Quantifying gene expression in individual cells can substantially improve our understanding about complex genetically engineered cell products such as chimeric antigen receptor (CAR) T cells. Here we designed a single-cell RNA sequencing (scRNA-seq) approach to monitor the delivery of a CD19-CAR gene via lentiviral vectors (LVs), i.e., the conventional vesicular stomatitis virus (VSV)-LV and the CD8-targeted CD8-LV. LV-exposed human donor peripheral blood mononuclear cells (PBMCs) were evaluated for a panel of 400 immune response-related genes including LV-specific probes. The resulting data revealed a trimodal expression for the CAR and CD8A, demanding a careful distribution-based identification of CAR T cells and CD8+ lymphocytes in scRNA-seq analysis. The fraction of T cells expressing high CAR levels was in concordance with flow cytometry results. More than 97% of the cells hit by CD8-LV expressed the CD8A gene. Remarkably, the majority of the potential off-target cells were in fact on-target cells, resulting in a target cell selectivity of more than 99%. Beyond that, differential gene expression analysis revealed the upregulation of restriction factors in CAR-negative cells, thus explaining their protection from CAR gene transfer.

In summary, we provide a work allowing a better understanding and improvements.

INTRODUCTION

In the time since Gross et al. generated genetically modified T cells to express a chimeric antigen receptor (CAR), many advances have been made including the development of second-generation CARs, which has led to impressive clinical benefit, particularly for patients suffering from B cell lymphoma and acute lymphoblastic leukemia (ALL), through targeting the CD19 antigen.1-5 Further improvements, aiming at on one hand the expansion of CAR T cells to the treatment of other cancer entities including solid tumors and on the other hand the simplification of the manufacturing process, are the focus of ongoing research. Especially the complex manufacturing process, which results in a patient-specific, highly complex mixture of various T cell types, requires novel single-cell-based analysis tools to allow a better understanding and improvements.
~12 days. The first half includes T cell activation, incubation with vector particles, followed by vector entry and genomic integration, and finally the expression and cell surface transport of the CAR. The second half is mainly expansion of the CAR T cells to reach sufficient numbers for transplantation. The transduction process contains many unknowns on the cellular and molecular levels, such as why certain T cells become CAR-positive and others do not or what consequences the vector particle exposure has for T cells. Questions like this can now be addressed by single-cell RNA sequencing (scRNA-seq). However, as an emerging tool in the field, only a few studies have analyzed CAR T cells via scRNA-seq so far. These studies focused mainly on the diversity of CAR T cell phenotypes in pre-infusion products and correlated these to activities in patients or investigated the consequences of different CAR signaling domains and antigenic stimulation. None of these studies has looked closely into the process of LV-mediated transduction. Our scope was to establish a methodology that thoroughly investigates differences between transduced and non-transduced cells during product generation and profiles the consequences of different vector types applied. We have set up a nanowell-based scRNA-seq approach for LV-mediated CAR delivery making use of the high selectivity of CD8-LV for CD8+ T cells. In particular, we have performed a targeted gene amplification analysis of untransduced CD8-LV- and VSV-LV-generated CAR T cell products expanded for a short period of time. The CAR T cells were detected via 3’-targeted amplification of the transgene with customized primers annealing within the WPRE region. This approach enabled us to accurately associate changes in cellular gene expression caused by the CAR and/or exposure to the LV particles and demonstrate that CD8-LV has near-perfect selectivity for its target cells.

