

Manipulation of MAP3K Signaling to Improve CAR-T Cell Therapy

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Introduction

- Chimeric Antigen Receptor (CAR)-T cell therapy has made remarkable strides in the treatment of patients with B-cell malignancies and certain hematological cancers.
 - Although CAR-T cells confer tumor antigen specificity, success with solid tumors has been limited, partly due to the metabolically hostile tumor microenvironment (TME).¹
 - Mitogen Activated Protein (MAP) kinase kinase kinase (MAP3K) is a crucial triple kinase that mediates CAR-T cell efficacy and is also a promising target for improving the effectiveness of CAR-T cell therapy in solid tumors.²
 - MAP3K is a highly labile protein because its degradation is ubiquitin-dependent. This, however, can be prevented by the deletion of its N-terminal portion.³ Due to MAP3K's essential role in CAR-T cell therapy, there is a clear rationale for further investigating its mechanistic role as well as developing methods to increase its stability.
 - This study aims to construct a CAR-T cell that expresses a stable form of MAP3K lacking its N-terminal region (MAP3KΔN) with the hope of further improving the antitumor efficacy of CAR-T cell therapy in solid tumors.
- We **hypothesize** that MAP3KΔN expression will promote CAR-T cell activation and metabolic fitness in the TME of solid tumors.

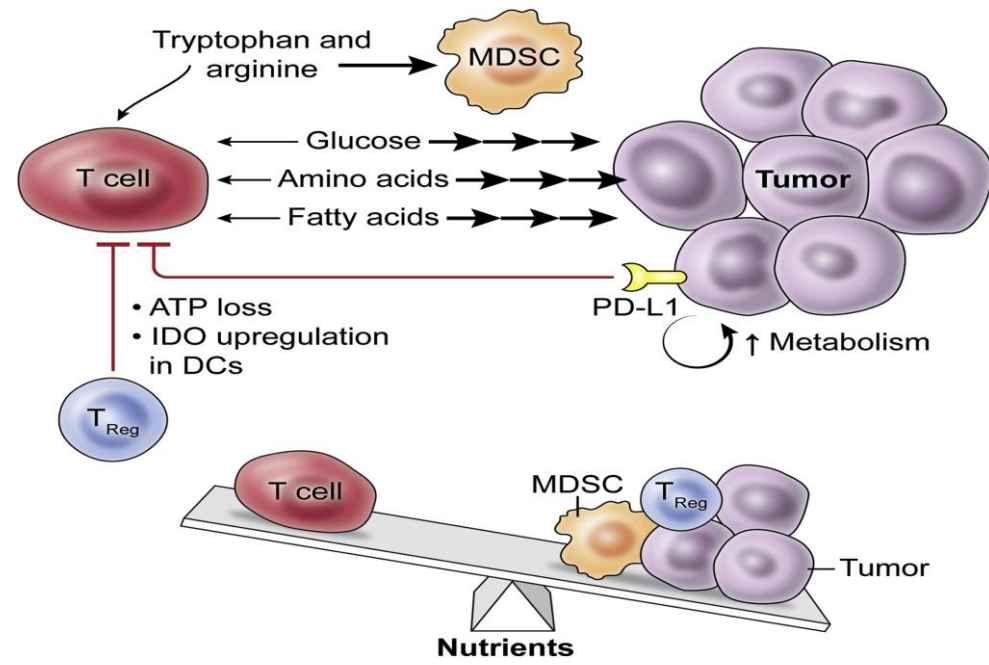


Figure 1. CAR-T cells face metabolic challenge in solid tumor microenvironment. CAR-T cells become metabolically exhausted in the hostile solid tumor microenvironment (TME) that includes the presence of immunosuppressive molecules (TGFβ, IL-10, etc.) and cells (Tregs, MDSCs, etc.) and lead to CAR T-cell hypofunctionality.⁴

