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Highly Efficient and Selective CAR-Gene Transfer Using CD4- and CD8-Targeted Lentiviral Vectors

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Chimeric antigen receptor (CAR)-modified T cells have revealed promising results in the treatment of cancer, but they still need to overcome various hurdles, including a complicated manufacturing process. Receptor-targeted lentiviral vectors (LVs) delivering genes selectively to T cell subtypes may facilitate and improve CAR T cell generation, but so far they have resulted in lower gene delivery rates than conventional LVs (vesicular stomatitis virus [VSV]-LV). To overcome this limitation, we studied the effect of the transduction enhancer Vectofusin-1 on gene delivery to human T cells with CD4- and CD8-targeted LVs, respectively, encoding a second-generation CD19-CAR in conjunction with a truncated version of the low-affinity nerve growth factor receptor (ΔLNGFR) as reporter. Vectofusin-1 significantly enhanced the gene delivery of CD4- and CD8-LVs without a loss in target cell selectivity and killing capability of the generated CAR T cells. Notably, delivery rates mediated by VSV-LV were substantially reduced by Vectofusin-1. Interestingly, a transient off-target signal in samples treated with Vectofusin-1 was observed early after transduction. However, this effect was not caused by uptake and expression of the transgene in off-target cells, but rather it resulted from cell-bound LV particles having ΔLNGFR incorporated into their surface. The data demonstrate that gene transfer rates in the range of those mediated by VSV-LVs can be achieved with receptor-targeted LVs.

INTRODUCTION

Nowadays, the combination of gene therapy and immunotherapy for the treatment of cancer becomes a true and realistic addition to classical surgery, radiotherapy, or chemotherapy. In 2017, two therapies relying on the genetic manipulation of autologous T cells with a chimeric antigen receptor (CAR) for the treatment of advanced blood cancers were granted marketing authorization by the U.S. Food and Drug Administration (FDA) and recently also the European Commission (EC).[1] Lentiviral vectors (LVs) are one of the most applied vehicles for CAR gene delivery into human T cells.[2]

Commonly, LVs are pseudotyped with the glycoprotein of the vesicular stomatitis virus (VSV-G) or with the envelope proteins of γ-retroviruses (γ-RVs), such as murine leukemia virus (MLV) or gibbon ape leukemia virus (GALV), and, more recently, the baboon endogenous retrovirus (BaEV), resulting in vector particles with a broad tropism mediating gene transfer into basically all human cell types.[3] In contrast, receptor-targeted LVs aim at delivering genes exclusively to a cell population of choice. This can be achieved by permanent ablation of natural receptor binding of the pseudotyped envelope proteins and genetic fusion of a targeting ligand recognizing a specific cell surface antigen.[4] With this concept, LVs exclusively targeting distinct cell types of the hematopoietic system, tumor cells, pluripotent cells, or cells of the CNS were already generated.[4] Of these, LVs retargeted to human CD4 and CD8, respectively, were proven to deliver genes selectively to the respective T cell subtypes *in vitro* and *in vivo*.[5–7]

While the CD8-targeted vector (CD8-LV) was pseudotyped with modified envelope proteins of Nipah virus (NiV) fused to a CD8-specific single chain variable fragment (scFv), the CD4-targeted LV (CD4-LV) contained modified envelope proteins of measles virus (MV) fused to a CD4-specific designed ankyrin repeat protein (DARPin).[5,6] Both LVs have been shown to deliver reporter genes as well as therapeutic genes, including T cell receptors (TCRs) and CARs, with high selectivity into the targeted T lymphocyte cell population.[6,7] In this respect, CD8-LV and CD4-LV could mediate subtype-specific T cell manipulation without the need for prior cell separation, and, thus, they facilitate and improve CAR T cell generation. However, before these LVs can be applied to manufacturing, their gene delivery rates must be improved to come close to those of VSV-LVs.

Transduction enhancers are commonly used to boost vector cell entry by facilitating efficient co-localization of vectors and cells. These

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culture additives can be cationic polymers, lipids, or peptides (e.g., polybrene, protamine sulfate) or even small molecules (e.g., fibronectin).[8] More recently, Vectofusin-1, a histidine-rich cationic amphipathic short peptide, was discovered promoting the transduction of human CD34-positive hematopoietic stem cells with various pseudotyped LVs.[9] Interestingly, for human T and B cells, enhanced transduction using Vectofusin-1 was observed for LVs pseudotyped with the envelope protein of GALV, but not VSV-G.[10]

Here we investigated the effect of Vectofusin-1 on CD4-LV and CD8-LV along with VSV-LV and LVs pseudotyped with the glycoprotein of BaEV (BaEV-LV) for the delivery of a second-generation CD19-CAR to human T cells. To facilitate the detection of the CAR transgene, the transfer vector encoded a truncated version of the low-affinity nerve growth factor receptor (ΔLNGFR) as reporter. We show that Vectofusin-1 significantly enhanced gene delivery mediated by BaEV-LV and, notably, also CD4-LV and CD8-LV, without compromising target cell selectivity. We detected, however, a transient delivery of ΔLNGFR as protein to target and off-target cells upon vector particle addition.

RESULTS

Vectofusin-1 Augments CAR-Gene Transfer Rates by CD4- and CD8-Targeted LVs

Four types of CD19-CAR-encoding LVs were produced, besides targeted CD4-LV and CD8-LV also the non-targeted VSV-LV and BaEV-LV, each in several batches. All vectors encoded a second-generation CD19-specific CAR[11] and the reporter protein ΔLNGFR linked by a P2A cleavage site (Figure 1A). The generated LV particles were used to transduce interleukin-2 (IL-2)-stimulated human peripheral blood mononuclear cells (PBMCs) in the presence or absence of Vectofusin-1. Transduction was analyzed 12 days later using flow cytometry by staining the cells for the reporter ΔLNGFR as well as CD4 or CD8.