RESULTS
Setting up the system
Human donor peripheral blood mononuclear cells (PBMCs) were used as the cell source for CAR T cell generation. The cells were activated and then incubated with CD8-LV or VSV-LV or left untransduced as control. After 6 days of cultivation in the presence of interleukin (IL)-7/IL-15, the cells were analyzed by flow cytometry and processed for scRNA-seq (Figure 1A). Vector doses were optimized such that a similar fraction of CD8+ CAR T cells was obtained with both vector types, while also leaving a significant fraction of cells CAR-negative (Table 1). According to flow cytometry, CD8-LV had generated 22.5% CAR T cells, all of which were CD8-positive. Thus, about one-third of the CD8 cells had been converted to CAR T cells (Figure 1B). With VSV-LV, similar fractions of CD8+ and CD4+ CAR T cells were obtained (Figure 1B). Thus, CAR T cell generation had worked out as expected. For scRNA-seq, we chose a microwell-based system for single-cell isolation and processing. Rather than utilizing a whole transcriptome approach, we opted for a targeted gene panel covering 399 human genes relevant for immune response to achieve high resolution of the sequencing results, thus allowing highly sensitive detection of differentially expressed genes. To detect CAR-positive T cells by scRNA-seq, the WPRE element adjacent to the 3' LTR was identified as being at an ideal distance from the poly(A) tail to allow sensitive detection of mRNA transcribed from the integrated vector genome (Figure 1C).
we used as thresholds for gating of the populations (Figure S3). For CD8Ahigh T cell-mediated killing.14 The absence of B cells in the transduced samples thus confirmed the peaks of the modes as well as the antimodes, which we used as thresholds for gating of the populations (Figure S3). For CD8Ahigh cells, this resulted in the most confined population of CD8 cells in UMAP plots (Figure 2B). On the other hand, CD8Alow and CD8Aneg cells comprised the rest of immune cell populations, including CD4 cells. While less pronounced, a similar tendency for a trimodal distribution was also observed for CAR T cells as determined by the CAR transgene expression (Figure 2B; Figure S3).

Next, we compared these data to the flow cytometry results generated from identical cell samples. Interestingly, we observed concordant CAR T cell frequencies between flow cytometry and scRNA data when we subset for CARhigh and CD8Aneg in the scRNA plots. Then, 23.2% CAR T cells were identified by scRNA with CD8-LV (Figure 2C). Notably, our subsetting approach showed that 97.5% of CARhigh cells were CD8Ahigh cells in the CD8-LV sample (Figure 2D) and 2.5% of CARhigh (in total 59 cells) were CD8Aneglow. These potential off-target cells consisted of 54.2% γδ T cells, 6.8% CD4/CD8 double-positive cells, 3.4% NKT cells, and 25.4% CD4 cells (Table 2; Figure S4). Of all these, only 28.8% were CD8Areg and CD8Dreg double-negative cells (Table 2). Importantly, a close match in the frequencies of CD8 and CD4 CAR T cells between flow cytometry and scRNA analysis was also observed for CAR T cells generated with VSV-LV (Figure 2C), which nicely validated our gating strategy.

The CARlow-expressing cells consisted of all identified immune cells, including CD8, CD4, γδ T, and NKT cells (Figures 2A and 2B). Comparison of their expression profile with that of CARreg cells revealed no significant differences between these two cell populations, whereas significant differences were obvious between CARlow and CARhigh cells (Figure S5). We therefore combined them with the CARreg cells in one group (CARreglow) for further analysis. Notably, CARlow cells were substantially different from untransduced cells, which had not been exposed to vector particles (Figures S5A and S5B).

### Differentially expressed genes in the CD8+ populations

By analyzing the isolated cell subsets of CD8 T cells, as described above, we observed alterations of gene expression profiles across the populations. The differentially expressed genes for each group identified by expression analysis are shown in the heatmap plots in Figure 3A. Intriguingly, we observed differences for each group identified by expression analysis in the untransduced control sample (Figure 2A; Figure S2D). Presence of residual B cells during the first days of PBMC cultivation was expected, and their absence was previously demonstrated to be due to CAR T cell-mediated killing.14 The absence of B cells in the untransduced control samples thus confirmed the killing activity of the generated CAR T cells.