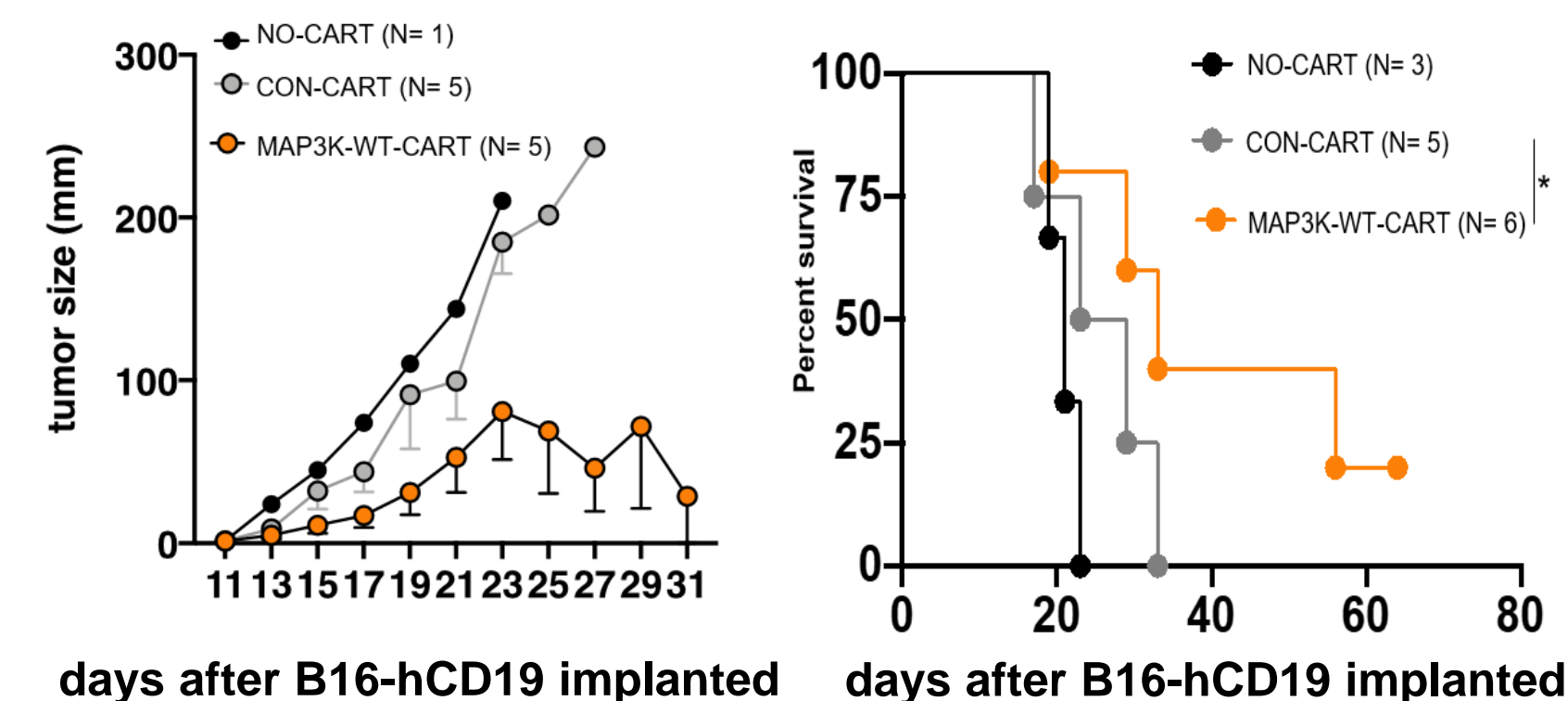


Figure 2. MAP3K expression enhances CAR-T cell antitumor response, which dramatically improves cancer prognosis. This MAP3K regulates T cell metabolism via an NF-κB-independent mechanism that involves stabilization of hexokinase 2 (HK2), which is a rate-limiting enzyme of the glycolytic pathway and is required for metabolic reprogramming of activated T cells. However, MAP3K is subject to ubiquitin-dependent degradation.

