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Application Note

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CAR-T Cell Generation: From Optimized Plasmid Design and Lentiviral Vector Production to Killing Assay

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Abstract

Gene-modified cell therapies, such as CAR-T cells, are among the most promising advanced therapy medicinal products (ATMP) in the fight against cancer. These therapies typically use lentiviral vectors to transfer a gene of interest and modify a patient's cells. The raw materials used during lentiviral vector manufacturing must be carefully selected based on different parameters such as quality, performance, and supplier.

This application note explores the use of e-Zyvec® DNA assembly technology to generate a CD19 CAR LV plasmid transgene, which can be used in combination with FectoVIR® -LV to produce lentiviral particles. The study examines the generation of ex vivo CAR-T cells and their capacity to kill antigen-positive target cells.

Introduction

Cell and gene therapies are at the forefront of biomedical innovation, offering unprecedented opportunities to treat and potentially cure many diseases that were once considered intractable. Among the most promising advances in this field is chimeric antigen receptor (CAR)-T cell therapy, which has revolutionized the treatment of certain cancers. CAR-T therapy harnesses the body's immune cells, equipping them with synthetic receptors that specifically target and destroy cancer cells. This approach has shown remarkable efficacy, particularly in the treatment of hematological malignancies, and has become a beacon of hope for patients with few other therapeutic options.

This study aimed to generate CAR-T cells using a novel approach that involves the construction of various plasmid vectors expressing a fusion protein CAR, which serves as a marker for tracking transduction efficiency. Utilizing the innovative e-Zyvec® plasmid assembly technology provided by Polyplus, we designed and assembled multiple prototypes of plasmids. These were subsequently encapsulated within lentiviral (LV) vectors produced using the FectoVIR®-LV reagent, which has been demonstrated to support the production of high titers. The resulting vectors were used to transduce a population of CD4+ CD8+ T cells isolated from peripheral blood, which were then expanded and assessed for their ability to eliminate cancer cells through a cytotoxicity assay.

This application note describes our methodology, from the initial plasmid design and LV vector production to the generation, expansion, and functional validation of the CAR-T cells. We then present our findings, which highlight the efficacy of our approach in producing CAR-T cells with potent antitumor activity, and discuss the implications of these results for advancing CAR-T cell therapies.