When we plotted normalized gene expression data for CD8A-expressing cells, a trimodal distribution of CD8Aneglow cells was within the expected range as determined by fragment analysis (Figure S1A). Low-quality cells (185 in total), which passed the Seven Bridges pre-processing filtering steps but showed too low or too high content of targeted genes and RNA molecule numbers, were eliminated from the analysis (Figure S1B). For the initial computational analysis, post-processed samples were merged in one Seurat object and principal component analysis was performed (Figures S1C and S2A). Unsupervised clustering identified 14 clusters (Figure S2B). The expression of the major T cell-associated markers was analyzed across the clusters, and, subsequently, clusters were merged depending on their identity (Figure S2C). The expected cell types such as CD4, CD8, γδ T cells, and some mixed population of natural killer T (NKT) cells were present, while transduced and untransduced samples showed similar cellular compositions (Figure 2A; Figure S2D). The only exception was a small cluster of B cells in the untransduced control sample (Figure 2A; Figure S2D). Presence of residual B cells during the first days of PBMC cultivation was expected, and their absence was previously demonstrated to be due to CAR T cell-mediated killing.14 The absence of B cells in the transduced populations thus confirmed the killing activity of the generated CAR T cells.

The majority of the differentially expressed genes, 130 out of 151 either up- or downregulated, were identified when comparing untransduced with CARhigh cells (Figure 3C, left). From these, 70 genes

| Sample | MOI | Particles/cell | VCN | Viability |
|--------|-----|---------------|-----|-----------|
| Untransduced | 1.1 | n.d. | n.d. | 62.7% |
| CD8-LV | 0.045 | 12 × 10^3 | 0.91 ± 0.71 | 69.3% |
| VSV-LV | 33.3 | 5.8 × 10^3 | 3.94 ± 1.76 | 74.6% |

n.d., non-detectable.

aVCN was measured in replicates from different samples generated using different batches of LVs on three donors, including the batch and donor used in the scRNA-seq experiment (untransduced n = 3, CD8-LV n = 7, VSV-LV n = 14) (mean ± standard deviation). bDetermined by BD Rhapsody scanner upon staining with calcein AM and DRAQ7, before single-cell seeding. cAs determined on MOLT cells. dVCN was quantified on DNA extracted from whole samples and extrapolated on CD8 cells with the pool of primers from the Immune Response Panel (BD Biosciences) and the customized WPRE primers. Quality analysis of the generated cDNA libraries was within the expected range as determined by fragment analysis (Figure S1A). Low-quality cells (185 in total), which passed the Seven Bridges pre-processing filtering steps but showed too low or too high content of targeted genes and RNA molecule numbers, were eliminated from the analysis (Figure S1B). For the initial computational analysis, post-processed samples were merged in one Seurat object and principal component analysis was conducted, choosing the first 40 components for further analysis and uniform manifold approximation and projection (UMAP) plotting (Figures S1C and S2A). Unsupervised clustering identified 14 clusters (Figure S2B). The expression of the major T cell-associated markers was analyzed across the clusters, and, subsequently, clusters were merged depending on their identity (Figure S2C). The expected cell types such as CD4, CD8, γδ T cells, and some mixed population of natural killer T (NKT) cells were present, while transduced and untransduced samples showed similar cellular compositions (Figure 2A; Figure S2D). The only exception was a small cluster of B cells in the untransduced control sample (Figure 2A; Figure S2D). Presence of residual B cells during the first days of PBMC cultivation was expected, and their absence was previously demonstrated to be due to CAR T cell-mediated killing.14 The absence of B cells in the transduced samples thus confirmed the killing activity of the generated CAR T cells.
were shared between the comparisons of untransduced with either CAR\textsuperscript{high} or CAR\textsuperscript{neg/low}, 33 genes were shared in all of the comparisons, including CAR\textsuperscript{neg/low} with CAR\textsuperscript{high}, whereas 43 were unique, comprising the biggest difference in that group of comparison. On the other hand, by comparing the whole CD8 cell groups between control and CD8-LV or VSV-LV, we found that most of the differentially expressed genes were shared between the vectors, when comparing control with transduced samples (100 out of 149), whereas 29 genes were commonly identified in all of the three comparisons (Figure 3C, right). Gene set enrichment analysis with the Gene
expression of interferon-lating factor (GM-CSF) (Figure 4A).

