Bundesinstitut für Impfstoffe und biomedizinische Arzneimittel
Federal Institute for Vaccines and Biomedicines

Paul-Ehrlich-Institut

Monitoring early transcriptional profiles of CAR T cell products generated with conventional or CD8-targeted lentiviral vectors*

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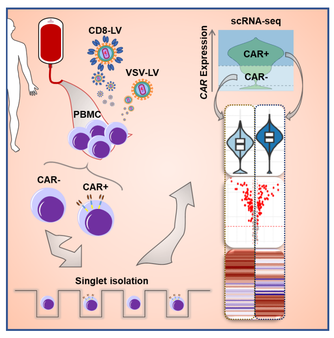
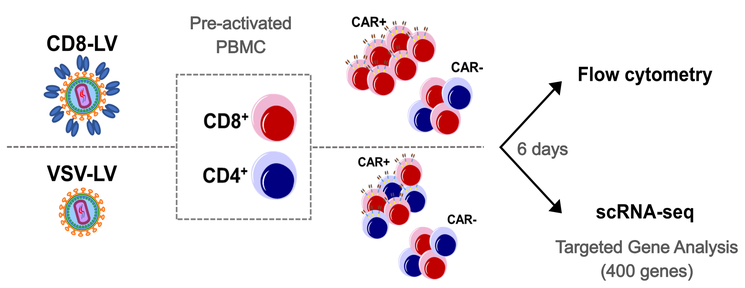
Abstract



The complicated and multifactorial manufacturing process of chimeric antigen receptor (CAR) T cell products involving gene delivery often by lentiviral vectors (LVs) can potentially affect clinical efficacy and emerge issues ranging from compromised product quality to post-transfusion side effects. Investigating gene expression profiles via single-cell RNA sequencing (scRNA-seq) can substantially improve our understanding about CAR T cell products.

In this study, we designed an approach to monitor early events of transduction on CD19-CAR T cells generated with either the conventional VSV-LV or the CD8α-targeted CD8-LV. LV-exposed human donor PBMC were evaluated using a panel of 400 immune-response related genes including LV-specific probes. The resulting data revealed a tri-modal expression for the *CAR* and *CD8A* demanding for 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 agreement with flow cytometry results. More than 97% of the cells transduced by CD8-LV expressed the *CD8A* gene. Remarkably, the majority of the potential off-target cells were in fact on -target cells identified by the expression of *CD8B* resulting in a target cell selectivity of above 99%. This is the first study proving the excellent specificity of receptor-targeted LVs by scRNA-seq. Transduced CD8+CAR+ cells had an increased expression of genes associated with proliferation, activation, cell exhaustion, as well as regulatory genes of TCR signaling and inhibitors of apoptosis. Moreover, we identified genes associated with negative regulation of T cell activation and proliferation, along with IFN-induced and pro-apoptotic genes to be upregulated in non-transduced CD8+CAR- cells. The data suggest gene profile alterations and the activation of intrinsic mechanisms possibly influencing the transduction efficiency of LVs. Recent whole transcriptome analysis confirmed the initial findings and appears to provide further insights.

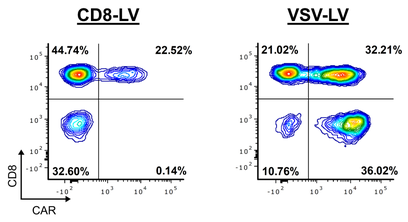
Experimental design



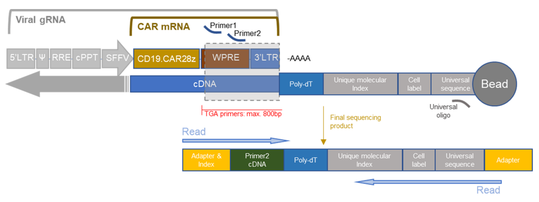
Human donor PBMC were used as cell source for CAR T cell generation. The cells were activated and then incubated with CD8-LV or VSV-LV, or, left untransduced as control. After six days of cultivation in presence of IL7/IL15, the cells were analyzed by flow cytometry and processed for scRNA sequencing, evaluating a panel of 400 immune-related genes (**Targeted Gene Analysis—TGA**).