Materials and Methods

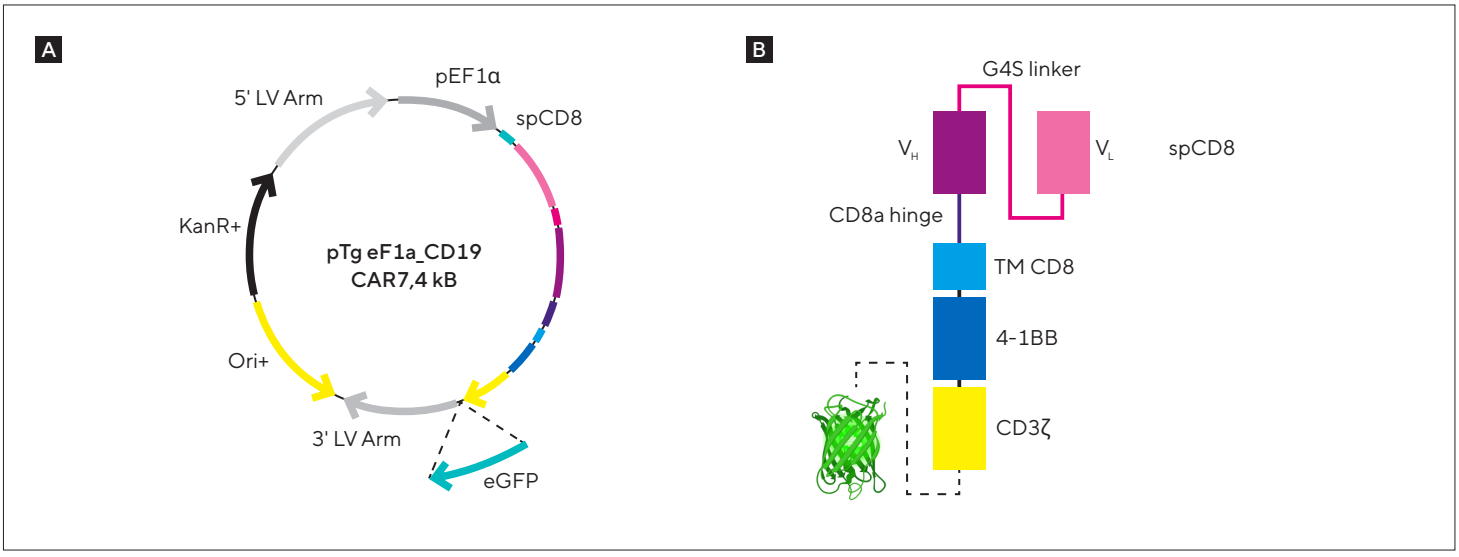
Plasmid Design

Five LV plasmids Transgenes were built using our proprietary e-Zyvec® DNA assembly technology. This technology consists of the de novo assembly of linear DNA bricks, which allow us to design fully tailor-made plasmids containing only desired sequences.

The designs were conducted using our proprietary software, defining the DNA bricks to build and assemble plasmids. Each DNA brick was obtained through high-fidelity polymerase chain reaction (PCR) with Q5® Hot-Start Polymerase (New England Biolabs) following the supplier's recommendations regarding primer and template quantity and thermocycling programs. The DNA bricks were then purified using the Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (Cytiva). Afterward, the DNA bricks of interest were put together in equimolar proportions in nuclease-free H_2O. Our optimized assembly reaction mix was then added to the DNA bricks. The assembly thermocycling program was developed in-house.

The assembly mix was then transformed in NEB® Stable Competent E. coli (High Efficiency) [Ref C3040] using thermal shock, following the supplier's recommendations. The transformation mix was then plated on Kanamycin-resistance selection plates and incubated overnight at 30 °C. The following day, clones for each plasmid were selected and amplified in a 3.5 mL liquid culture from 12 to 16 hours at 30 °C and under 250 rpm shaking. Plasmids were then extracted using the DNA purification NucleoSpin® Plasmid kit (Macherey-Nagel) following the supplier's recommendations. Finally, each plasmid was verified through restriction profiles and Illumina next-generation sequencing. The plasmids contain the following features (Figure 1).

Figure 1: *Schematic Representation of the CD19 CAR LV pTransgene (pTg eF1a_CD19 CAR) and the Obtained CD19 CAR*