We found increased levels of CAR-mediated activities (Figure 4A), which can trigger both T cell apoptosis as well as a TH1-phenotype as exemplified by upregulated IL-12 receptor (IL12RB2) (Figure 4B) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (CSF2) (Figure 4C) as well as an unaltered expression of interferon-γ (IFNG) and STAT4 (Figure S8). Unchanged expression of TNF (Figure S8) and reduced levels of FAS (Figure 4D), which can trigger both T cell apoptosis as well as increased levels of IER3, are well in line with an anti-apoptotic protein profile in CARhigh cells (Figure 4D). The expression of several exhaustion and immune checkpoint markers (ENTPD1, LAG3, HAVCR2, LAT2, CTLA4) (Figure 4E) accompanied by the increase in co-stimulatory markers (ICOS, CD70, CD27) (Figure 4A) indicated early exhaustion of the CARhigh CD8 cells, potentially as a result of CAR tonic signaling. Finally, we found the mitogen-activated protein (MAP) kinase phosphatase-2 (DUSP4), which is known to inactivate MAP and extracellular signal-regulated kinase (ERK) kinases as well to promote TH1 response, to be significantly upregulated in CARhigh cells (Figure 4F).

Besides being caused by CAR activities, differences between CARneg/low and CARhigh cells may have been caused by intrinsic factors present in particular cells preventing proper transduction by the LVs. Among these is the IL-2 receptor alpha subunit (IL2RA), which we found to be downmodulated in the CARneg/low cells not only compared to CARhigh cells but also compared to untransduced cells (Figure 4A), suggesting that their low activation level contributed to being protected from gene transfer. In line with this, PIK3IP1 implicated in inhibition of T cell activation was upregulated in the CARneg/low population (Figure 4A), further confirming the low activation status of these cells. Although only slightly upregulated, both interferon-induced transmembrane proteins covered by the panel, IFITM2 and IFITM3, were significantly higher in CARneg/low cells than in untransduced or CARhigh cells (Figure 4F).

Besides changes in gene expression in the CARhigh cells only, we identified several genes that were up- or downregulated in both CARhigh and CARneg/low cells compared with untransduced cells. Thus, these changes were most likely due to the exposure to LV vectors. This referred to the negative regulators of proliferation and inhibitors of T cell activation CD37 and PIK3IP1, respectively (Figure 4A), as well as co-stimulatory and phenotype markers such as CD27, CD7, CD62L (SELL), and TC7 (Figures 4A and 4G). Typical markers for apoptosis induction (CASP5) (Figure 4D) and exhaustion (LIF, C10orf54) (Figure 4E) were also induced upon exposure to LV particles. Of particular interest in this context are antiviral response factors. Indeed, Btg1, a cell cycle regulator, the CD11c gene (ITGAX), IFITM3, and in part also IRF4 were all upregulated upon exposure to LVs (Figures 4F and 4H).

