep the organs in alcohol until paraffin-embedded slices are prepared from the fixed tissues.

Cell isolation from PB and hematopoietic tissues ● Timing 4 h
▲ CRITICAL At least two trained people are necessary for organ and blood processing.
85 Take the blood from Step 81 and proceed with preparation for FACS analysis (A). Also isolate mononuclear cells from spleen (B) and bone marrow (C).

(A) Preparation of peripheral blood for final analysis
(i) Centrifuge blood from Step 81 at 300g for 5 min at room temperature.
(ii) For plasma collection, transfer the upper phase to a 1.5-ml tube and centrifuge at 14,000g for 5 min at 4 °C. Transfer the supernatant to a fresh 1.5-ml tube and store at −80 °C.
(iii) Wash the remaining blood cells with 1 ml of PBS without Mg²⁺/Ca²⁺ and centrifuge at 300g for 5 min at 4 °C.
(iv) Add 700 µl of 1× RBC lysis buffer for 10 min at room temperature (dark). If a different lysis buffer is used, the incubation time can differ (2–10 min). Please follow the individual manufacturer’s instructions.
(v) Perform a washing step as indicated in Step 85A(iii).
(vi) Resuspend the pellet in 1 ml of PBS without Mg²⁺/Ca²⁺ and count cells after trypan blue staining (as described in Step 43). Note that the cell suspension should be diluted 20-fold with PBS for cell counting in a 96-well plate.
(vii) Place 1 × 10⁶ cells in a FACS tube and 2 × 10⁶ cells in a 1.5-ml tube for qPCR for further analysis (Step 86A and B).
■ PAUSE POINT Cells can be stored on ice for 1–2 h before proceeding with Step 86. Freeze the remaining cells at −150 °C in a freezing medium.

(B) Mononuclear cell isolation from spleen
▲ CRITICAL As an alternative to Step 85B(iv–xiv), resuspend the cell pellet in 1 ml of RBC lysis buffer, incubate for 5 min at room temperature and dilute the lysis buffer by adding 5 ml of PBS without Mg²⁺/Ca²⁺ before centrifugation.
(i) Place a 40-µm cell strainer on top of a 50-ml Falcon tube.
(ii) Put the spleen on the filter and use the plunger base of a syringe to mash the spleen on the filter while pouring PBS without Mg²⁺/Ca²⁺ through the strainer until the spleen is completely dissociated.
(iii) Centrifuge at 300g for 10 min at 4 °C.
(iv) Discard the supernatant and resuspend the pellet in 3 ml of PBS without Mg²⁺/Ca²⁺.
(v) Put 2 ml of Lymphoprep solution in a 15-ml tube.
(vi) Slowly add 3 ml of cell suspension on top of the Lymphoprep.
▲ CRITICAL Gently pipette the cells to avoid mixing the two phases.
(vii) Centrifuge at 850g for 20 min at 20 °C (acceleration: 1; deceleration: 0).
(viii) Carefully aspirate the upper phase, leaving 2–3 ml above the ring of mononuclear cells.
(ix) Carefully aspirate the ring of mononuclear cells with a 10-ml pipette and put it into a new 50-ml tube.
(x) Fill up the tube with PBS without Mg²⁺/Ca²⁺.
(xi) Spin at 300g for 10 min at 4 °C.
(xii) Resuspend the pellet with 5–10 ml of PBS without Mg2+/Ca2+ to reach ~1–5 × 10^6 cells/ml.
(xiii) Count cells with trypan blue solution (as described in Step 43). Note that the cell suspension should be diluted 20-fold with PBS for cell counting in a 96-well plate.
(xiv) Place 1 × 10^6 cells in a FACS tube and 2 × 10^6 cells in a 1.5-ml tube for qPCR for further analysis (Step 86A and B).

- **PAUSE POINT** Cells can be stored on ice for 1–2 h before proceeding with Step 86. Freeze the remaining cells at −150 °C in a freezing medium.