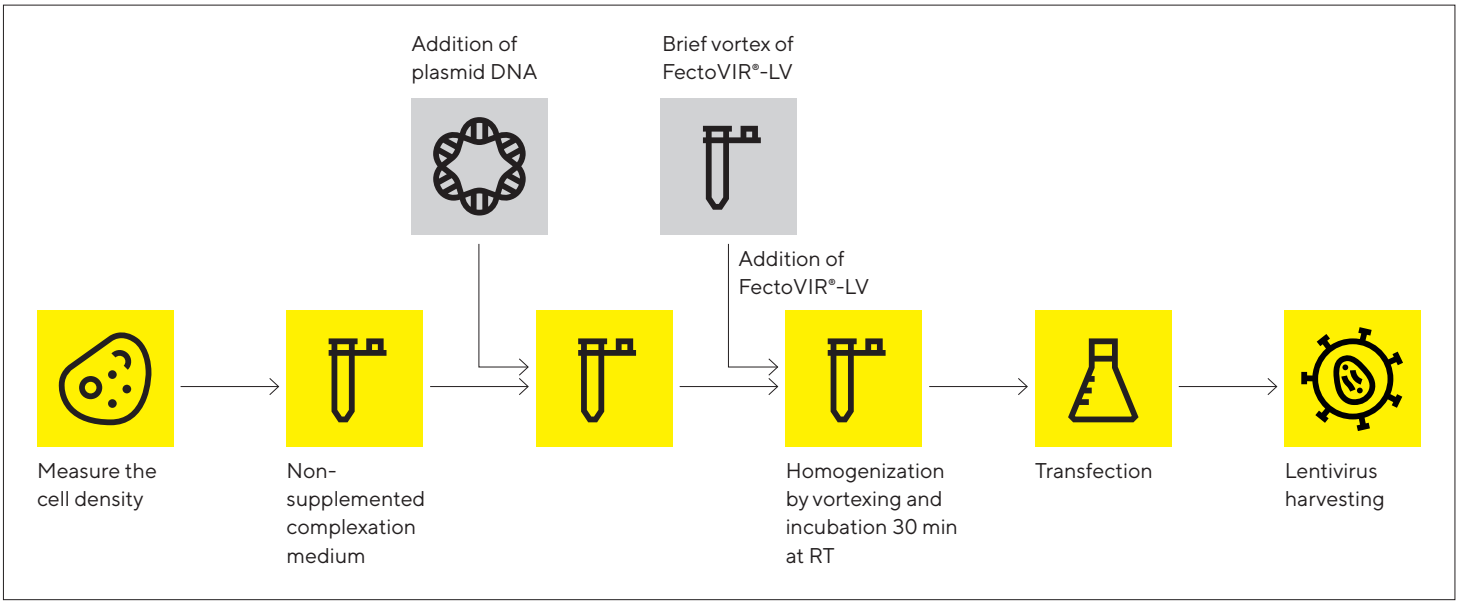
Note. A) 5'LV Arm contains the Rous Sarcoma Virus promoter, HIV-1 LTR and Psi sequence, RRE, and cPPT/CTS. B) The Elongation-Factor 1α promoter allows transcription of the CD19 CAR. The latter is composed of the CD8 signal-peptide, the light and heavy chains of the CD19 single-chain variable fragment linked with G4S peptide, the hinge and trans-membrane domain of CD8, the intracellular signaling domains 4-1BB and CD3ζ. For the three constructs, there is the enhanced GFP linked with IRES, T2A, or G4S peptide. The 3'LV arm contains HIV-1 ΔU3LTR and SV40-PA signal. Our optimized origin of replication and optimized Kanamycin resistance cassette is also present.

Lentiviral Vector Production

LVs were produced using FectoVIR®-LV and a 4-plasmid system (ratio 1:2:1:3 pALD_Rev-K: pALD_GagPol-K: pALD_VSVG-A: pTg eF1a_CD19 CAR or pTg eF1a_CD19 CAR-GFP or pALD_GFP-A from Aldevron as a positive control for GFP expression) in a 125 mL shake flask with HEK293T cells cultivated in suspension.

The transfection mix was incubated for 30 minutes at room temperature, using the recommended conditions (1:1 ratio of DNA:transfection reagent, 1 µg of DNA per million cells, 5% complexation volume) and LV were harvested 72 hours post-transfection.

Figure 2: *FectoVIR®-LV Protocole for Lentivirus Production*



Lentivirus Titration

Human CD4+ and CD8+ T cells were isolated from peripheral blood using a positive selection kit and cryopreserved. After thawing, cells were activated with TransAct™ T cell reagent (Miltenyi Biotec) in I10 media (Gibco™ IMDM with 1% penicillin/streptomycin, 1% L-Glutamine, and 10% FBS) in the presence of IL-2 (20 UI/106 cells, PeproTech®). The cells were seeded in triplicates in 100 µL medium into 96-well plates. Twenty-four hours after seeding, thawed LV vectors were diluted in a 4-fold serial dilution and cells were transduced by adding 100 µL medium containing diluted lenti-CD19 CAR (scFv-41BB-CD3ζ), lenti-CD19 CAR-GFP, or lenti-GFP viral particles and an enhancer of transduction Synperonic F108 (1 µg/µL, Sigma-Aldrich). Cells were fed on day 4 and the expression of CD19 CAR was analyzed by flow cytometry using the Guava® easyCyte™ (Cytek®) on day 6.