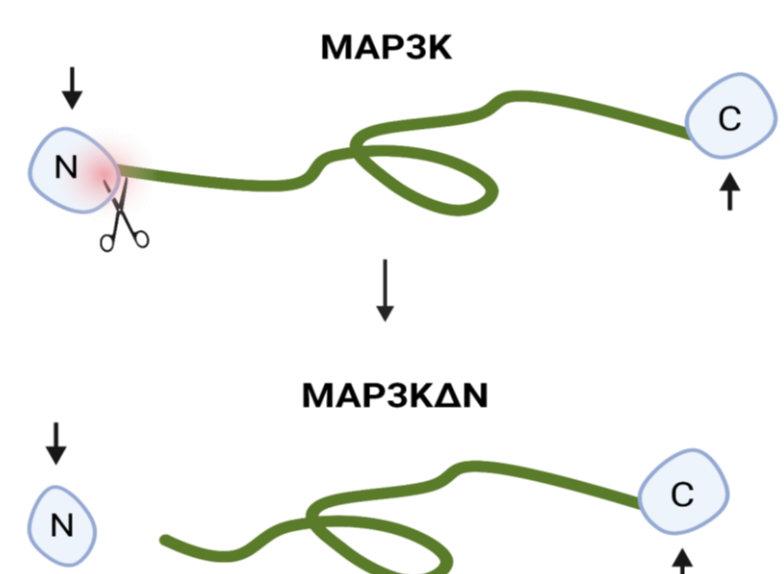


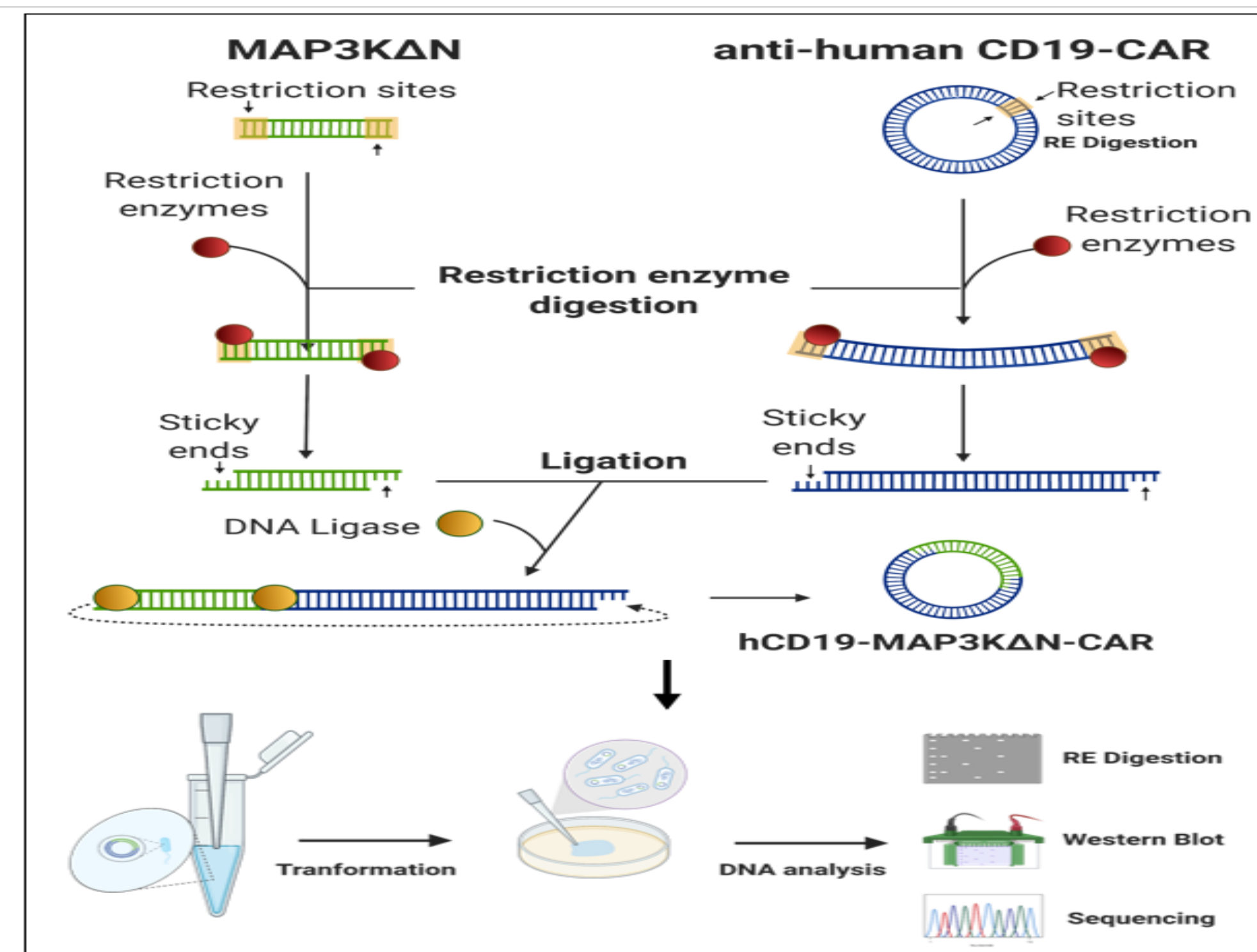
Figure 3. Stabilization of MAP3K by deletion of its N-terminal end. Under normal conditions, tumor necrosis factor receptor-associated factor 3 (TRAF3) physically associates with MAP3K via the N-terminal region, which mediates ubiquitination and proteasomal degradation. This can be prevented by deletion of its N-terminal portion.