In the presence of Vectofusin-1, the number of CD4-positive cells transduced by CD4-LV was enhanced more than 2-fold from 22% to 57% (Figure 1B). This effect of Vectofusin-1 was even more pronounced for CD8-LV, where the transduction rate of CD8-positive cells increased from 32% to 87% (Figure 1B). Importantly, receptor specificity was retained for both retargeted vectors. The low amount of stained receptor-negative ΔLNGFR-positive cells was at the background level of untransduced cells (Figure 1B). In contrast, transduction with VSV-LV in the presence of Vectofusin-1 was diminished when compared to the sample without transduction enhancer (Figure 1B). With Vectofusin-1 only, 21% and 13% of the CD4- and CD8-positive cells were transduced, while without Vectofusin-1 transduction rates of 52% and 33% were achieved, respectively. The highest overall transduction rate was achieved using BaEV-LV, reaching 62% and 45% of CD4- and CD8-positive cells in the absence and 79% and 66% in the presence of Vectofusin-1, respectively.

To investigate whether this observed effect of Vectofusin-1 was independent from the used donor, the PBMC activation conditions, or the particular vector batch, transduction was performed several times with varying donors, under two different PBMC stimulation protocols, and with two to five vector productions for each vector type. Notably, the different vector types contained reproducibly similar particle numbers (Table S1).

The transduction experiments revealed that Vectofusin-1 improved transgene delivery for CD4-LV, CD8-LV, and BaEV-LV substantially and was detrimental for VSV-LV (Figure 1C). On average, transduction of CD4- or CD8-positive T cells in the presence of Vectofusin-1 with CD4-LV or CD8-LV increased from approximately 50% to 80%, with no impact on vector specificity. For BaEV-LV, enhancements from about 60% to 85% for CD4-positive cells and 50% to 85% for CD8-positive cells were observed. In contrast, 75% of CD4-positive and 55% of CD8-positive cells were transduced with VSV-LV in the absence of Vectofusin-1, which decreased to 40% ($CD4^+$) and 30% ($CD8^+$) in the presence of Vectofusin-1.

When we analyzed the cells by flow cytometry for ΔLNGFR expression at earlier time points than day 12, we detected ΔLNGFR-positive cells in some samples that were transduced with CD4-LV or CD8-LV not only in target receptor-positive but also in target receptor-negative cells. One example in which this effect was particularly pronounced is shown in Figure S1. The off-target signals disappeared after prolonged cultivation of the cells. For CD4-LV, we observed about 60% of CD4-negative cells showing a signal for ΔLNGFR on day 5 post-transduction, which disappeared to background level on day 13. Similarly, for CD8-LV, about 20% ΔLNGFR-positive but CD8-negative cells were present on day 5, which decreased to 0.8% ΔLNGFR-positive, CD8-negative cells on day 13. The transient off-target staining for ΔLNGFR in the presence of Vectofusin-1 was also observed in some samples for CD4-CD8 double-negative cells, which were likely monocytes, B lymphocytes, or granulocytes (Figure S1C). Notably, these cells were present in all PBMC cultures to a donor variable amount to the same extent at early and late analysis time points (Figure S1D). Death of ΔLNGFR-positive cells other than T cells between days 5 and 13 during cultivation could, therefore, be excluded as explanation for the absence of ΔLNGFR-positive off-target cells at day 13. Rather, transfer of ΔLNGFR as protein from packaging cells to PBMCs could be an explanation for these observations (see below).

Vectofusin-1 Did Not Impair the Killing Capability of the Generated CAR T Cells

We next assessed whether the transduction enhancer had an influence on the viability, proliferation capacity, or phenotype of the transduced T cells. No significant difference in T cell viability was observed upon transduction with receptor-targeted VSV- or BaEV-pseudotyped particles (Figure S2A). With regard to proliferation capacity, Vectofusin-1 appeared to slightly impact the growth of PBMCs at least in some samples, including untransduced cells (Figure S2B). Analysis of the amount of naive (T_N), stem cell memory (T_{SCM}), effector memory (T_{EM}), central memory (T_{CM}), or effector (T_{EFF}) T lymphocytes present in the CD8-positive or CD4-positive PBMC fraction 13 days