CAR T Cell Generation and Expansion

Human CD4+ and CD8+ T cells from peripheral blood from two donors were isolated using a positive selection kit and cryopreserved. After thawing, cells were activated with TransAct™ T cell reagent in I10 media in the presence of IL-2. The cells were seeded in triplicates in 1 mL medium into 24-well plates. Twenty-four hours after seeding, cells were transduced by adding 1 mL medium containing lenti-CD19 CAR (scFv-41BB-CD3ζ) viral particles to achieve a final MOI of 10 and an enhancer of transduction Synperonic F108. Cells were passaged on day 4, 6, 8, and 11. Expression of CD19 CAR was analyzed by flow cytometry on day 13 using the CD19 CAR FMC63 antibody (Miltenyi Biotec).

In Vitro Cytotoxicity Assay

NALM-6 and K562 cells were stained with CellTrace Violet™ (Invitrogen) and co-cultivated with transduced T cells at different effector-to-target (E:T) ratios for 24 hours. Wells containing non-transduced (NT) cells or only labeled NALM-6 cells served as controls. CAR-mediated cytotoxicity was determined by analyzing the residual alive CellTrace Violet™ target cells at each E:T ratio by flow cytometry and the amount of TNF-α secreted in the supernatant by ELISA (DuoSet Human TNF-a, BioTechne).

Figure 4: *CAR-T Cells and Target Cells Interaction in a Co-Culture Assay*

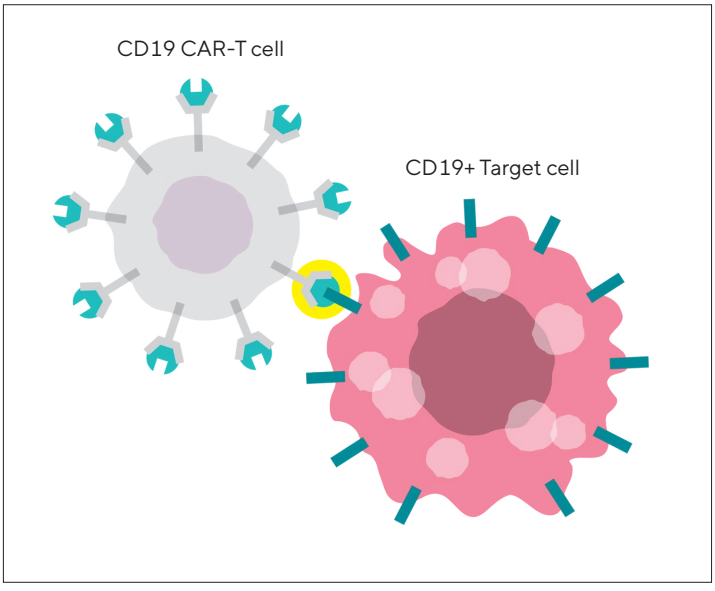
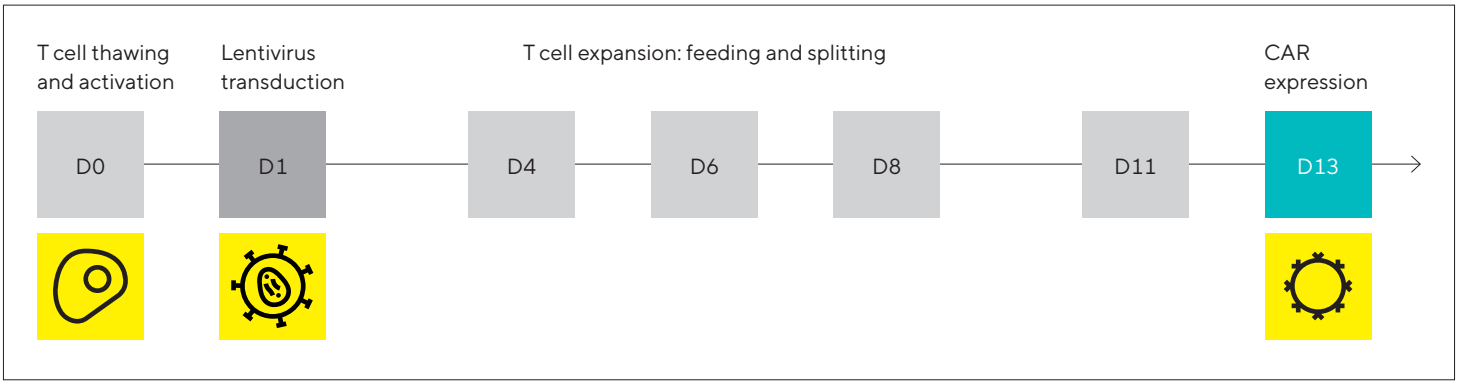