Detection of CAR T cells

By flow cytometry

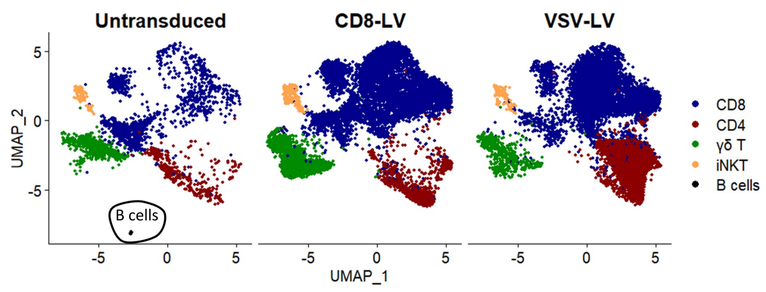


By scRNA-seq

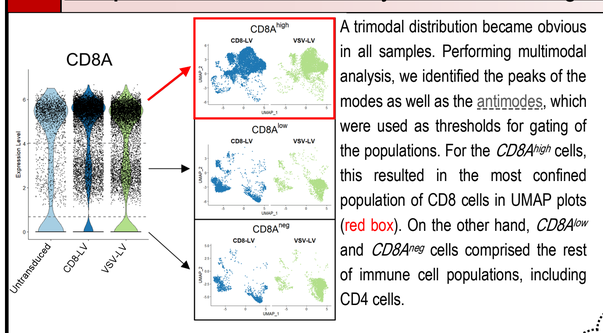


High selectivity of CD8-LV is confirmed by scRNA seq

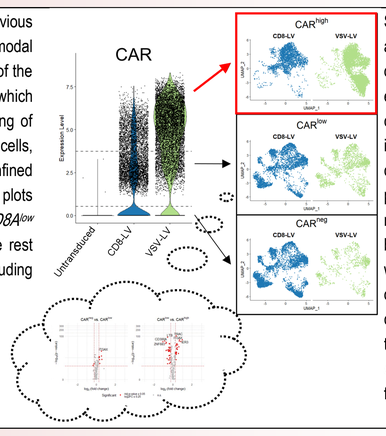
UMAP plots: CAR-mediated killing of remnant B cells in transduced samples



Computational multimodal analysis and subsetting strategy for distinguishing CD8 CAR T cells

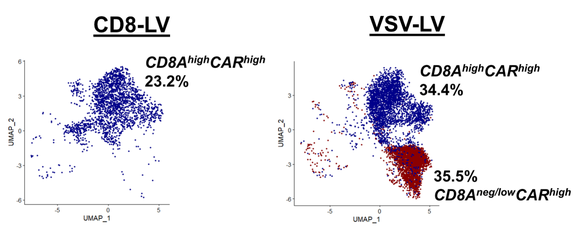


A trimodal distribution became obvious in all samples. Performing multimodal analysis, we identified the peaks of the modes as well as the antimodes, which were used as thresholds for gating of the populations. For the *CD8A*^{high} cells, this resulted in the most confined population of CD8 cells in UMAP plots (red box). On the other hand, *CD8A*^{low} and *CD8A*^{neg} cells comprised the rest of immune cell populations, including CD4 cells.



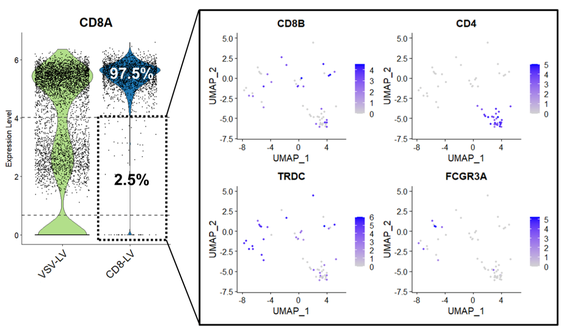
Similarly, a trimodal distribution was also observed for CAR T cells as determined by the CAR transgene expression. The *CAR*^{low} expressing cells consisted of all identified immune cells, including non-targeted cells. Comparing their expression profile with that of *CAR*^{neg} cells revealed no significant differences between these two cell populations, while significant differences were obvious between *CAR*^{low} and *CAR*^{high} cells (volcano plots). We have therefore combined them with the *CAR*^{neg} cells in one group (*CAR*$^{neg/low}$) for further analysis.

CAR T cell frequency matches flow cytometry & CD8-LV highly selective for CD8 cells



We observed concordant CAR T cell frequencies between flow cytometry and scRNA data, when we subset for *CAR*^{high} and *CD8A*^{high} in the scRNA plots.