Looking closer into particular genes of CD8+ cells that show a significant level of up- or downmodulation between the different settings, we grouped them into topics related to CAR activities and vector-host interactions (Figure 4). With respect to T cell activation, upregulation of CD70, ICOS, and JUNB (part of the AP-1 transcription factor) selectively in the CARhigh cells is most likely a result of the CAR-mediated activities (Figure 4A). CARhigh CD8 cells favored a TH1-phenotype as exemplified by upregulated IL-12 receptor (IL12RB2) (Figure 4B) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (CSF2) (Figure 4C) as well as an unaltered expression of interferon-γ (IFNG) and STAT4 (Figure S8). Unchanged expression of TNF (Figure S8) and reduced levels of FAS (Figure 4D), which can trigger both T cell apoptosis as well as increased levels of IER3, are well in line with an anti-apoptotic profile in CARhigh cells (Figure 4D). The expression of several exhaustion and immune checkpoint markers (ENTPD1, LAG3, HAVCR2, LAT2, CTLA4) (Figure 4E) accompanied by the increase in co-stimulatory markers (ICOS, CD70, CD27) (Figure 4A) indicated early exhaustion of the CARhigh CD8 cells, potentially as a result of CAR tonic signaling. Finally, we found the mitogen-activated protein (MAP) kinase phosphatase-2 (DUSP4), which is known to inactivate MAP and extracellular signal-regulated kinase (ERK) kinases

Table 2. Identification of CARhighCD8Aneg/low cells after CD8-LV mediated transduction

| Cell type          | Marker genes                  | Cell number |
|--------------------|-------------------------------|-------------|
| CD4 T cells        | CD4neg/CD8Bneg                | 15          |
|                    | CD8Aneg                       | 7           |
|                    | CD8Bneg                       | 8           |
| CD8 T cells        | CD8Bneg                       | 5           |
|                    | CD8Aneg                       | 5           |
| CD4/CD8 T cells    | CD4neg/CD8Bneg                | 4           |
|                    | CD8Aneg                       | 2           |
|                    | CD8Bneg                       | 2           |
| NKT                | CD8neg/CD8Alow                | 1           |
|                    | CD8Bneg/CD8Aneg               | 2           |
|                    | TRDCneg                       | 32          |
| γδ T               | CD8Bneg/CD8Alow               | 7           |
|                    | CD8neg/CD8Aneg                | 7           |
|                    | CD8Bneg/CD8Aneg               | 9           |
| Remaining cells    | CD8Aneg/CD4neg/CD8Aneg        | 1           |
|                    | CD8Aneg                       | 1           |

Numbers in bold indicate total counts of the subfractions listed below, respectively.

Ontology (GO) Biological Process database revealed enrichment of genes especially related with cytokine-mediated signaling, T cell activation and immune response, and regulation of cell proliferation or apoptosis (Figure S7).
conclusions drawn from the data. In this study, we have therefore started to follow early time points in CAR T cell generation, especially focusing on the transduction process with the final goal to improve the identification of the various subpopulations and to better understand why particular cells are becoming CAR T cells and others not.

Key for our analysis was the use of CD8-LV, which is known to be highly selective for the CD8+ T cells in human PBMCs. This facilitated identification of the transduced CD8+ T cells and their comparison to CAR T cells generated with the conventional VSV-LV. We decided to perform a targeted gene amplification and sequencing...
analysis covering ~400 genes to establish our methodological approach. In particular, comparing to a typical whole transcriptome experiment requiring 50,000 reads/cell, we managed to efficiently sequence with ~4,000 reads/cell without compromising the sequencing saturation and depth. This allowed us to increase the processed cell number by up to 10-fold compared to a typical whole transcriptome analysis (WTA) run and yet have a high resolution of the expressed genes.

Our approach identified a trimodal distribution not only in the expression of the CAR gene but also for CD8A. Notably, previous studies have not described this. Since the expression profiles of CARlow and CARnegT cells were basically identical, they were merged for the downstream analyses (Figure S2A). The situation appears more complicated with the CD8Alow population. Indeed, the PBMCs used in this study include besides T lymphocytes also natural killer (NK) cells and dendritic cells (DCs). However, almost all CD8Alow cells were CD3+, thus rather excluding the presence of NK cells and DCs as explanation (Figure S2D). Instead, it is likely that transient fluctuations in mRNA levels that do not immediately convert in loss of the encoded proteins have accounted for this.