Discussion and Conclusion

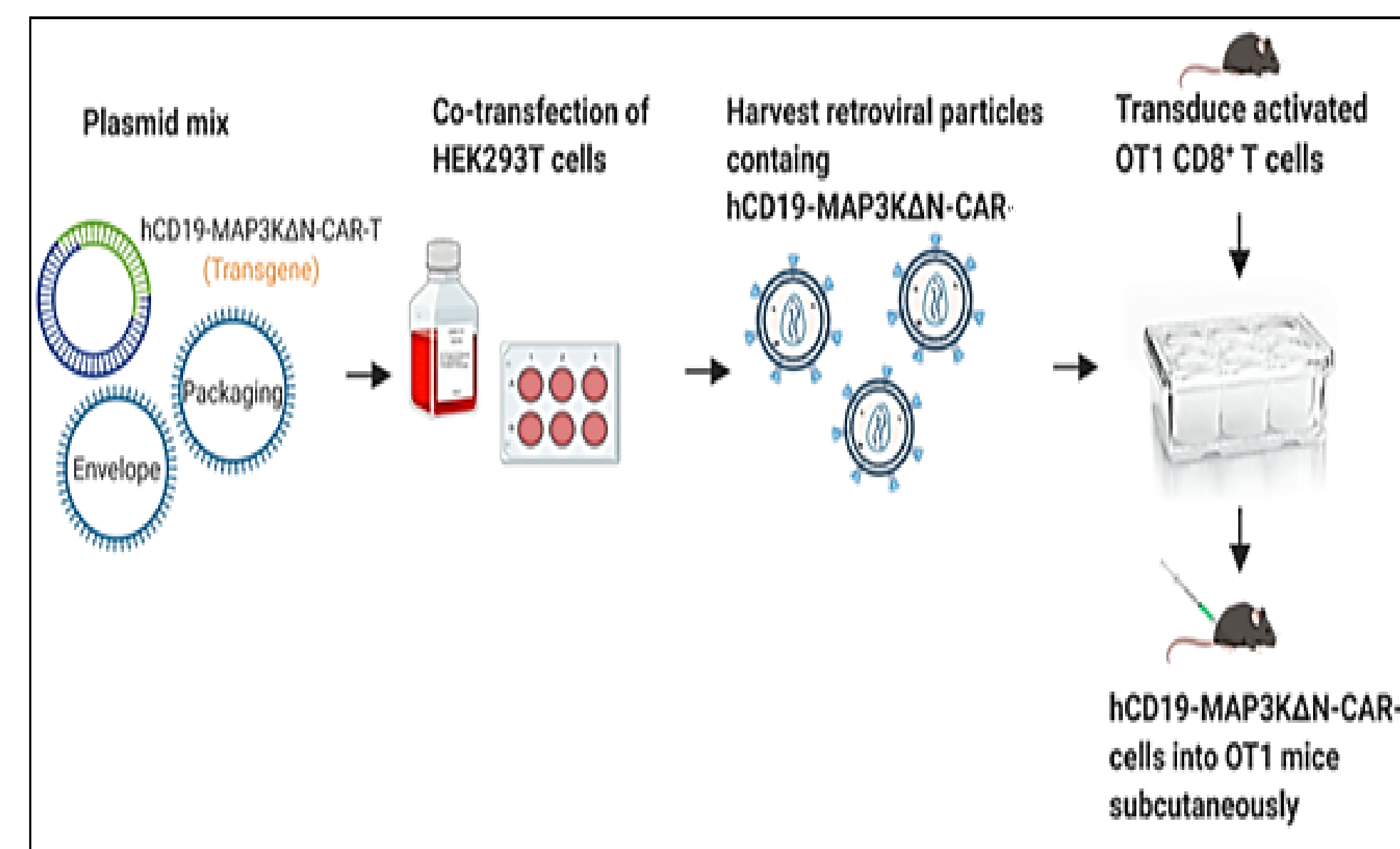
- We have found evidence to reject our hypothesis that MAP3KΔN will promote CAR-T cell efficacy due to avoidance of degradation. However, MAP3K^{WT} leads to a notable decrease in tumor growth *in vivo* consistent with our previous observations. Several points remain to be addressed to define cause-effect relationships and we propose further directions to enhance this promising immunotherapy.
- We have concluded that expression of MAP3K^{WT}, but not MAP3KΔN, profoundly improves the antitumor function of CAR T cells.
- Our results suggest two hypotheses:
 - Optimal rather than excessive T cell activation is critical for potential therapeutic applications using MAP3K expression.
 - Deletion of the N-terminal portion of MAP3K impairs its role in facilitating T cell metabolic functions.