(C) **Mononuclear cell isolation from bone marrow**

(i) Put the femur into a 10-cm dish. Note that the tibia can also be processed using this procedure.
(ii) Remove the muscles and residual tissue surrounding the femur with sterile forceps and scissors.
(iii) Cut the femurs at both ends with sharp sterile scissors. Use a 23- or 25-gauge needle and a 10-cc syringe filled with ice-cold PBS without Mg2+/Ca2+ to flush the bone marrow onto a 40-µm nylon cell strainer placed in a 50-ml Falcon conical tube until the flow-through turns transparent.
(iv) Dissociate the bone marrow through the cell strainer with a 5-ml plunger and wash the strainer with PBS without Mg2+/Ca2+.
(v) Centrifuge cells at 300g for 10 min at 4 °C. Discard the supernatant. If the pellet has a strong red color, this indicates contamination with high numbers of RBCs. To remove RBCs, resuspend the cell pellet with 1 ml of RBC lysis buffer. Incubate for 5 min at room temperature and dilute the lysis buffer by adding 5 ml of buffer before doing another centrifugation.
(vi) Resuspend the pellet with 2 ml of PBS without Mg2+/Ca2+.
(vii) Count bone marrow cells with a hemocytometer with trypan blue (as described in Step 43). Note that the cell suspension should be diluted 20-fold with PBS for cell counting in a 96-well plate.
(viii) Place 1 × 10^6 cells in a FACS tube and 2 × 10^6 cells in a 1.5-ml tube for qPCR for further analysis (Step 86A and B).

- **PAUSE POINT** Cells can be stored on ice for 1–2 h before proceeding with Step 86. Freeze the remaining cells at −150 °C in a freezing medium.

**Analysis of in vivo samples**

86 Detect in vivo–generated CAR T cells via FACS (A) and qPCR (B). FACS provides phenotypic characterization of CAR T cells, whereas qPCR is more sensitive and provides the vector copy number by detection of genomic integration of the CAR. We usually use both methods for quantification and characterization of in vivo–generated CAR T cells.

(A) **FACS-based phenotypic characterization of in vivo–generated CAR T cells**

- **Timing 2 h for staining, 3 h for measurement**

  - **CRITICAL** Use antibody panel 2 (Table 2) for the following steps. Note that fluorophores can be exchanged dependent on the laser configuration of the flow cytometer used. The panel can be extended by, for example, adding anti-human CD20 (LT20), anti-human CD69 (FN50), anti-human CD71 (AC102) and anti-human TIM3 (F38-2E2). Naive and stem cell subsets may be additionally identified by including CD45RO. In this case, naïve T cells are identified as CD45RA^+ CD62L^+ CD45RO^−, whereas stem cells are CD45RA^− CD62L^+ CD45RO^+.

  (i) Wash the purified cells from Step 85A, B and C (there should be 1 × 10^6 cells from each organ in separate microns) twice with 500 µl of FACS wash buffer and pellet cells at 400g for 5 min at 4 °C.

  (ii) Resuspend the cell pellet with blocking solution (tube 1) and incubate for 10 min at 4 °C.

  (iii) Add 50 µl of prepared antibody mixture (tube 2) or isotype control mixture (tube 3) into the respective well and incubate for 30 min in the dark at 4 °C.

  - **CRITICAL** Fluorescent minus one controls should be included in case of a spread/spillover of one channel into the other. Fluorescent minus one controls enable all ambiguity to be removed from the compensated plots and help to distinguish false positive from actual positive signals.

  (iv) For compensation, use Ultracomp eBeads. This is necessary to compensate spillover signals from channels with overlapping fluorescence spectra. Add 1 µl of each mAb to a drop of the compensation beads in microns.

- **CRITICAL** Vortex the beads before use.
(v) Wash cells with 500 µl of FACS wash buffer.
(vi) Centrifuge at 400 \(g\) for 5 min at 4 °C and discard the supernatant carefully.
(vii) Repeat this procedure (Step 86A(v and vi)).
(viii) Add 100 µl of viability dye (tube 4) to each well and incubate for 20 min in the dark at 4 °C.
(ix) Perform the washing step as indicated in Step 86A(v–vii).
(x) Add 100 µl of FACS fixation buffer and resuspend the pellet.
(xi) Proceed to sample acquisition by flow cytometry and analysis by FlowJo or FCS express software (Fig. 6).

**TROUBLESHOOTING**

(B) Determine the number of vector copies associated with gDNA extracted from huNSG mouse tissues by qPCR  
**Timing** 2 h for enrichment of cells, 3–4 h for isolation of gDNA and 3 h for qPCR

(i) Enrich \(2 \times 10^6\) cells from bone marrow or spleen for a CD4\(^+\) or CD8\(^+\) cell population with the respective MicroBeads (Miltenyi) according to the manufacturer’s protocol. This allows the selectivity of the LV for the CD8\(^+\) or the CD4\(^+\) population by qPCR to be confirmed later in the procedure.
**PAUSE POINT** Cells can be frozen at −80 °C as a pellet before further analysis. Perform the enrichment before freezing the cells.