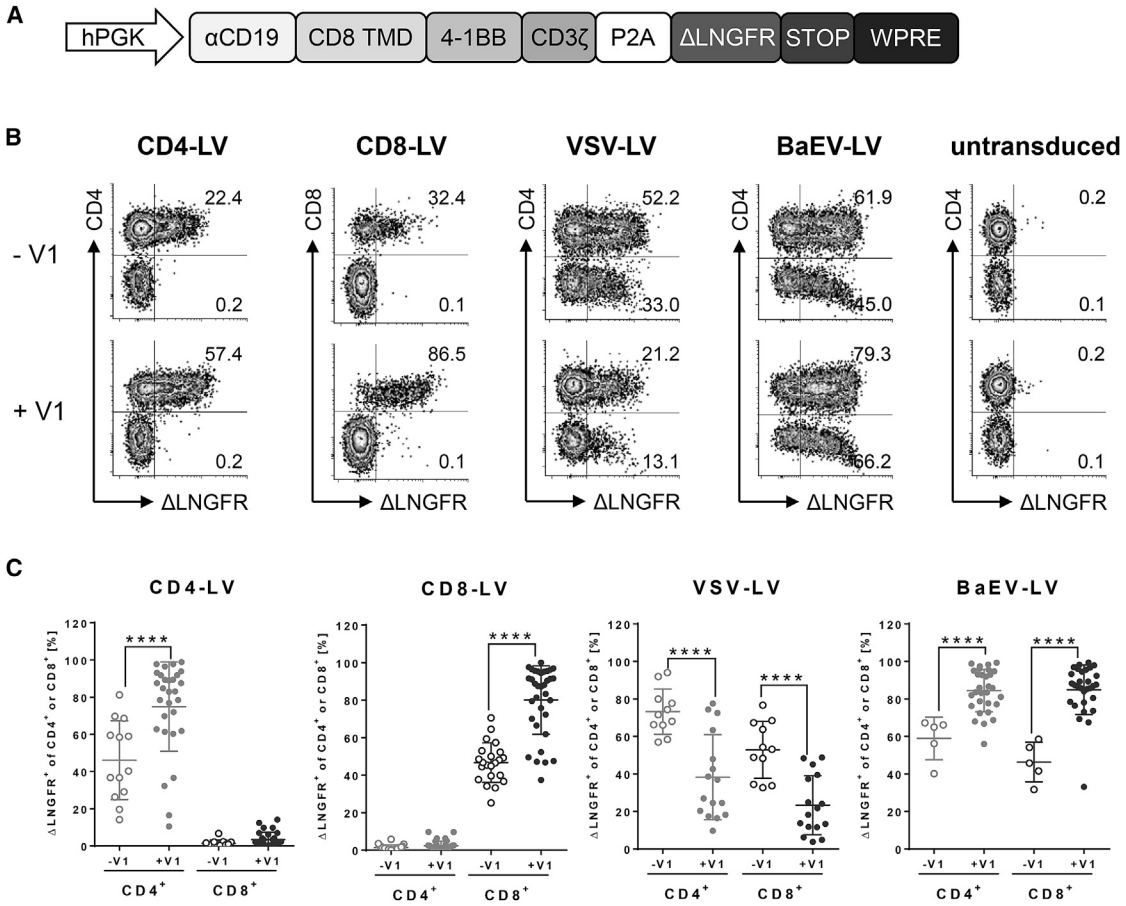

Figure 1. CAR Gene Delivery with Different Lentiviral Vectors in the Presence and Absence of Vectofusin-1

(A) Schematic representation of the CAR construct. The second-generation CAR consists of a CD19-scFv derived from the murine monoclonal antibody FMC63, a human CD8-derived hinge and transmembrane domain, the 4-1BB co-stimulatory domain, and a CD3ζ-signaling domain. The reporter protein ΔLNGFR is connected to the CAR gene by a P2A peptide self-cleavage sequence. Transgene expression is under the control of the human phosphoglycerate kinase (PGK) promoter. The woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) encoded after a stop codon promotes enhanced transgene expression. (B) IL-2-stimulated human PBMCs were transduced with 5 μL CD4-LV, CD8-LV, or VSV-LV or 1 μL BaEV-LV in the presence or absence of Vectofusin-1 (V1) or left untransduced. Then 12 days later, flow cytometry was performed after staining the cells for the expression of CD4, CD8, and ΔLNGFR. Numbers in the dot plots refer to the percent of ΔLNGFR-positive cells within the $CD4^+$ or $CD8^+$ cells, respectively. (C) Each dot represents an independent transduction experiment. Transduction rates were determined after 9–13 days as in (B). PBMCs of 6–8 different donors, stimulated either with IL-2 alone or a combination of IL-7 and IL-15, were transduced with 5 or 2.5 μL CD4-LV or CD8-LV, 5 μL VSV-LV, or 1 or 5 μL BaEV-LV. Two to five batches of vector stock were used (see Table S1 for details). Flow cytometry analysis was performed staining cells for CD4, CD8, LNGFR, and viability. Results for viability are presented in Figure S2A. Individual results as well as mean values, SDs, and p values are shown. Unpaired t test or Mann-Whitney test was performed to determine significance. ****$p < 0.0001$.

after transduction with and without Vectofusin-1 revealed that Vectofusin-1 or the transduction process per se only minimally altered the resulting T cell phenotype ratios, if at all (Figure S2C).

In a next step, we analyzed whether Vectofusin-1 impacts the functionality of the generated CAR T cells with respect to killing of CD19-positive tumor cells. For this purpose, CAR T cells were generated with CD8-LV in the presence or absence of Vectofusin-1. Equal amounts of CAR^+ cells were then incubated with carboxyfluorescein succinimidyl ester (CFSE)-labeled CD19-positive Nalm-6 cells for 4 h at different effector-to-target ratios. Target cell lysis was determined by flow cytometry. Both CAR T cell products were able to kill the Nalm-6 tumor cells and showed a significantly higher killing activity compared to untransduced T cells (Figure 2). Importantly, no significant difference in target cell lysis was observed for CAR T cells generated with or without Vectofusin-1 (Figure 2). This result demonstrates that Vectofusin-1 has no negative influence on the cytotoxicity of CAR T cells.

ΔLNGFR Is Present on the Surface of LV Particles and Transferred to Cells as Protein

To further characterize the off-target signals seen early after transduction, PBMCs were incubated with vector particles for 1 h at 4°C, followed by staining for LNGFR and flow cytometry analysis. Under

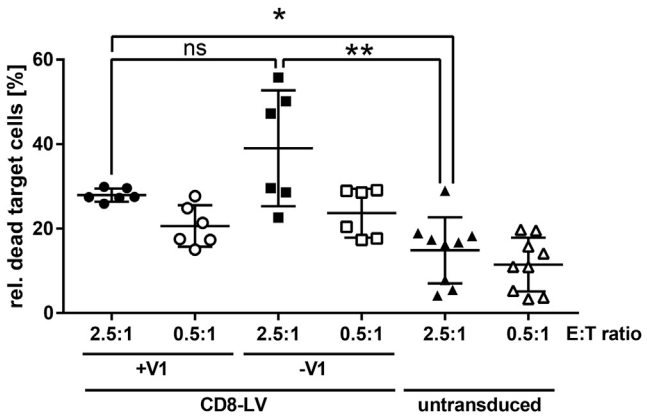

Figure 2. CAR T Cells Generated in the Presence of Vectofusin-1 Are Functional

A 4-h killing assay of Nalm-6 cells was performed to analyze the cytotoxic function of CAR T cells transduced with CD8-LV in the presence or absence of Vectofusin-1 (V1). CAR T cells were generated from activated PBMCs (either IL-2 or IL-7 and IL-15 stimulated) using 5 μL CD8-LV. After the determination of CAR expression, CAR T cells were mixed with CFSE-labeled Nalm-6 cells in different effector-to-target ratios as indicated and incubated for 4 h at 37°C. The relative percentage of dead target cells was calculated based on the amount of dead target cells measured by flow cytometry as a CFSE-positive, viability dye-positive cell population. The killing assay was performed twice in three technical replicates. Individual results as well as mean values, SDs, and p values are shown. One-way ANOVA was performed to determine significance. *$p < 0.05$, **$p < 0.005$; ns, not significant.

these conditions transduction and gene expression are not possible. Signals for ΔLNGFR were detected on receptor-positive and -negative cells in the presence or absence of Vectofusin-1 (Figure 3A; Figure S3). After incubation with CD4-LV, on average 5% of the CD4-negative cells and 77% of the CD4-positive cells were ΔLNGFR positive in the absence of Vectofusin-1. In the presence of the transduction enhancer, a significant increase of ΔLNGFR on CD4-negative cells was observed, while no significant difference was present for the CD4-positive compartment.