Methods and Experimental Design

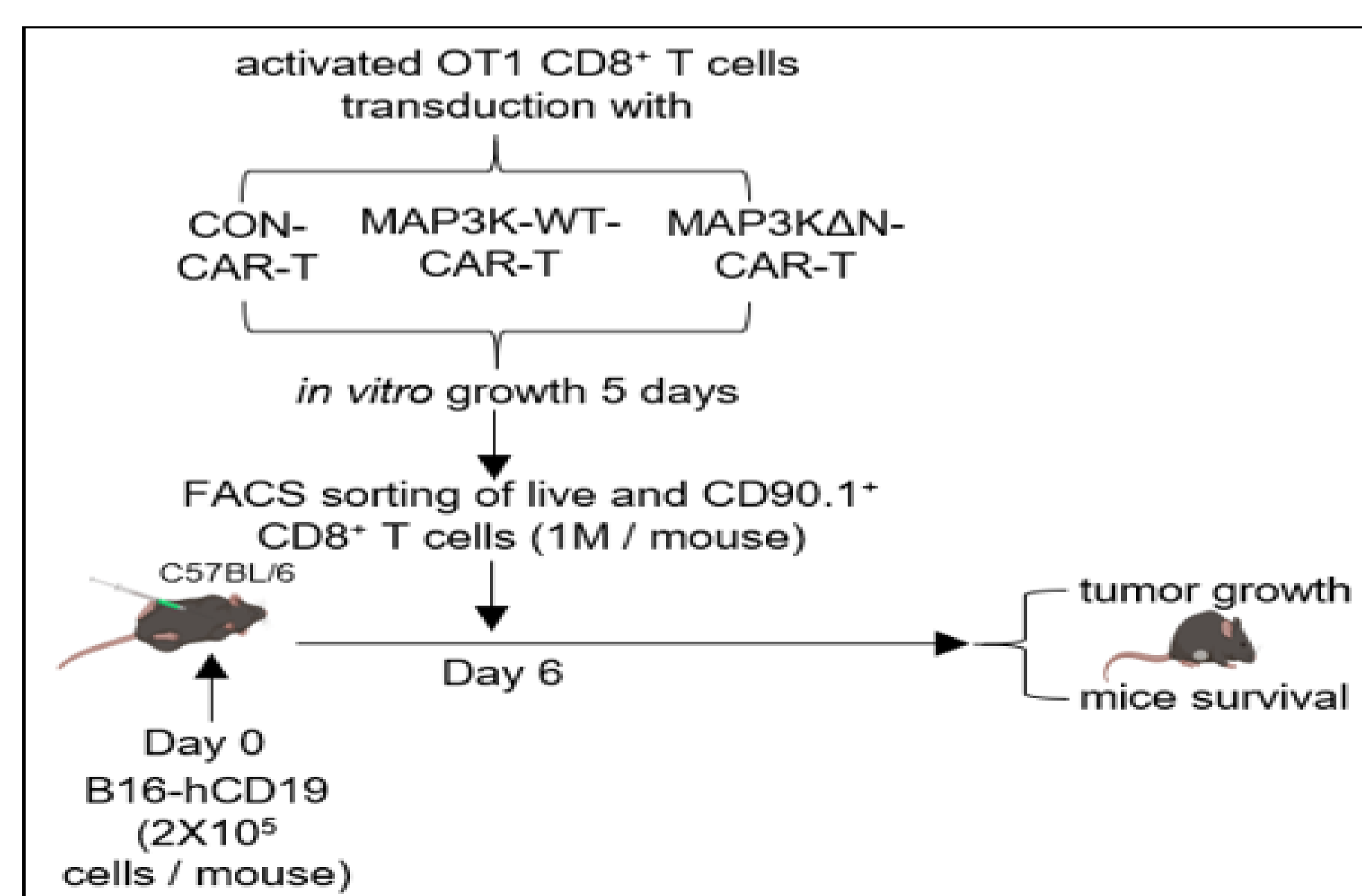
1. Construct anti-human CD19 CAR expressing MAP3KΔN through molecular cloning