**CRITICAL** Start with $2 \times 10^6$ cells from each bone marrow or spleen sample as a starting material before enrichment to obtain $\sim 1 \times 10^5$ cells for DNA isolation.

(ii) Isolate high–molecular weight gDNA from the enriched cells using the DNeasy kit, according to the manufacturer’s instructions. Elute DNA with 200 µl of elution buffer and determine DNA concentration using Nanodrop.

(iii) Mix 12.5 µl of LightCycler 480 Probes Mastermix with a 0.2 µM concentration of each primer and a 0.2 µM concentration of each probe in a total volume of 15 µl per sample.

**CRITICAL** Dilute with DNase- and RNase-free water at all steps.

**CRITICAL** We use WPRE to quantify CAR gene transfer and human albumin as a housekeeping gene. Alternatively, other primers and target sequences can be used.

(iv) Transfer 15 µl of the reaction mixture into the wells of a 96-well PCR plate and then add 10 µl of sample containing 100 ng of DNA to the plate without mixing to achieve a total volume of 25 µl. As a control, transfer 10 µl of serial dilutions (from $1 \times 10^2$ to $1 \times 10^0$ molecules/µl) to the plate containing the reaction mixture. As a negative control, add 10 µl of DNase and RNase-free water to the plate.

**CRITICAL** All samples and controls should be measured in duplicates or, preferably, triplicates.

(v) Seal the plate and centrifuge at 20 g for 1 min at 4 °C to mix samples with the reaction mixture.

**CRITICAL** $1 \times 10^7$ molecules in total have been added to your sample when using a volume of 10 µl of the standard plasmid with a concentration of $1 \times 10^6$ molecules/µl per well. This has to be taken into account during analysis with the LightCycler 480 software, because you have to provide the total amount of standard.

(vi) Perform qPCR with the LightCycler 480 Instrument II (5 min at 95 °C, 45 cycles of 10 s at 95 °C and 40 s at 60 °C). Use a fluorescein-amidite (FAM)-labeled probe to detect albumin (excitation: 465 nm; emission: 510 nm). A probe with a Cy5-fluorophore enables CAR detection via WPRE (excitation: 618 nm; emission: 660 nm). Analyze data with the LightCycler 480 software using absolute quantification.

**Troubleshooting**

Troubleshooting advice can be found in Table 3.

| Step | Problem | Possible reason | Solution |
|------|---------|-----------------|----------|
| 2    | Packaging cells and/or vector stock contaminated with microorganisms | Wrong handling of material | Take care not to move your hand or material above an opened cell culture plate. Be very cautious with your materials such as tips and media bottles. Exchange all materials required for transfection |
| 23 and 32 | Low gene transfer activity on target cells | Inefficient particle formation and release from packaging cells | Determine particle number in vector stock. If $<5 \times 10^{11}$/ml, improve transfection efficiency as explained above (Step 19) |
|       |         | Absence/low level of target receptor | Check expression of the target receptor via flow cytometry. See Fig. 4a as an example for sufficient receptor density. Use an alternative cell line for quantification of gene transfer activity |
|       |         | The vector stock contains a sufficient number of vector particles ($>5 \times 10^{11}$/ml), but they are inactive in gene transfer ($<2 \times 10^6$ t.u./ml) | Check concentration step: determine gene transfer activity in unconcentrated harvest (taken in Step 10). Loss of gene transfer activity during concentration should not be more than twofold. Check the identity and functionality of all plasmids used, especially surface expression and receptor binding activity of the MV-H– or NiV-G–encoding plasmids |

Table continued
### Table 3 (continued)