Similar results were observed for CD8-LV: ΔLNGFR signals were detected on off-target cells that were incubated with CD8-LV in the presence of Vectofusin-1 (on average 64%), which were significantly lower without Vectofusin-1 (on average 4.5%). Interestingly, on-target binding of CD8-LV to CD8-positive cells was also slightly increased in the presence of Vectofusin-1 (Figure 3A). For VSV-LV, no significant difference in ΔLNGFR signals upon vector binding was observed for either CD4- or CD8-positive cells (Figure 3A). These results demonstrated that target-receptor specificity was high for both, CD4-LV and CD8-LV, in the absence of Vectofusin-1, while the addition of the transduction enhancer led to the detection of off-target signals after pure vector binding. Interestingly, in the presence of Vectofusin-1, binding to off-target cells reached the same level as binding of vector particles to on-target cells for all LV variants (Figure 3A).

Next, we determined the kinetics of the appearance of ΔLNGFR signals on the surface of lymphocytes. For this purpose, vector particles and cells were incubated for 30 s and up to 60 min in the presence and absence of Vectofusin-1. Interestingly, the percentage of ΔLNGFR-positive cells was similar for each time point and was overall low in the absence and high in the presence of Vectofusin-1 (Figure 3B). Already after 30 s, ΔLNGFR-positive T cells were detected in the absence and more so in the presence of Vectofusin-1. These signals only slightly elevated over time in the presence of the transduction enhancer for both vector types (Figure 3B). Again, binding efficiency to CD4- and CD8-positive cells was comparable in the presence of Vectofusin-1, while only target cells were bound in the absence of the transduction enhancer (Figure S4).

To address whether ΔLNGFR was incorporated into vector particles, a ΔLNGFR-specific ELISA was performed. To quantify the amount of detected ΔLNGFR on vector particles, recombinant LNGFR protein was used as a standard. ΔLNGFR molecules were detected in all stocks of vector particles encoding for the CD19-CAR in conjunction with ΔLNGFR as reporter, but not in stocks encoding GFP (Table 1; Figure S5). Calculation of the total amount of ΔLNGFR molecules revealed between 169 and 350 ΔLNGFR molecules per particle (Table 1).

Next, we were interested whether vector particle binding to off-target cells required the envelope proteins used for LV pseudotyping. To study the role of the different envelope components, vector particles displaying either only the attachment protein, CD4-MV-H or CD8-NiV-G, or only the fusion protein, MV-F or NiV-F, were generated (Figure S6). In addition, bald particles harboring no envelope proteins were produced. The CD19-CAR-ΔLNGFR construct was used as transfer vector plasmid. Notably, bald particles harbored by far the highest amount of ΔLNGFR per particle (Table S3). Also particles carrying only one type of the paramyxoviral glycoproteins contained more ΔLNGFR than CD4-LV or CD8-LV, respectively (Table 1; Table S3). Vector particle binding to PBMCs was investigated after co-incubation for 1 h at 4°C in the presence or absence of Vectofusin-1. Vector binding was detected by staining for ΔLNGFR and flow cytometry analysis. As expected, vector particles without attachment proteins (bald LV, MV-F-LV, or NiV-F-LV) were very inefficient in cell binding in the absence of the transduction enhancer (Figure 4). In contrast, sole presentation of a retargeted attachment protein on vector particles (CD4-H-LV or CD8-G-LV) was sufficient to selectively detect ΔLNGFR on target cells in the absence of Vectofusin-1 (Figure 4). Interestingly, in the presence of Vectofusin-1, particle binding to, and thus ΔLNGFR detection on, PBMCs was enhanced for all vector types, without any preference for CD4- or CD8-positive cells (Figure 4), and it was comparable to the binding rates observed with CD4-LV and CD8-LV particles (Figure 3). These results demonstrate that Vectofusin-1-mediated attachment of particles to cells is independent of the used envelope proteins.

DISCUSSION

The data presented in this manuscript show that gene transfer into T lymphocytes by receptor-targeted vectors based on paramyxoviral glycoproteins can be significantly improved using the transduction

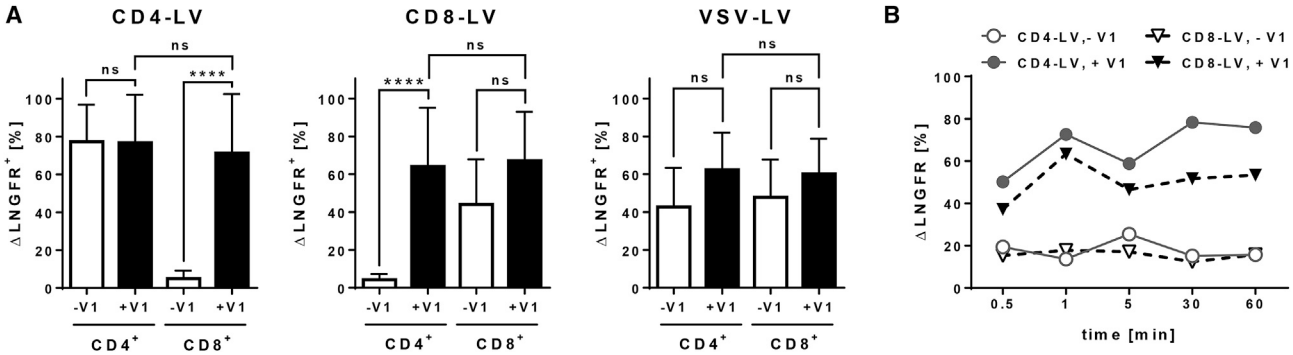

Figure 3. Vectofusin-1 Mediates ΔLNGFR Protein Transfer to Non-target Cells

IL-2-stimulated human PBMCs (1×10^5) were incubated with 10 μL of the indicated vector type in the presence or absence of Vectofusin-1 (V1) at 4°C. (A) After 1 h, the amount of ΔLNGFR on CD4- or CD8-positive cells was analyzed by flow cytometry. A representative set of dot plots is presented in Figure S3. Mean values and SDs of at least 8 replicates are shown. Statistical analyses were performed using unpaired t tests. *$p < 0.05$, ***$p < 0.0005$, ****$p < 0.0001$; ns, not significant. (B) Kinetic analysis of ΔLNGFR transfer to T cells. Incubation times ranged from 30 s to 60 min.