In the current study, we have evaluated the selectivity of a receptor-targeted vector by both flow cytometry and scRNA-seq, thus on protein and mRNA levels. The obtained data on one hand were in perfect agreement with flow cytometry-based selectivity analysis and on the other hand confirmed the high, near-absolute selectivity of CD8-LV for its target cells (Figures 1B and 2D). Of the 59 (2.5%) potential off-target cells, 9 were in fact CD8+ or double-positive cells based on the expression of CD8B. Of the remaining 50 cells, 32 were γδ T cells, of which only 9 express neither CD8A nor CD8B; 2 cells were NKT cells; 15 were CD4 cells, of which 8 were negative for CD8 markers whereas 7 had CD8Alow expression (Table 2). This leaves just 17 cells that could be true off-target cells and thus an on-target rate of 99.28%. However, even these few cells could be target cells when considering the above-mentioned mRNA fluctuation. Combining barcoded antibody staining with expression profiling could be a straightforward next step to clarify this issue.

Our study revealed interesting differences in expression of particular genes between the groups. The data revealed transcriptomic alterations upon exposure to vector particles and expression of the CAR as the result of proper gene delivery and transduction. Thus, the exposure of cells to any of the two LVs resulted in alterations of their gene expression profiles, regardless of whether the cells were eventually properly transduced by the vector or not. These findings suggest that under the given experimental conditions exposure of T cells to LV particles results in stronger gene expression profile alterations than presence of the CAR. While CD8+ CARhigh cells exhibited an activated TH1 phenotype and an overall profile well in accordance with that observed in previous studies (Figure 4), CD8 CARneglow cells expressed genes that potentially restricted cell viability, phenotype, and viral entry. These cells had upregulated genes involved in inhibition of T cell activation (PIK3IP1) and proliferation (CD37, BTG1) as well as promoting pyroptosis (CASP5), a type of programmed cell death resulting in inflammatory cytokine release (Figures 4A, 4D, and 4H). Particularly remarkable was the observation that two restriction factors (IFITM2, IFITM3) directly implicated in preventing viral entry were significantly increased in those cells that remained CARneg despite having been exposed to LVs (Figure 4F). The two interferon-induced transmembrane proteins expressed from IFITM2 and IFITM3 reside in endosomal compartments and inhibit the fusion of the viral envelope with the endosomal membrane, a process characteristic for the pH-dependent entry pathway of VSV-LV. IFITM3 has been investigated most and was recently identified as the main target to improve gene transfer into hematopoietic stem cells through the use of resveratrol as transduction enhancer. Although our data certainly support this strategy also for T lymphocytes, they also suggest that not only intracellular localization of IFITMbs but also subtle changes in their overall expression levels may determine whether a particular T cell becomes properly transduced.

Differences in gene profiles between the cells exposed to CD8-LV versus VSV-LV were also observed, although we previously demonstrated that CAR T cells generated with each of the two vector types were equally active in killing tumor target cells. These might be a consequence of the viral entry pathway used, toxicity of VSV-LV or the transduction of CD4 cells by VSV-LV thus affecting CD8 cells in a paracrine cytokine secretion-driven manner. CCL4 (MIP-1B), a major HIV suppressive factor, was highly upregulated in CD8 cells of the CD8-LV sample, indicating a possible antiviral response against the Nipah virus-derived envelope of CD8-LV (Figure 4C). In addition, CCR7 and CD62L were upregulated upon VSV-LV exposure, indicating a more pronounced central memory phenotype in these CAR T cells than in those generated with CD8-LV (Figure 4G). This is in line with granulysin (GNYL) increase in the CD8-LV sample, which is indicative for a more cytotoxic activity of these CAR T cells (Figure 4I). Although a central memory phenotype is beneficial for CAR T cell persistence, previous phenotype comparisons between CAR T cells generated with VSV-LV versus CD8-LV did not reveal significant differences. In the same direction, cathepsin-D peptidase (CSTD) was significantly increased in the CD8-LV sample (Figure 4H). It prevents oxidative stress-induced cell death, a feature relevant for CD3/CD28-activated T cells, in which the glycolysis pathway is elevated because of a skew to effector