Figure 3: *CAR-T Cell Generation and Expansion*

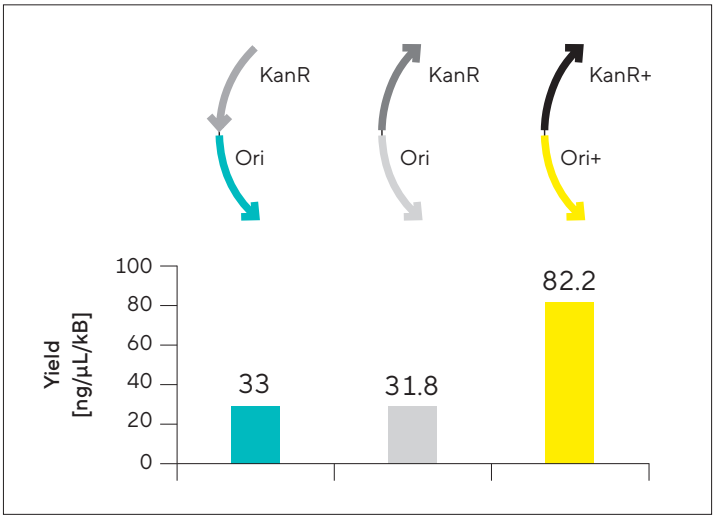


Results

Plasmid Design

We used our e-Zyvec® DNA assembly technology to modulate and optimize different parts of the plasmids. First, several combinations of bacterial genetic features (Origin of replication—Ori and antibiotic resistance—KanR) were tested to improve plasmid amplification rate. Figure 5 shows that our optimized Ori+ (yellow) and KanR+ (black) cassettes increase plasmid yield by almost 3-fold. Then, we modularly built plasmids encoding various versions of a CD19-CAR differing by the presence or absence of an eGFP reporter or the use of different linkers (IRES, T2A, or G4S peptide) between CAR and eGFP (not shown). For the purpose of the study, five plasmids were generated simultaneously under 4 weeks from design to plasmid sequencing.