enhancer Vectofusin-1. Transduction levels achieved with CD4-LV and CD8-LV are now comparable to those possible with VSV-LV. In contrast to the latter, however, transduction with CD4-LV and CD8-LV is highly selective for the respective T lymphocytes. This high selectivity as well as target cell killing by the delivered CD19-CAR was not compromised by Vectofusin-1. In line with this, its influence on T cell phenotype, viability, and proliferation capacity of PBMCs was, if detectable at all, only marginal.

Since particle numbers in stocks of CD4-LV and CD8-LV were very similar to those of VSV-LV, their reduced gene delivery activity in the absence of Vectofusin-1 must be due to inefficient contact with the membrane of cultured cells. Vectofusin-1 was previously shown to assemble into nanofibrils that associate with viral particles, leading to their sedimentation and, thus, an increased local concentration at the cell surface.[12] This activity of Vectofusin-1 might explain why, already 30 s after vector addition to cells, a high amount of cell-bound vector particles was observed in the presence of the transduction enhancer. This effect was independent of the used envelope protein.

Previous studies investigating the influence of Vectofusin-1 on the transduction of hematopoietic stem and progenitor cells (HSPCs) suggested that Vectofusin-1 promotes both the adhesion and the fusion steps of VSV-LV and GALV-LV with the plasma membrane of $CD34^+$ cells.[9] The CD4- and CD8-targeted LVs applied in our study revealed that Vectofusin-1 mediates receptor-independent association of vector particles with cells, while in its absence only target receptor-positive cells were bound. Off-target signals present at early time points after transduction disappeared after prolonged cultivation. This was not due to death of transduced off-target cells but rather due to the removal and degradation of the cell-bound vector particles over extended cell cultivation. Notably, this forced binding of receptor-targeted vectors to target-receptor-negative cells by Vectofusin-1 was not accompanied with transduction (Figure 5). Thus, close vector-cell contact is not sufficient to activate the MV- or NiV-derived fusion proteins on the vector surface to induce fusion of the vector particle and host cell membranes.

Interestingly, while Vectofusin-1 was shown to be beneficial for the transduction of HSPCs with VSV-LV,[9] it did not promote gene transfer into T lymphocytes, as demonstrated by our own as well as previous work.[10] This discrepancy in T lymphocyte transduction by Vectofusin-1 might be explained by the different vector particle entry pathways. For CD4- and CD8-LV as well as for LV pseudotyped with glycoproteins from other retroviruses, such as GALV, host cell binding and fusion occur at the host cell plasma membrane in a pH-independent manner.[13–15] In contrast, VSV-LV is endocytosed after host cell binding before fusion with endosomal vesicles under low pH and release into the cytoplasm.[16] Although we cannot completely exclude that differences in the amounts of gene transfer active particles present in the different vector stocks contributed to the oppositional effect of Vectofusin-1 on VSV-LV compared to the receptor-targeted LVs, it is more likely that the different entry modes are more relevant. Accordingly, if closely related isomers of Vectofusin-1 were used for the transduction of HSPCs with VSV-LV, again, no or even a negative impact was observed, which was not the case for GALV-pseudotyped LVs.[10] Possibly, Vectofusin-1 reduces the endocytosis rate of cell surface-bound vector particles. In this respect, cell-specific differences in endocytosis were observed for nanoparticles dependent on the size and surface properties of those particles.[17–19] Although it is well established that the inhibition of endocytosis impairs gene delivery with VSV-LV but improves that of receptor-targeted LVs,[20] further studies will have to prove this hypothesis.

ΔLNGFR is not only frequently used as a reporter protein for CAR molecules but also as a cell tracking marker for other therapeutic transgenes.[21] In addition, ΔLNGFR could be used as a marker to analyze the behavior of vector cell attachment in the presence and absence of Vectofusin-1, as it is incorporated in the particle surface of all generated vectors, including VSV-LV. Incorporation of

Table 1. ΔLNGFR Is Present on the Surface of LV Particles

LV Vectors[a]	Particle (Number/mL)	ΔLNGFR in 0.1 μL Vector Solution (ng)	ΔLNGFR Molecules per Vector Particle
CD4-LV	1.40×10^{12}	3.01 ± 0.97[b]	288
	1.35×10^{12}	2.76 ± 0.39[c]	274
CD8-LV	4.85×10^{11}	0.64 ± 0.22[b]	175
	5.20×10^{11}	1.26 ± 0.04[c]	323
	1.13×10^{12}	1.43 ± 0.33[b]	169
VSV-LV	4.42×10^{11}	1.19 ± 0.39[b]	358
	1.18×10^{11}	2.08 ± 0.40[c]	239

[a]For CD4-LV and VSV-LV two and for CD8-LV three individual vector batches were assessed.
[b]n = 9
[c]n = 2

cytosolic proteins, like GFP, in retroviral vector particles is also possible and responsible for classical protein transfer, so-called pseudotransduction, shortly after vector cell fusion.[22] Upon detailed vector-cell-binding studies and incorporation analysis, we found that the presence of ΔLNGFR shortly after transduction is not due to classical protein transfer. Detection of ΔLNGFR as soon as 30 s after co-incubation of PBMCs with vector particles at 4°C argues against membrane fusion having mediated the transfer of ΔLNGFR from the packaging cells to the PBMCs. Rather, protein transfer was mediated by cell-bound vector particles (Figure 5). This effect was enhanced by Vectofusin-1, which prolonged the period that vector particles are associated with cells without membrane fusion, at least on receptor-negative cells.