Figure 4. Violin plots for genes of interest
Representative differentially expressed genes in CD8+ subpopulations clustered into topics related to CAR activities and vector-host interactions. (A) T cell activation. (B) TH1 phenotype. (C) Cytokines and chemokines. (D) Apoptosis. (E) Exhaustion and immune checkpoint. (F) Antiviral response. (G) Memory phenotype. (H) Miscellaneous. (l) Cytotoxicity. KW, Kruskal-Wallis multiple comparison test. Wilcoxon rank-sum test performed for pairwise comparisons. p values adjusted based on Bonferroni; ns, non-significant; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. The bars in box plots indicate the 25th to 75th percentile range and the median value is shown with a horizontal line.
phenotype leading to production of reactive oxygen species (ROS). In the VSV-LV sample, a lysosomal lipase (LIPA) was downregulated, which correlates with the endosomal-related pH-dependent entry pathway of VSV-LV (Figure 4H).

Although further investigation will be required before final conclusions on up- or downregulation of particular genes can be drawn, we have here successfully established a scRNA-seq workflow for CAR T cells, generated with conventional or receptor-targeted LV, and managed to distinguish transduced from untransduced cells by implementing a customized pair of primers. Based on the distribution of gene expression, we propose a subsetting method for distinguishing CAR T cells in scRNA-seq analysis, wherever applicable.

MATERIALS AND METHODS

Cell culture

HEK293T (ATCC CRL-11268) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma-Aldrich, Munich, Germany) supplemented with 2 mM glutamine (Sigma-Aldrich) and 10% fetal calf serum (FCS) (Biochrom, Berlin, Germany). MOLT 4.8 cells were cultured in RPMI 1640 (Biowest, Nuaille, France) supplemented with 10% FCS and 2 mM glutamine. All cell cultures were incubated at 37°C with 5% CO2 and 90% humidity.

Generation of lentiviral vectors

A detailed protocol describing the generation of T cell-targeted LVs was recently published. In brief, second-generation LVs were produced as previously described by co-transfecting 2 × 107 HEK293T cells per T125 flask with a plasmid cassette, consisting of packaging plasmid (pCMVΔR8.9) and transfer plasmid (pSEW-mycCD19-CAR-28z), in the presence of linear polyethylenimine (PEI). For the generation of VSV-pseudotyped LV we co-transfected the plasmid encoding the envelope G-glycoprotein (pMD2.G) and for the CD8-LV the plasmids (9B11, Cell Signaling Technology, Danvers, MA, USA) and with a monoclonal antibody to C (clone BW264/56, Miltenyi Biotec), anti-CD8 (clone BW135/80, Miltenyi Biotec), and anti-c-myeloid differentiation related protein 8 (M-DRAQ7, BD Biosciences). Single cells were then customized by BD Bioscience technology, using the Immune Response Signal Miner. Primers binding within 800 bp away from the signal sequence for the CAR gene, in CAR T cells, were in silico predicted and confirmed by sequencing the poly(A) signal site within the 3'-UTR of the transfer vector plasmid (Poly(A) Signal Miner). Primers binding within 800 bp away from the poly(A) signal, thus within the WPRE, were then customized by BD Bioscience technology.

Flow cytometry

Cells were re-suspended, washed with buffer (PBS, 5% FCS, 2 mM NaHCO3), and stained for 30 min at 4°C with the antibodies anti-CD3 [fluorescein] (clone BW264/56, Miltenyi Biotec), anti-CD8 [VioBlue] (clone BW135/80, Miltenyi Biotec), and anti-c-myeloid differentiation related protein 8 (M-DRAQ7, BD Biosciences). Results were analyzed in FCS Express v.6 (De Novo Software, Pasadena, CA, USA).