CAR^{high} cells



Notably, our subsetting approach showed that 97.5% of *CAR*^{high} cells were *CD8A*^{high} cells in the CD8-LV sample, while 2.5% of *CAR*^{high} cells (in total 59 cells) were *CD8A*$^{neg/low}$. Of the 59 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 did neither express *CD8A* nor *CD8B*; 2 cells were NKT cells; 15 were CD4 cells, from which 8 were negative for CD8 markers, while 7 had *CD8A*^{low} expression. This leaves just 17 cells that could be true off-target cells and thus an **on-target rate of 99.28%**.

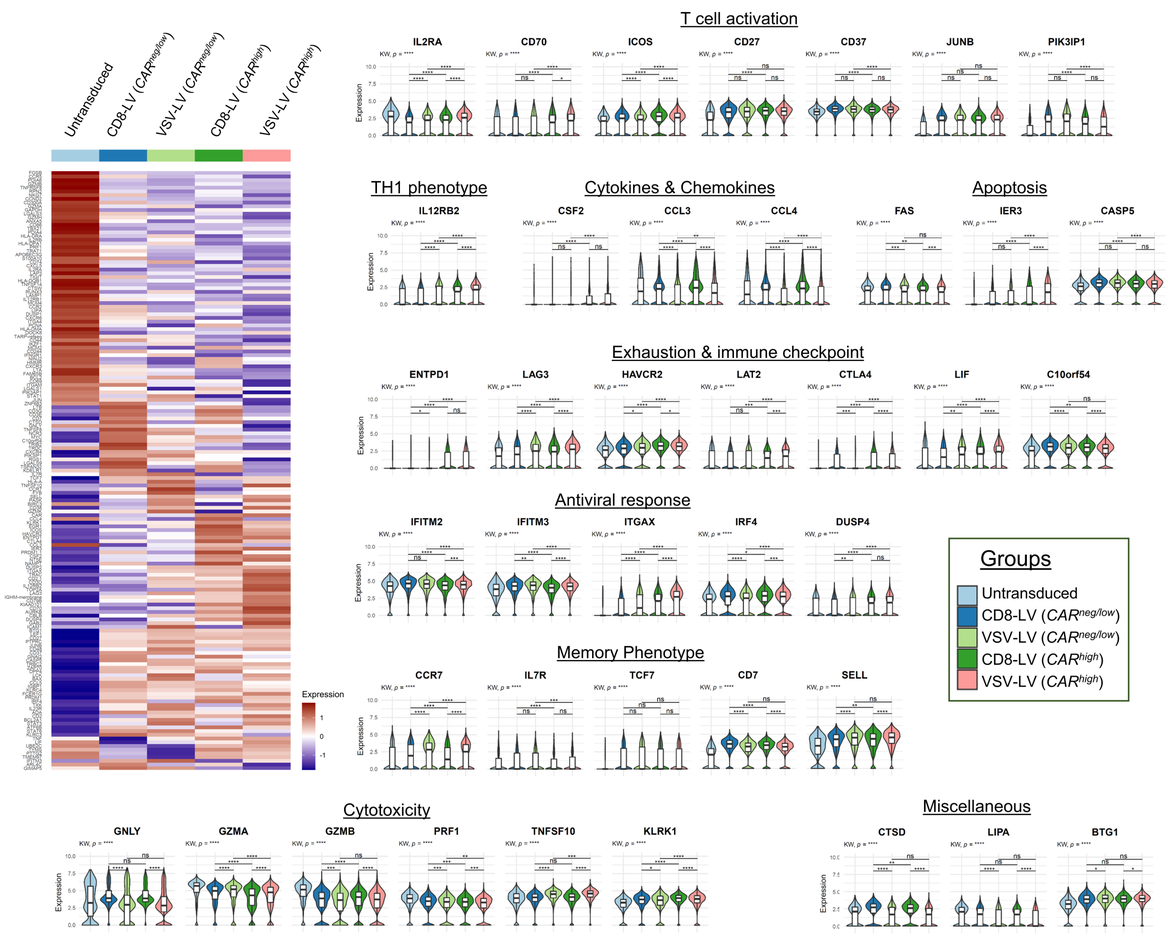
Identification of *CAR*^{high}*CD8A*$^{neg/low}$ cells after CD8-LV mediated transduction