The incorporation of heterologous viral glycoproteins but also non-viral transmembrane proteins into LV particles expressed in the producer cell line has been described before.[20,23,24] For envelope proteins derived from viruses, such as MV or NiV, cytoplasmic tail truncations have to be performed to allow efficient incorporation into LV particles.[25,26] MV F or NiV F proteins, e.g., have just 5–6 residual C-terminal amino acids when used for pseudotyping.[5,25] This is similar to the ΔLNGFR construct in which the entire cytoplasmic domain has been deleted except for 8 amino acids. This phenomenon is also known for HIV-1 or other enveloped viruses, which can acquire a variety of host cell proteins during particle formation.[27]

In principle, the incorporation of transmembrane proteins, which are not encoded on the transgene but expressed during vector production into fusion-competent LV particles, offers the opportunity to transiently transfer proteins into the host cell membrane upon vector fusion. This technique might be used to equip cells for a short term with additional receptors or signaling molecules. Interestingly, our data also suggest that CARs may become incorporated into the envelope of LV particles. However, detection of the CD19-CAR molecule in our vector stocks was only possible by western blot analysis, suggesting a lower incorporation rate than for ΔLNGFR (Figure S7). Given that the CAR contains a long cytoplasmic tail comprising the 4-1BB and CD3zeta domains (154 amino acid [aa]), this was not surprising. However, it is a possibility with potentially also extensive consequences for the production of CAR-encoding LV particles.

As a therapeutic outlook, CD8- and CD4-targeted LV particles may facilitate and improve the generation of CAR T cells in the future, since only the desired cell population of choice will be transduced.[4] T cell purification steps as they are currently typically applied during the manufacturing process of CAR T cells may then become obsolete. Beyond that, however, also other therapeutic applications in the genetic modification of lymphocytes may profit from targeted gene delivery to lymphocyte subsets.[28]

MATERIALS AND METHODS

Ethics Statement

The research has been approved by the Ethics Committee of the University Hospital Frankfurt, Germany. Written informed consent was obtained from each donor.

Cell Lines and Primary Cells

HEK293T (ATCC: CRL-11268) cells were grown in DMEM (Biowest, Nuaillé, France), supplemented with 10% fetal calf serum (FCS) (Biochrom, Berlin, Germany) and 2 mM L-glutamine (Sigma-Aldrich, Munich, Germany). Molt.4.8 and Nalm-6-EBFP-luc cells were kindly provided by Dr. Martin Pule, University College of London (subsequently called Nalm-6 cells), and A301 cells were grown in RPMI 1640 (Biowest, Nuaillé, France), supplemented with 10% FCS and 2 mM L-glutamine.

Human PBMCs were isolated from the fresh blood of healthy donors or buffy coats purchased from the German blood donation center (DRK-Blutspendedienst Hessen, Frankfurt, Germany). 1×10^7 PBMCs/6-well were activated for 72 h (IL-2 stimulation) or 48 h (IL-7 and IL-15 stimulation) in RPMI 1640, supplemented with 10% FCS, 2 mM L-glutamine, 0.5% streptomycin and penicillin, 25 mM HEPES (Sigma-Aldrich, Munich, Germany), 3 μg/mL anti-CD28 antibody (clone 15E8), 1 μg/mL pre-coated anti-CD3 antibody (clone OKT3), and 100 U/mL IL-2 or 25 U/mL IL-7 and 50 U/mL IL-15. During and following transduction, cells were cultivated in the same medium without CD3 antibody and CD28 antibody. All cytokines and antibodies were from Miltenyi Biotec (Bergisch Gladbach, Germany).

LV Generation, Concentration, and Titration

Vector particles were generated by transient transfection of HEK293T cells using polyethylenimine (PEI; Sigma-Aldrich, Munich, Germany) and second-generation packaging plasmids, as described before.[5,6,29] In brief, 1 day before transfection, $1.5–2 \times 10^7$ cells were seeded into a T175 flask. In total, 35 μg DNA was added to 2.3 mL DMEM without additives and combined with 2.2 mL DMEM containing 140 μL 18 mM PEI solution. The transfection solution was mixed and incubated for 20 min at room temperature. The cell medium was replaced by 10 mL DMEM, supplemented with 15% FCS and 3 mM L-glutamine, before the transfection