| Step | Problem | Possible reason | Solution |
|------|---------|----------------|----------|
| 32   | Low transfection efficiency (<80% determined with GFP-encoding transfer vector plasmid) | Insufficient contact between vector particles and target cells under given culture conditions Inactivation of vector particles through high endocytosis rate of target receptor Condition of Lenti-X 293T | Check target receptor binding of the vector particles<sup>33</sup> Transduction enhancers like Vectofusin<sup>33</sup> substantially increase transduction of target cells in vitro (Step 29). In addition, spinfection (Step 30) enhances gene transfer<sup>33</sup> Block endocytosis (e.g., by NH₄Cl or bafilomycin) to enhance gene delivery activity<sup>54</sup> Culture conditions (Step 1): monitor the condition and health of the cells daily. Identify the best source (and lot) of FBS experimentally. Cells must not be cultivated for >20 passages. Cells should not be grown to overconfluence. Split cells twice a week and seed at a 1:8–1:10 dilution. Seeding of Lenti-X 293T (Step 2): cells must be distributed uniformly over the plate. Time of transfection (Step 2): cells should be ≥75% confluent by the time of performing the transfection and should not be growing in 3D clusters. During transfection (Steps 3 and 7): avoid cells drying out when kept in low volumes of media. Low volumes should be used for a maximum of 1 h 30 min. Avoid detaching cells when adding the transfection mixture onto the cells. Poor plasmid quality (e.g., too high a level of endotoxin and RNA) Wrong plasmid ratios | |
| 74   | Low and/or slow humanization | Busulfex conditioning is not strong enough Insufficient purity of human CD34<sup>+</sup> cells used for injection Too few living human CD34<sup>+</sup> cells injected | Calibrate the Busulfex dose carefully according to the weight of the mice (Step 33) and do not inject the CD34<sup>+</sup> cells earlier than 30–36 h after Busulfex injection (if CD34<sup>+</sup> cells are injected too soon, they will be eliminated). Use human CD34<sup>+</sup> cells with a purity of >90%, containing less than 3% T cells and B cells. Make sure that a minimum of 7 × 10⁴ living CD34<sup>+</sup> cells is injected (Step 56). Injecting ≥1 × 10⁵ cells speeds up humanization | |
| 86A(xi) | No signal detected by FACS for in vivo-generated CAR T cells | Inactive vector stock Inadequate FACS staining and analysis | Improve production conditions as described above. Low signal intensity observed either because of insufficient antibody or quenching of fluorochrome. Perform antibody titration and use an optimal amount of antibody (Step 86A(ii)). To avoid quenching of fluorochrome, measure samples within 3 d after staining. For storage longer than 1 d, resuspend samples in FACS wash buffer after fixing. Set up a positive control, ideally a mixture of CAR<sup>+</sup> and CAR<sup>−</sup> T lymphocytes in serial dilutions. 1% CAR<sup>+</sup> should be readily detectable with the staining protocol. DNA might have been lost in preparation (Step 86B (ii)). Determine the DNA concentration and be sure to use an accurate amount of DNA for qPCR. Make sure that DNA is of sufficient quality by gel electrophoresis | |
| 86B(vi) | No detection of CAR T cells by qPCR | Inadequate qPCR due to poor DNA quality Inadequate qPCR due to poor design of primers and probe When a positive signal is observed from FACS, this could be a false-positive signal When a negative signal is observed from FACS, this could be the result of an inactive vector stock | Redesign primers and probe using appropriate software. Compensate FACS settings using beads as well as single stainings to avoid spillovers (Step 86A(iv)) Improve production conditions as described above | |
Timing

**Production of targeted LVs**
Step 1, seed Lenti-X 293T cells: Day 1
Steps 2–8, transfection of Lenti-X 293T cells: Day 2
Step 9, media change: Day 3
Steps 10–14, harvesting of LV-containing supernatant and concentration via sucrose cushion: Days 4 and 5
Steps 15–18, resuspension of LV pellet and preparation of aliquots: Day 5

**Quantification of particle number and gene transfer activity of LVs**
Step 19, determine particle number via NTA: ~1 h per stock
Steps 20–23, gene transfer activity on target receptor–positive cell lines: 3–4 d
Steps 24–32, gene transfer activity on primary cells: 8–11 d

**Humanization of mice**
Steps 33–37, mice conditioning with sublethal injection of Busulfex: 1 h (for 20 mice)
Steps 38–46, cell preparation for CD34+ cell activation: 40–50 min
Steps 47–49, cell preparation for injection: 1 h
Steps 50–58, transplantation of human cells: 1 h (for 20 mice)
Human immune system reconstitution: 2–4 months
Steps 59–74, determination of the humanization level in the PB: 2–3 h

**In vivo CAR T cell generation**
Steps 75–77, IL7 conditioning: 45 min (for 20 mice)
Step 78, in vivo administration of the vector: 1 h
Step 79, CAR-T longitudinal analysis: 1–8 weeks
Steps 80–84, euthanasia and organ collection: 2 h (for 20 mice)

**In vivo sampling and analyses**
Step 85A–C, cell isolation from PB and hematopoietic tissues: 4 h
Step 86A, FACS-based phenotypic characterization of in vivo–generated CAR T cells: 2 h for staining (i–x), 3 h for measurement (xi)
Step 86B, determination of the number of vector copies associated with gDNA extracted from huNSG mouse tissues by qPCR: 2 h for enrichment of specific cell populations (i), 3–4 h for isolation of gDNA (ii)
Step 86B, determination of vector copy number in gDNA by qPCR: 3 h (iii–vi)