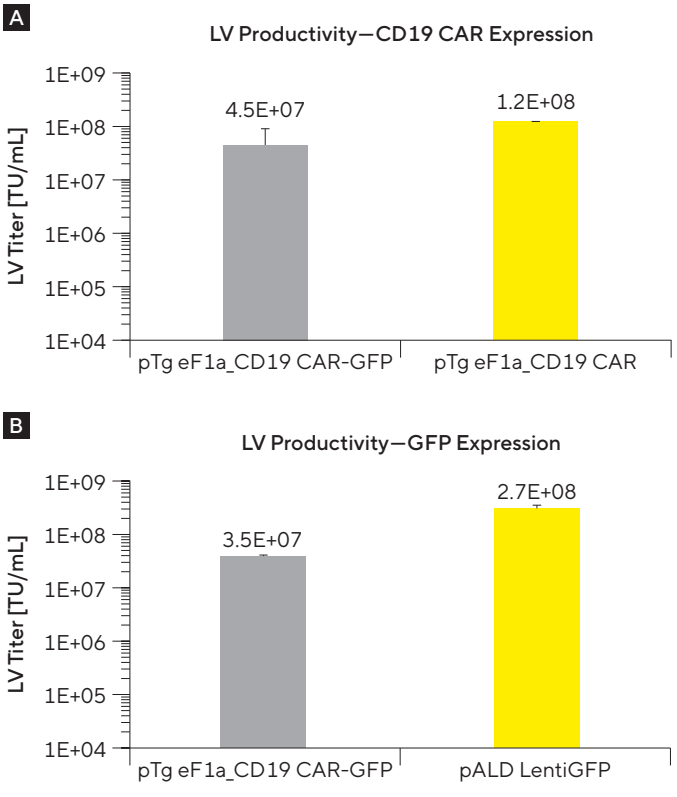
Figure 5: *Plasmid Yield Obtained With Different Bacterial Backbones*



Highly Productive Lentiviral Vector Manufacturing With FectoVIR®-LV

Functional titers were measured using CD19 CAR expression (Figure 6A) or GFP expression (Figure 6B) on transduced CD4+ CD8+ T cells. FectoVIR®-LV demonstrates a high productivity for CD19 CAR, CD19 CAR-GFP, and GFP LV vector manufacturing. When evaluating the LV titer evaluated by the CD19 CAR expression, a higher titer is observed with the CD19 CAR LV encoding for only CD19 CAR protein (1.2E+08 TU/mL) compared to CD19 CAR-GFP LV encoding for both CD19 CAR and GFP protein (4.5E+07 TU/mL), perhaps due to a competition of expression between the GFP and the CD19 CAR for some cells transduced with the CD19 CAR-GFP LV. Regarding the LV titer evaluated by GFP expression, a higher titer is also observed with the positive control pALD_GFP-a encoding for only GFP protein (2.7E+08 TU/mL) compared to CD19 CAR-GFP LV encoding for both proteins (3.5E+07 TU/mL). In both cases, FectoVIR®-LV allows the collection of a large amount of potent LV vectors, leading to the success of CD19 CAR-T cell generation.

Figure 6: *Lentivirus Productivity Evaluated by CD19 CAR or GFP Expression on CD4+ CD8+ T Cells*



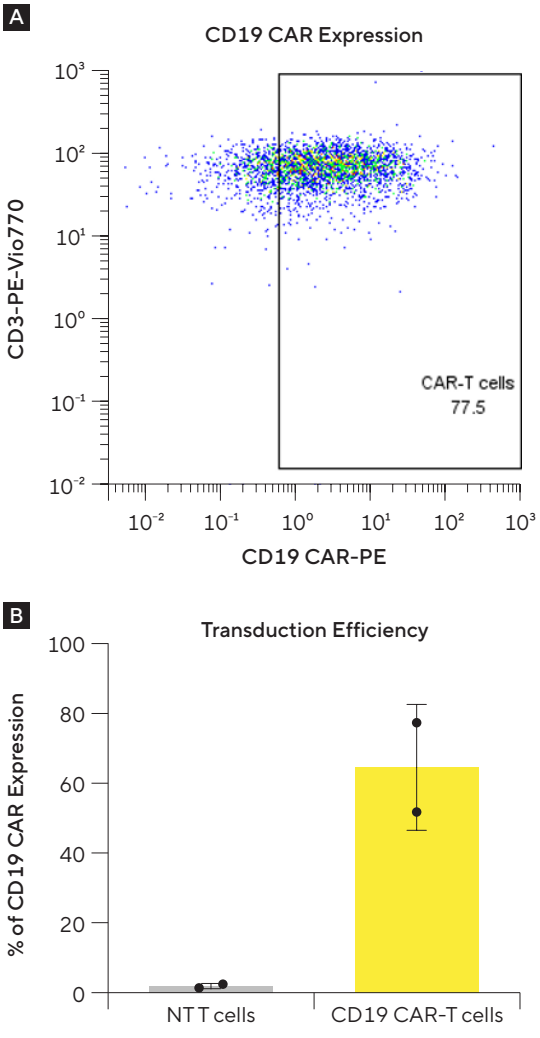
Note: Results are presented as Transduction Unit (TU)/mL.