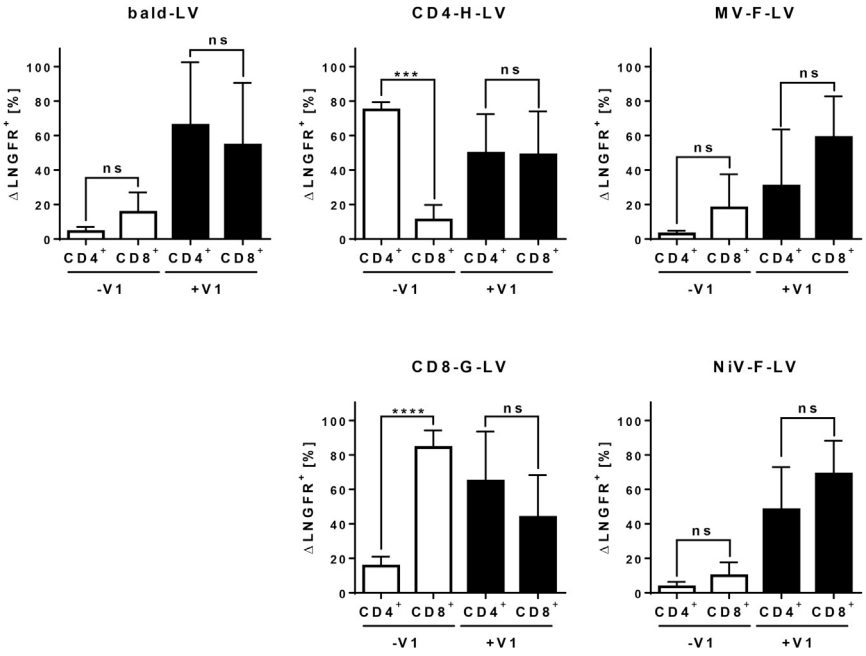

Figure 4. Transfer of ΔLNGFR in the Absence of Envelope Glycoproteins

10-μL stocks of bald LV particles, CD4-LVs without MV F protein (CD4-H-LV) or H (MV-F-LV), or CD8-LV without NiV F protein (CD8-G-LV) or G (NiV-F-LV) were added to IL-2-stimulated PBMCs (1×10^5 cells) in the presence or absence of Vectofusin-1 (V1). After 1 h at 4°C, the amount of ΔLNGFR on CD4- and CD8-positive cells was analyzed by flow cytometry, respectively. The binding assay was performed at least 6 times showing mean values, SDs, and p values. Statistical analyses were performed using unpaired t tests. ***$p < 0.0005$, ****$p < 0.0001$; ns, not significant. A graphical explanation for vector composition is provided in Figure S6.

solution was added to HEK293T cells. Then 4–6 h later, the medium was replaced by DMEM with 10% FCS and 2 mM L-glutamine. At 2 days after transfection, the cell culture supernatant was collected, and released vector particles were concentrated over a 20% sucrose cushion at 4,500 × *g* for 24 h. The supernatant was discarded, and pellets were resuspended in 60 μL Dulbecco's PBS (Lonza, Cologne, Germany) per T175 flask.

Plasmid ratios for the generation of CD4- and CD8-receptor-targeted vector particles as well as particles pseudotyped with the VSV glycoprotein G or two modified baboon envelope glycoproteins were described previously[5,6,29] and can be found in Table S2. The transfer plasmid used for packaging encodes for a polycistronic expression cassette containing the CD19-CAR sequence,[11] followed by a P2A element-linked ΔLNGFR under the control of a phosphoglycerate kinase (PGK) promoter. Notably, based on the co-expression of ΔLNGFR and the CAR construct, detection of ΔLNGFR can be used as surrogate marker for the expression of CAR molecules on the cell surface. All vectors were titrated on A301 (CD4-LV) or Molt.4.8 (CD8-LV, CD4-LV, VSV-LV, and BaEV-LV) cells, respectively, as described previously,[5] using a LNGFR-specific antibody for detection. Particle numbers were determined using an HIV-1 p24 antigen ELISA Kit (ZeptoMetrix, Buffalo, NY, USA), according to the manufacturer's instructions, by assuming that there is an average of 2,000 molecules of p24 present per viral particle[30] and that the molecular mass of p24 is 25.587 kDa for HIV-1.[31] This equates to 1.18×10^{10} viral particles/1 μg p24.

PBMC Transduction

4×10^4 activated PBMCs were seeded in a 96-well plate in 100 μL of the normal culture medium containing the appropriate cytokines. The transduction enhancer Vectofusin-1 (Miltenyi Biotec, Bergisch Gladbach, Germany) was used according to the manufacturer's instructions. In brief, Vectofusin-1 was diluted in RPMI without additives to a final concentration of 40 μg/mL in 50 μL. 1, 2.5, or 5 μL vector stock was filled up with RPMI without additives to the same volume. For vector particle numbers refer to Table S1. Diluted Vectofusin-1 and diluted vector solutions were mixed and incubated for 5–10 min at room temperature. Subsequently, the transduction mix was added to the cells. When the transduction was performed without Vectofusin-1, 1, 2.5, or 5 μL of each vector was diluted in 100 μL RPMI without additives and directly added to the cells. For spinfection, centrifugation at 850 × *g* and 32°C for 90 min was performed. Afterward, the PBMCs were gently resuspended by pipetting. PBMCs received fresh complete medium every other day after transduction until cell analysis. Notably, after initial activation, no re-stimulation with extraneous antigen or antibody cocktail was performed. Transgene expression was determined by flow cytometry 7–13 days post-transduction if not specified otherwise. Cytotoxic activity was analyzed in a flow cytometry-based cytotoxicity assay 14 or 15 days post-transduction.

Cytotoxicity Assay

Cytotoxic activity of CD8-LV-transduced, CD19-CAR-expressing PBMCs was determined using CD19-positive Nalm-6 cells. To reduce activity of the PBMCs and enhance their cytotoxic function upon antigen presentation, the cytokine treatment of transduced PBMCs was reduced by half 11 or 12 days post-transduction. Then 48 h later, the cells were analyzed for ΔLNGFR expression by flow cytometry before they were used in killing assays on the next day.

5×10^4 or 1×10^4 CAR-positive T cells or untransduced T cells were coincubated with 1×10^4 Nalm-6 cells, which were previously labeled with CellTrace CFSE (Thermo Fisher Scientific), according to the manufacturer's instructions. To compensate for variations of transduction efficiency, the effector cell population was normalized to an absolute T cell number by the addition of untransduced PBMCs. Nalm-6 cells cocultured without effector cells were used as a control.

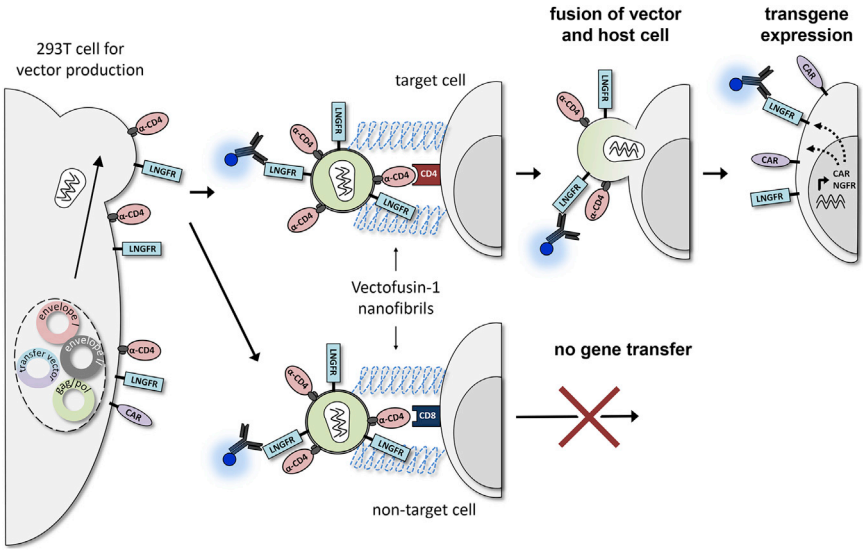

Figure 5. Working Model for the Transfer of ΔLNGFR from Packaging Cells to Target and Non-target Cells