**Anticipated results**
Successful completion of this protocol results in in vivo–generated CD4+ or CD8+ CAR T cells present in the blood and tissues of humanized mice. These CAR T cells carry the genetic information for the CD19-CAR stably integrated in their genome. This genetic modification is restricted to the target receptor–expressing cell type, human CD4+ T cells for CD4-LV and CD8+ T cells for CD8-LV, respectively. Any other human or mouse cells present in the animals do not become genetically modified. When we follow this protocol, ~2–14% of human CD8+ T cells (with CD8-LV) or CD4+ T cells (with CD4-LV) convert into CAR T cells in NSG mice transplanted with human hematopoietic stem and progenitor cells. Most of the converted CAR T cells carry a single vector copy, and the cells are found in the blood, spleen and bone marrow of the mice. Notably, these numbers can go up to 50% when working with PBMC-transplanted NSG mice. As a consequence of CAR T cell generation, the CD19+ target cells of the CAR become completely eliminated in both mouse models. Properly humanized mice and vector stocks with sufficient activity are key to achieving this result. It is recommended that animals with ≥40% humanization (i.e., 40% human CD45+ cells of total CD45+ cells) are used for the CAR T cell generation part of the protocol. However, by following this procedure, a humanization of >80% is expected. CD4-LV and CD8-LV stocks produced according to the procedure should contain ≥5 × 1011 particles/ml and 107 t.u./ml as determined on target receptor–positive cell lines (Fig. 4), whereas target receptor–negative cells should not become genetically modified. CAR T cells can be generated in 10–15 mice from a total volume of ~2.5 ml per
vector stock. Any therapeutic or gene of interest can be packaged into CD4-LV or CD8-LV using the provided protocol. However, yields of vector particles and gene transfer activities might differ. This holds true also for LVs targeted to surface receptors other than CD4 or CD8, for which the same protocol for vector stock generation can be followed.

**Reporting Summary**

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Source data for Figs. 4 and 5 are provided. Data shown in Fig. 6 were previously published in ref. 12.

**Code availability**

Two NTA software scripts, the continuous flow script and the stop flow script, have been deposited in figshare (10.6084/m9.figshare.13221602) and are available as Supplementary Information.