Single-cell RNA isolation, library preparation, and sequencing

Single-cell isolation and mRNA processing were conducted with the BD Rhapsody platform (BD Biosciences) according to the manufacturer’s instructions (Doc IDs: 214062 and 210968, BD Biosciences). For viability evaluation, cell suspensions were incubated for 5 min at 37°C with 10 μM calcein AM (Thermo Fisher Scientific) and 1.5 μM DRAQ7 (BD Biosciences). Single cells were then captured in nanowell-containing cartridges; cellular mRNA was released after lysis and captured by poly(dT)-coated magnetic beads. Beads were subsampled to yield ~31,000 total cells.

Reverse-transcribed cDNA was amplified with the Immune Response Panel (cat. 633750, BD Biosciences) of primers covering 399 genes. For detection of mRNA derived from the CAR gene in CAR T cells, we in silico predicted and confirmed by sequencing the poly(A) signal site within the 3’-UTR of the transfer vector plasmid (Poly(A) Signal Miner). Primers binding within 800 bp away from the poly(A) signal, thus within the WPRE, were then customized by BD Bioscience technology.
Biosciences (Franklin Lakes, NJ, USA) and added to the Immune Response Panel.

Libraries were indexed uniquely, and their quality and final length were assessed with the high-sensitivity NGS kit with the Fragment Analyzer (Agilent, Santa Clara, CA, USA). Data were analyzed in ProSize 2.0 (Advanced Analytical Technologies, Heidelberg, Germany) (Figure S1A). Libraries were then quantified by Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific), pooled in a ratio defined by the cell number of each sample and the required sequencing depth, and loaded into a NextSeq High Output flowcell at 1 pM, spiked with 20% PhiX, and sequenced in NextSeq 550 (Illumina, San Diego, CA, USA).

Data analysis
The raw FASTQ files were processed with the bioinformatics pipeline of Seven Bridges Genomics (Charlestown, MA, USA). Overall, 21,527 sequences were processed with a mean sequencing depth of 4,304 reads per cell. Recursive substitution error correction (RSEC)-adjusted molecule count matrices were generated and processed in R (v.4.0.3) with Seurat.44 Thresholds for filtering the low quality of cells and the number of principal components chosen for the UMAP analysis are shown in Figures S1B–S1D. The filtering metrics as well as the final number of putative cells per sample are listed in Table S1. Wilcoxon rank-sum test for differential gene expression analysis was performed with the FindAllMarkers and FindMarkers functions, applying the thresholds of $\log_{2}(fold \ change) \pm 0.25$ and minimum fraction of expressing cells 25%. Violin plots were constructed with ggplot2 (v.3.3.3), and Kruskal-Wallis non-parametric test and Wilcoxon rank-sum pairwise test were performed with rstatix (v.0.7.0).45 GO Biological Process enrichment analysis on differentially expressed genes was performed with the Independent Enrichment Analysis tool of Appyters collection by devo/geo/query/acc.cgi?acc=GSE184895).48

Data availability
The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE184895 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE184895).48

SUPPLEMENTAL INFORMATION
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AUTHOR CONTRIBUTIONS
Conceptualization: C.J.B., F.T.C., and F.B.T. Data curation: F.T.C. Formal analysis: F.T.C. and E.A. Funding acquisition: C.J.B. and C.C. Investigation: F.T.C. and E.A. Methodology: C.J.B., F.T.C., and F.B.T. Project administration: C.J.B. Resources: C.J.B. and C.C. Supervision: C.J.B. Visualization: F.T.C. and E.A. Writing – original draft: F.T.C., E.A., and C.J.B. Writing – review & editing: C.J.B. and C.C.

DECLARATION OF INTERESTS
C.J.B. is listed as co-inventor of the patent for the CD8-targeted lentiviral vector. All the other authors declare no competing interests.

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