Generation of High CD19 CAR-T Cells

Production of autologous CAR-T cells begins with the isolation of T cells from the patient, followed by ex vivo genetic modification with LV vectors and expansion of cells before they are injected back into the patient. In this study, we mimicked the CAR-T process to show that our manufactured CD19 CAR LV are able to transduce T cells and that these T cells still carry the CAR transgene at the end of the ex vivo expansion.

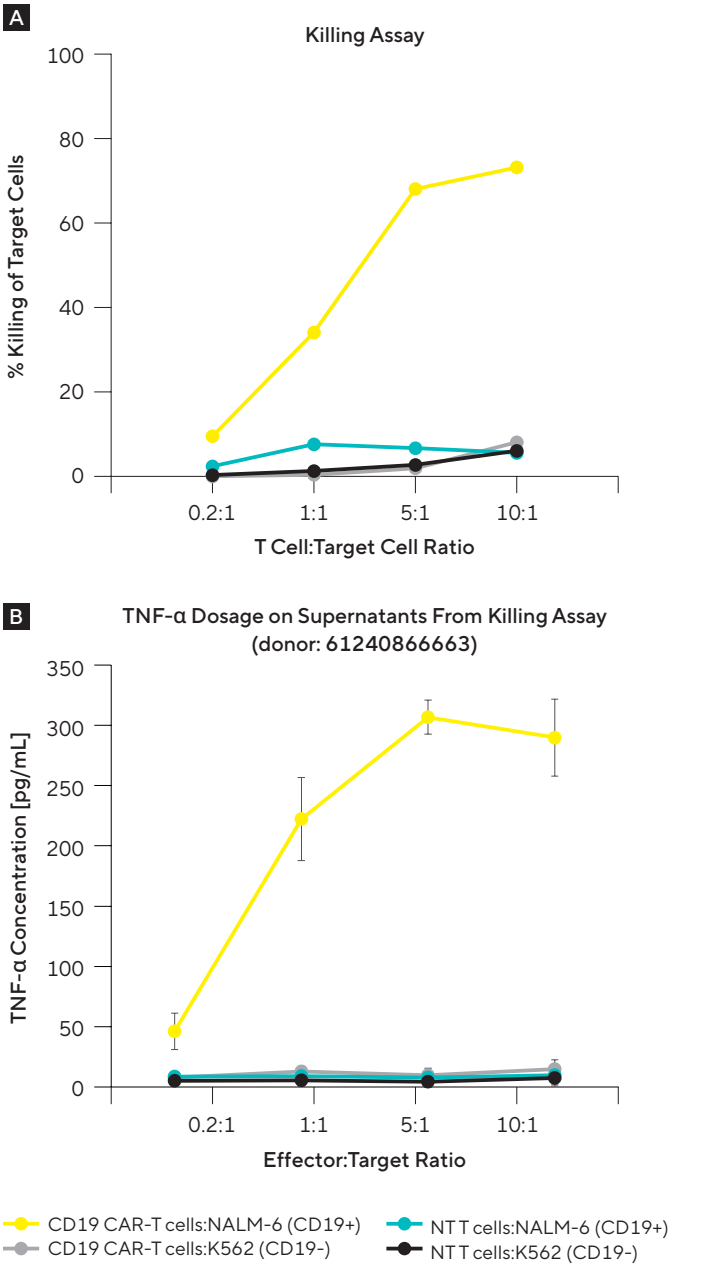
The expression of the inserted CD19 CAR gene was analyzed by flow cytometry on day 13. Up to 77.5 % of T cells at the end of the cultivation period expressed the CD19 CAR gene on their surface with a mean of 60 % for two donors.