**References**

1. Radek, C. et al. Vectofusin-1 improves transduction of primary human cells with diverse retroviral and lentiviral pseudotypes, enabling robust, automated closed-system manufacturing. *Hum. Gene Ther.* **30**, 1477–1493 (2019).
2. Zhang, Z., Qiu, S., Zhang, X. & Chen, W. Optimized DNA electroporation for primary human T cell engineering. *BMC Biotechnol.* **18**, 4 (2018).
3. Dayball, K., Millar, J., Miller, M., Wan, Y. H. & Bramson, J. Electroporation enables plasmid vaccines to elicit CD8+ T cell responses in the absence of CD4+ T cells. *J. Immunol.* **171**, 3379–3384 (2003).
4. Naldini, L. Gene therapy returns to centre stage. *Nature* **526**, 351–360 (2015).
5. June, C. H. & Sadelain, M. Chimeric antigen receptor therapy. *N. Engl. J. Med.* **379**, 64–73 (2018).
6. Gattinoni, L., Klebanoff, C. A. & Restifo, N. P. Paths to stemness: building the ultimate antitumour T cell. *Nat. Rev. Cancer* **12**, 671–684 (2012).
7. Fraietta, J. A. et al. Determinants of response and resistance to CD19 chimeric antigen receptor (CAR) T cell therapy of chronic lymphocytic leukemia. *Nat. Med.* **24**, 563–571 (2018).
8. Xu, Y. et al. Closely related T-memory stem cells correlate with in vivo expansion of CAR.CD19-T cells and are preserved by IL-7 and IL-15. *Blood* **123**, 3750–3759 (2014).
9. Frank, A. M. & Buchholz, C. J. Surface-engineered lentiviral vectors for selective gene transfer into subtypes of lymphocytes. *Mol. Ther. Methods Clin. Dev.* **12**, 19–31 (2018).
10. Agarwal, S., Weidner, T., Thalheimer, F. B. & Buchholz, C. J. In vivo generated human CAR T cells eradicate tumor cells. *Oncoimmunology* **8**, e167161 (2019).
11. Pfeiffer, A. et al. In vivo generation of human CD19-CAR T cells results in B-cell depletion and signs of cytokine release syndrome. *EMBO Mol. Med.* **10**, e9158 (2018).
12. Agarwal, S. et al. In vivo generation of CAR T cells selectively in human CD4+ lymphocytes. *Mol. Ther.* **28**, 1783–1794 (2020).
13. Buchholz, C. J., Friedel, T. & Büning, H. Surface-engineered viral vectors for selective and cell type-specific gene delivery. *Trends Biotechnol.* **33**, 777–790 (2015).
14. Funke, S. et al. Targeted cell entry of lentiviral vectors. *Mol. Ther.* **16**, 1427–1436 (2008).
15. Anliker, B., Longhurst, S. & Buchholz, C. J. Environmental risk assessment for medicinal products containing genetically modified organisms. *Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz* **53**, 52–57 (2010).
16. Zhou, Q. et al. T-cell receptor gene transfer exclusively to human CD8+ cells enhances tumor cell killing. *Blood* **120**, 4334–4342 (2012).
17. Zhou, Q. et al. Exclusive transduction of human CD4+ T cells upon systemic delivery of CD4-targeted lentiviral vectors. *J. Immunol.* **195**, 2493–2501 (2015).
18. Frank, A. M. et al. CD8-specific DARPinS improve selective gene delivery into human and primate T lymphocytes. *Hum. Gene Ther.* **31**, 679–691 (2020).
19. Bender, R. R. et al. Receptor-targeted Nipah virus glycoproteins improve cell-type selective gene delivery and reveal a preference for membrane-proximal cell attachment. *PLoS Pathog.* **12**, e1005641 (2016).
20. Weidner, T. et al. Genetic in vivo engineering of human T lymphocytes in mouse models. Preprint at https://protocolexchange.researchsquare.com/article/pex-1282/v1 (2020).
21. Seif, M., Einsle, H. & Löfler, J. CAR T cells beyond cancer: hope for immunomodulatory therapy of infectious diseases. *Front. Immunol.* **10**, 2711 (2019).
22. Akkina, R. New generation humanized mice for virus research: comparative aspects and future prospects. *Virology* **435**, 14–28 (2013).
23. Ito, M. et al. NOD/SCID/γc null mouse: an excellent recipient mouse model for engraftment of human cells. *Blood* **100**, 3175–3182 (2002).
24. Hiramatsu, H. et al. Complete reconstitution of human lymphocytes from cord blood CD34+ cells using the NOD/SCID/γcnull mouse model. *Blood* **102**, 873–880 (2003).

25. Ishikawa, F. Development of functional human blood and immune systems in NOD/SCID/IL2 receptor γ chain null mice. *Blood* **106**, 1565–1573 (2005).

26. Shultz, L. D. et al. Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2Rγnull mice engrafted with mobilized human hematopoietic stem cells. *J. Immunol.* **174**, 6477–6489 (2005).

27. Anthony-Gonda, K. et al. Multispecific anti-HIV duoCAR-T cells display broad in vitro antiviral activity and potent in vivo elimination of HIV-infected cells in a humanized mouse model. *Sci. Transl. Med.* **11**, eaav5685 (2019).

28. Kitchen, S. G. & Zack, J. A. Engineering HIV-specific immunity with chimeric antigen receptors. *AIDS Patient Care STDs* **30**, 556–561 (2016).

29. Morizono, K. et al. Lentiviral vector retargeting to P-glycoprotein on metastatic melanoma through intravenous injection. *Nat. Med.* **11**, 346–352 (2005).

30. Liang, M. et al. Targeted transduction via CD4 by a lentiviral vector uses a clathrin-mediated entry pathway. *Blood* **106**, 1565–1573 (2005).

31. Yang, H., Joo, K.-I., Ziegler, L. & Wang, P. Cell type-specific targeting with surface-engineered lentiviral vectors co-displaying OKT3 antibody and fusogenic molecule. *Pharm. Res.* **26**, 1432–1445 (2009).

32. Rasbach, A. et al. The receptor attachment function of measles virus hemagglutinin can be replaced with an autonomous protein that binds Her2/neu while maintaining its fusion-helper function. *J. Virol.* **87**, 6246–6256 (2013).

33. Frank, A. M. et al. Combining T-cell specific activation and in vivo gene delivery through CD3-targeted lentiviral vectors. *Blood Adv.* **4**, 5702–5715 (2020).