Potential mechanism of action of ΔLNGFR protein transfer illustrated for CD4-LV. For vector production, the envelope plasmids encoding for CD4-H and MV-F (shown in red and gray), the transfer plasmid encoding for CAR-ΔLNGFR (shown in blue to lilac), and the packaging plasmid (shown in green) are transfected into 293T cells. Upon protein expression, the encoded transmembrane proteins translocate to the 293T plasma membrane. This way, CD4-H and MV-F as well as ΔLNGFR, get efficiently incorporated into the viral surface of the budding vector particle. In the presence of Vectofusin-1, vector particles bind to CD4-positive (target) as well as CD4-negative (non-target) cells due to the stickiness of the transduction enhancer. Antibody staining for ΔLNGFR detects bound vector particles on receptor-positive as well as receptor-negative cells. Stable gene transfer, however, is restricted to target cells (top).

Coincubation was performed for 4 h at 37°C in a total volume of 200 µL RPMI medium, supplemented with 10% FCS, 1% L-glutamine, 0.5% streptomycin/penicillin, and 25 mM HEPES without cytokines. Afterward, the cell mixture was stained for dead cells using the fixable viability dye eFluor780, according to the manufacturer's instructions, and analyzed by flow cytometry. The percent of dead target cells was analyzed as the CFSE-positive, viability dye-positive cell population. The background level of dead target cells, incubated without T cells, was subtracted to calculate the relative amount of dead target cells.

Binding Assay

10 µL of various LV particles was diluted in 40 µL medium without additive and mixed with 2 µL Vectofusin-1 (final concentration 20 µg/mL). Afterward, the mix of vector and Vectofusin-1 was added to 1×10^5 IL-2-stimulated PBMCs/96-well. The plate was incubated for 1 h at 4°C. Subsequently, cells were washed twice with washing buffer (PBS, 2% FCS, and 0.1% NaN_3), before vector attachment was quantified by flow cytometry analyzing ΔLNGFR detection.

Flow Cytometry

Flow cytometry analysis was performed using the MACSQuant Analyzer 10 (Miltenyi Biotec, Bergisch Gladbach, Germany) flow cytometer, and data were analyzed by FCS Express 6 (*De Novo* Software, Glendale, CA, USA). To determine the percentage of transduced cells or cell-bound vector particles, staining of up to 1×10^5 PBMCs was performed. Viable cells were detected using the fixable viability dye eFluor780 (Life Technologies, Darmstadt, Germany), according to the manufacturer's instructions. To further characterize the PBMCs, cells were stained with a CD4-specific antibody (clone VIT4) labeled with VioBlue or phycoerythrin (PE)-Vio770 and a CD8-specific antibody (clone BW135/80) labeled with VioGreen or allophycocyanin (APC). The reporter protein ΔLNGFR, which is co-expressed with the CD19-CAR, was detected using a PE-labeled anti-LNGFR antibody (clone ME20.4-1.H4). After staining, cells were washed twice with washing buffer (PBS, 2% FCS, and 0.1% NaN_3) and fixed with PBS containing 1% formaldehyde prior to analysis. All antibodies were from Miltenyi Biotec (Bergisch Gladbach, Germany).

ELISA

A flat-bottom, 96-well MAXISORP microplate (Thermo Fisher Scientific, Darmstadt, Germany) was coated with 0.1 µL of different LV particles in 100 µL PBS-T (PBS and 0.05% Tween-20). After blocking the wells with PBS-B (PBS and 0.5% BSA), the biotinylated anti-LNGFR antibody (clone ME20.4-1.H4, Miltenyi Biotec, Bergisch Gladbach, Germany) was added. To quantify the amount of ΔLNGFR on the vector particles, wells were incubated with horseradish peroxidase (HRP)-conjugated streptavidin (Jackson ImmunoResearch Laboratories, Ely, UK) diluted 1:500 in PBS-T (PBS and 1% Tween-20). Thereafter, 1-Step Ultra TMB-ELISA substrate (Thermo Fisher Scientific, Darmstadt, Germany) was used to develop the color reaction. At the end, 1 N H_2SO_4 solution was added to stop the reaction, and measurement was performed at 450 nm and 630 nm by an ELISA reader. A serial dilution from 0 to 12.5 ng human recombinant NGFR (Sino Biological, Wayne, PA, USA) was used to draw standard curve. All protein or antibody incubation steps were performed for 1 h at room temperature, followed by washing the wells three times with PBS-T. Each experiment was repeated up to nine times with technical duplicates.

Statistical Analysis

Statistical analyses were performed with Prism 7 software (GraphPad, San Diego, CA, USA). Tests for statistical significance used the unpaired two-tailed Student's t test, Mann-Whitney test, or one-way ANOVA (Tukey's multiple comparisons test) as indicated; p values less than 0.05 were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2019.03.003.

AUTHOR CONTRIBUTIONS

A.J. and L.K. designed and performed experiments. L.K., A.J., J.H., and C.J.B. evaluated data. T.S. and I.C.D.J. contributed protocols and reagents and to writing of the manuscript. C.J.B. and J.H. conceived and designed the study, acquired grants, supervised work, and wrote the manuscript.

CONFLICTS OF INTEREST

C.J.B. is listed as an inventor on patents describing receptor-targeted LVs. T.S. and I.C.D.J. are full-time employees of Miltenyi Biotec GmbH. All other authors declare no conflicts of interest.

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