Cell type	Marker genes	Cell number
CD4 T cells	*CD4*^{pos}*CD8B*^{neg}	**15**
	CD8A^{low}	7
	CD8A^{neg}	→ 8
CD8 T cells	*CD8B*^{pos}	**5**
	CD8A^{low}	5
CD4/CD8 T cells	*CD4*^{pos}*CD8B*^{pos}	**4**
	CD8A^{low}	2
	CD8A^{neg}	2
NKT	*FCGR3A*^{pos}	**2**
	CD8B^{pos}*CD8A*^{low}	1
	CD8B^{neg}*CD8A*^{low}	1
γδ T	*TRDC*^{pos}	**32**
	CD8B^{pos}*CD8A*^{low}	7
	CD8B^{pos}*CD8A*^{neg}	7
	CD8B^{neg}*CD8A*^{low}	9
	CD8B^{neg}*CD8A*^{neg}	→ 9
Remaining cells	*CD8B*^{neg}*CD4*^{neg}*TRDC*^{neg}*FCGR3A*^{neg}	**1**
	CD8A^{low}	1

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

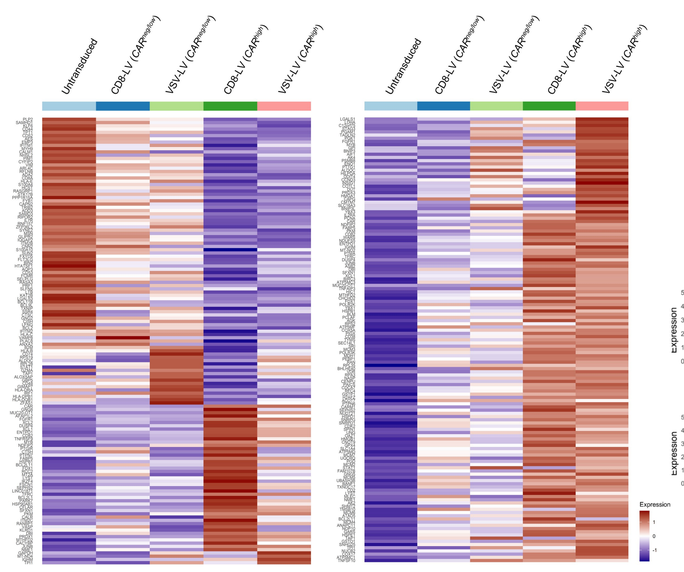
Differentially expressed genes in the CD8 populations

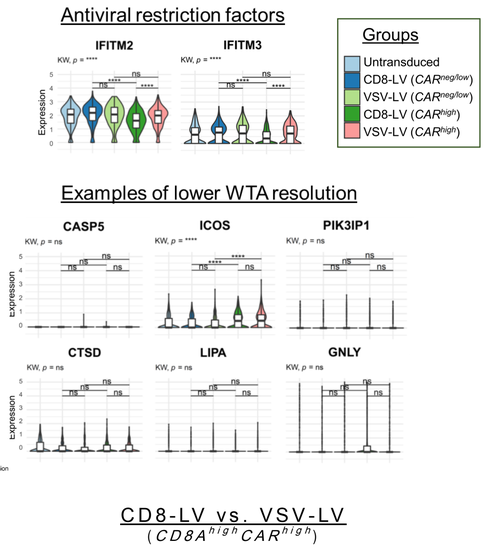
Altered phenotype, restricted cell viability and viral entry in CAR- cells compared to CAR+ T cells



These findings suggest that under the given experimental conditions the exposure of T cells to LV particles results in stronger gene expression profile alterations than presence or absence of the CAR in the particular LV-inoculated sample. While **CD8+ *CAR*^{high} cells** exhibited an activated TH1-phenotype and an overall profile well in accordance with that observed in previous studies, [1-3] **CD8 *CAR*$^{neg/low}$ 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*). 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 *CAR*^{neg} despite having been exposed to LVs.

Whole Transcriptome Analysis confirms TGA's findings





Conclusion

- CD8-LV confirmed to have high specificity for CD8 cells by scRNA-seq
- Non-transduced cells (*CAR*$^{neg/low}$) had elevated genes expressing restriction factors
- Bigger differences between control vs. LV-inoculated samples rather than *CAR*$^{neg/low}$ vs. *CAR*^{high}
- Differences observed between CAR T cells generated by either VSV-LV or CD8-LV
- Targeted gene scRNA-seq ameliorated high resolution gene expression analysis compared to WTA
- WTA confirmed some of the findings and provided further insights

STACCATO

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*Charitidis F.T., Adabi E., Thalheimer F.B., Clarke C., Buchholz C.J. (2021). Monitoring CAR T cell generation with a CD8-targeted lentiviral vector by single-cell transcriptomics. *Molecular Therapy Methods & Clinical Development 23*, 359-639

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