34. Smith, D. J. et al. Propagating humanized BLT mice for the study of human immunology and immunotherapy. *Sci. Transl. Med.* **11**, eaav5685 (2019).

35. Navaratnarajah, C. K., Generous, A. R., Yousaf, I. & Cattaneo, R. Receptor-mediated cell entry of paramyxoviruses: mechanisms, and consequences for tropism and pathogenesis. *J. Biol. Chem.* **295**, 2771–2786 (2020).

36. Rasbach, A. et al. The receptor attachment function of measles virus hemagglutinin can be replaced with an autonomous protein that binds Her2/neu while maintaining its fusion-helper function. *J. Virol.* **87**, 6246–6256 (2013).

37. Frank, A. M. et al. Combining T-cell specific activation and in vivo gene delivery through CD3-targeted lentiviral vectors. *Blood Adv.* **4**, 5702–5715 (2020).

38. Smith, T. T. et al. In situ programming of leukemia-specific T cells using synthetic DNA nanocarriers. *Nat. Nanotechnol.* **12**, 813–820 (2017).

39. Shultz, L. D., Ishikawa, F. & Greiner, D. L. Humanized mice in translational biomedical research. *Nat. Rev. Immunol.* **7**, 118–130 (2007).

40. Manz, M. G. Human-hematolymphoid-system mice: opportunities and challenges. *Immunity* **26**, 537–541 (2007).

41. Yahata, T. et al. Functional human T lymphocyte development from cord blood CD34+ cells in nonobese diabetic/Shi-scid, IL-2 receptor γ null mice. *J. Immunol.* **169**, 204–209 (2002).

42. Mhaidly, R. & Verhoeven, E. Humanized mice are precise tools for preclinical evaluation of CAR T and CAR NK cell therapies. *Cancers* **12**, 1915 (2020).

43. Brendel, C., Rio, P. & Verhoeyen, E. Humanized mice are precise tools for evaluation of hematopoietic gene therapies and preclinical modeling to move towards a clinical trial. *Biochem. Pharmacol.* **174**, 113711 (2020).

44. Tanaka, S. et al. Development of mature and functional human myeloid subsets in hematopoietic stem cell-engrafted NOD/SCID/IL2RγKO mice. *J. Immunol.* **188**, 6145–6155 (2012).

45. Huntington, N. D. et al. IL-15 trans-presentation promotes human NK cell development and differentiation in vivo. *J. Exp. Med.* **206**, 25–34 (2009).

46. Cosgun, K. N. et al. Kit regulates HSC engagement across the human-mouse species barrier. *Cell Stem Cell* **15**, 227–238 (2014).

47. McIntosh, B. E. et al. Nonirradiated NOD, B6, SCID IL2rγ−/−/KitWV/AyVj (NBSGW) mice support multilineage engraftment of human hematopoietic cells with KitWV mutations. *Exp. Hematol.* **48**, 41–49 (2017).

48. Rogova, A. et al. Human hematolymphoid system mice: current use and future potential for medicine. *Annu. Rev. Immunol.* **31**, 635–674 (2013).

49. Traggiai, E. et al. Development of a human adaptive immune system in cord blood cell-transplanted mice. *Science* **304**, 104–107 (2004).

50. Smith, D. J. et al. Propagating humanized BLT mice for the study of human immunology and immunotherapy. *Stem Cells Dev.* **25**, 1863–1873 (2016).

51. Münch, R. C. et al. DARPins: an efficient targeting domain for lentiviral vectors. *Mol. Ther.* **19**, 686–693 (2011).
55. Goujon, C. et al. SIVSM/HIV-2 Vpx proteins promote retroviral escape from a proteasome-dependent restriction pathway present in human dendritic cells. *Retrovirology* **4**, 2 (2007).
56. Berger, G. et al. A simple, versatile and efficient method to genetically modify human monocyte-derived dendritic cells with HIV-1-derived lentiviral vectors. *Nat. Protoc.* **6**, 806–816 (2011).
57. Friedel, T. et al. Receptor-targeted lentiviral vectors are exceptionally sensitive toward the biophysical properties of the displayed single-chain Fv. *Protein Eng. Des. Sel.* **28**, 93–106 (2015).
58. Zufferey, R., Nagy, D., Mandel, R. J., Naldini, L. & Trono, D. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nat. Biotechnol.* **15**, 871–875 (1997).
59. Folks, T. et al. Characterization of a continuous T-cell line susceptible to the cytopathic effects of the acquired immunodeficiency syndrome (AIDS)-associated retrovirus. *Proc. Natl Acad. Sci. USA* **82**, 4539–4543 (1985).
60. Hartmann, J. et al. A library-based screening strategy for the identification of DARPin ligands for receptor-targeted AAV and lentiviral vectors. *Mol. Ther. Methods Clin. Dev.* **10**, 128–143 (2018).