Figure 7: *Evaluation of the Transduction Efficiency*



Note. (A) Dot plot of transduction efficiency of one representative donor and (B) CD19 CAR expression on day 13 on non-transduced (NT) T cells and transduced T cells (n = 2 donors).

Figure 8: *CD19 CAR-T Cell Killing of Antigen-Positive Target Cells and Antigen-Specific Activation*



Note. Thawed CAR-T cells or non-transduced T cells were co-cultivated with CellTrace Violet-labeled NALM-6 or K562 cells in 0.2:1, 1:1, 5:1 and 10:1 effector to target ratios. After 24 hours, killing of target cells by CD19 CAR-T cells or non-transduced T cells was assessed by PI+ CellTrace Violet+ target cells (n = 2 donors). TNF-α production was quantified by ELISA in the supernatant (n = 1 donor).

Functionality of CD19 CAR-T Cells
Following cognate antigen recognition, CAR-T cells should be able to kill the antigen-bearing tumor cell. To mimic this and to verify the functionality of the generated CD19 CAR-T cells, we used the NALM-6 cell line, which expresses the CD19 antigen, and the K562 cell line, which does not express the CD19 antigen, as a negative control.

When co-cultivated, the expanded CD19 CAR-T cells kill the NALM-6 cells. This killing was not observed with antigen-negative target cells (K562) or with non-transduced T cells (Figure 8A). The maximal effect was measured using an E:T ratio of 10:1, representing an 80 % killing of the NALM-6 (mean of two donors). After the killing was observed by flow cytometry, cell supernatants were subsequently analyzed to assess the TNF-α production by T cells (Figure 8B). In the presence of NALM-6 cells, only CD19 CAR-T cells secrete TNF-α even from the lowest E:T ratio of 0.2:1 and with a maximal production of 300 pg/mL using the E:T ratio of 5:1.

Overall, this quantification demonstrates a clear antigen-specific activation of CD19 CAR-T cells as measured by secreted proteins in combination with the functional readout of killing antigen-expressing tumor cells.

Conclusion

In conclusion, the data presented in this study underscore the robustness and efficacy of the FectoVIR®-LV based system when combined with plasmids constructed using e-Zyvec® DNA assembly technology to produce high titers of potent lentiviral vectors. The successful generation of functional CD19 CAR-T cells, as demonstrated by their targeted cytotoxicity against antigen-bearing tumor cells, marks a significant milestone in the advancement of CAR-T cell therapies.

Our findings provide compelling evidence that the strategic integration of advanced plasmid engineering and lentiviral vector production techniques can lead to the development of highly potent CAR-T cells. This has important implications for the treatment of hematological malignancies, offering a potential pathway to improve patient outcomes and expand the therapeutic reach of CAR-T cell technology.

The methodologies and results discussed herein not only contribute to the scientific understanding of CAR-T cell generation but also highlight the importance of optimizing each step of the process to achieve clinical-grade therapeutic agents. As the field of cell and gene therapy continues to evolve, the insights gained from this research will inform future innovations and help streamline the transition from bench to bedside, ultimately enhancing the availability and efficacy of treatments for patients in need.

Moving forward, the continued refinement of CAR-T cell production processes, coupled with rigorous functional validation, will be paramount in addressing the remaining challenges and fulfilling the promise of CAR-T cell therapy as a standard of care for cancer patients.

For more information, visit
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