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Author contributions

T.W. drafted the protocol on LV vector stock generation and designed figures. S.A. drafted the protocol on CAR T cell detection, and S.P. and F.F. drafted the protocol on mouse humanization. G.B. and J.H. contributed to the writing of the manuscript. C.J.B. and E.V. supervised work, drafted the general parts and revised the manuscript.

Competing interests

E.V. and C.J.B are listed as inventors on patents on receptor-targeted LVs that have been licensed out. All other authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to E.V. or C.J.B.

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Agarwal, S., Weidner, T., Thalheimer, F. B. & Buchholz, C. J. *Oncoimmunology* **8**, e1671761 (2019): https://doi.org/10.1080/2162402X.2019.1671761
Agarwal, S. et al. *Mol. Ther.* **28**, 1783–1794 (2020): https://doi.org/10.1016/j.ymthe.2020.05.005
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Software and code

Policy information about availability of computer code

Data collection: FACS Diva Version 8.0.3; Nanosight NTA 3.3; LightCycler® 480 SW 1.5.1; MACSQuantify 2.6; ELISA Reader

Data analysis: FCS Express Version 6; FlowJo Version 10.0.8; GraphPad Prism 8; LightCycler® 480 SW 1.5.1

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**Life sciences study design**

All studies must disclose on these points even when the disclosure is negative.

| Sample size | For animal experiments we used sufficient numbers (usually \(n>5\)) to achieve statistical relevance. |
|-------------|----------------------------------------------------------------------------------------------|
| Data exclusions | Mice were excluded from further experiments, if humanization levels are \(<40\%\). |
| Replication | Representative data sets based on many biological replicas are shown. |
| Randomization | Not relevant, since all available mice were used for humanization. |
| Blinding | Blinding not relevant here, since the read-out for CAR T cell presence is quantified by FACS and qPCR which are independent from experimenter's influence. |

**Reporting for specific materials, systems and methods**

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### Materials & experimental systems

- n/a  Involved in the study
- Antibodies
- Eukaryotic cell lines
- Palaeontology and archaeology
- Animals and other organisms
- Human research participants
- Clinical data
- Dual use research of concern

### Methods

- n/a  Involved in the study
- ChIP-seq
- Flow cytometry
- MRI-based neuroimaging

**Antibodies**

- Antibodies used see Table 1 + 2
- Validation Antibodies as listed in Table 1 and 2 have been validated as described on the providers' websites. Additionally, absence of signals in antigen-negative control samples is routinously confirmed.

**Eukaryotic cell lines**

Policy information about cell lines

- Cell line source(s) see Materials in the manuscript
- Authentication Authentication certificates were obtained for the most important cell lines from the DSMZ (Germany).
- Mycoplasma contamination Cell lines are regularly checked for mycoplasma contaminations.
- Commonly misidentified lines (See ICLAC register) Contaminations were excluded by authentication.
Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

| Laboratory animals | See manuscript. |
|--------------------|-----------------|
| Wild animals       | The study did not involve wild animals. |
| Field-collected samples | The study did not involve samples collected from the field. |
| Ethics oversight   | NOD/SCIDgC-/- (NSG) mice were housed in the animal facility "Plateau de Biologie Expérimentale de la Souris (PBES)" (ENS de Lyon, Lyon, France). Experiments shown in Fig. 5 were performed in accordance with the European Union guidelines upon approval of the animal experimentation protocols by the local ethical and the French government (Authorization agreement number C2EA-15: CECCAPP, Lyon, France). Experiments shown in Fig. 6 were performed in accordance with the European Union guidelines upon approval of the animal experimentation protocols by the local ethical and the French government (Authorization agreement number C2EA-15: CECCAPP, Lyon, France). |

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Flow Cytometry

Plots

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

| Sample preparation | see manuscript |
|--------------------|----------------|
| Instrument         | FACS Diva Version 8.0.3; Nanosight NTA 3.3; LightCycler® 480 SW 1.5.1; MACSQuantify 2.6; ELISA Reader |
| Software           | FCS Express Version 6; FlowJo Verion 10.0.8; GraphPad Prism 8; LightCycler® 480 SW 1.5.1; Prism |
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| Gating strategy    | see manuscript |

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