2. Test expression by transient expression (transfection) in HEK293T cells and by infection (transduction) in OT1 CD8⁺ T cells



3. Generate mouse CAR-T cells by infecting CD8⁺ T cells with anti-hCD19 CAR-MAP3KΔN and examine tumor rejection using B16F10-hCD19 melanoma mouse models



Results

Restriction Enzyme Digestion of hCD19-MAP3KΔN-CAR

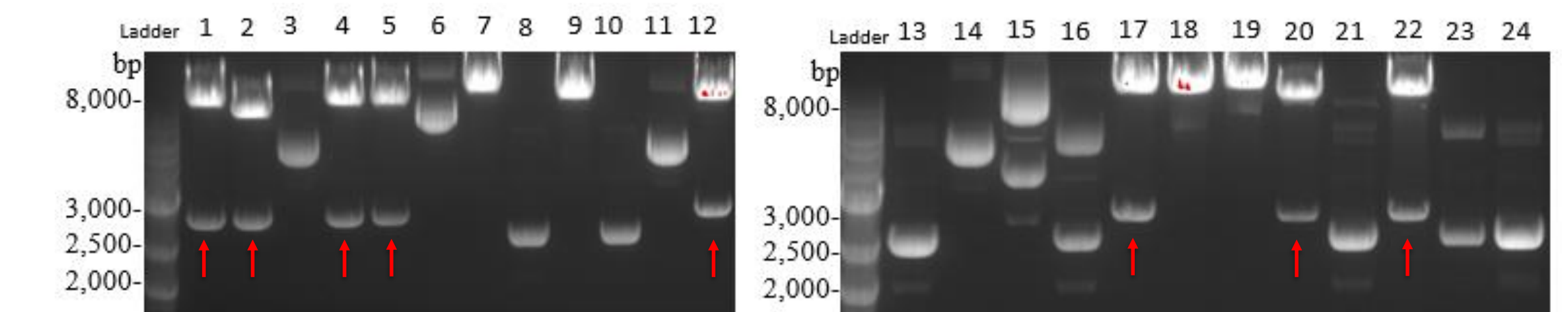


Figure 4. Restriction enzyme digestion confirmed successful synthesis of hCD19-MAP3KΔN-CAR (method 1). The patterns obtained for clones 1, 2, 4, 5, 12, 17, 20 and 22 showed the expected band sizes of approximately 3000 bp for MAP3KΔN and 8000 bp for hCD19-CAR.

Immunoblotting Analysis of HA tag in hCD19-MAP3KΔN-CAR



Figure 5. Immunoblotting analysis of HA protein tag expression in hCD19-MAP3KΔN-CAR plasmid (method 1). The plasmid containing the MAP3KΔN sequence has an additional HA tag and its detection allow us to confirm MAP3KΔN expression via immunoblotting. The presence of the protein HA associated with MAP3KΔN was identified in clones 1, 2, 4, 20, and 22. Additionally, Sanger sequencing confirmed the expected sequence for the plasmid (data not shown).

GFP Expression after OT1 Mouse CD8⁺ T Cells Transduction

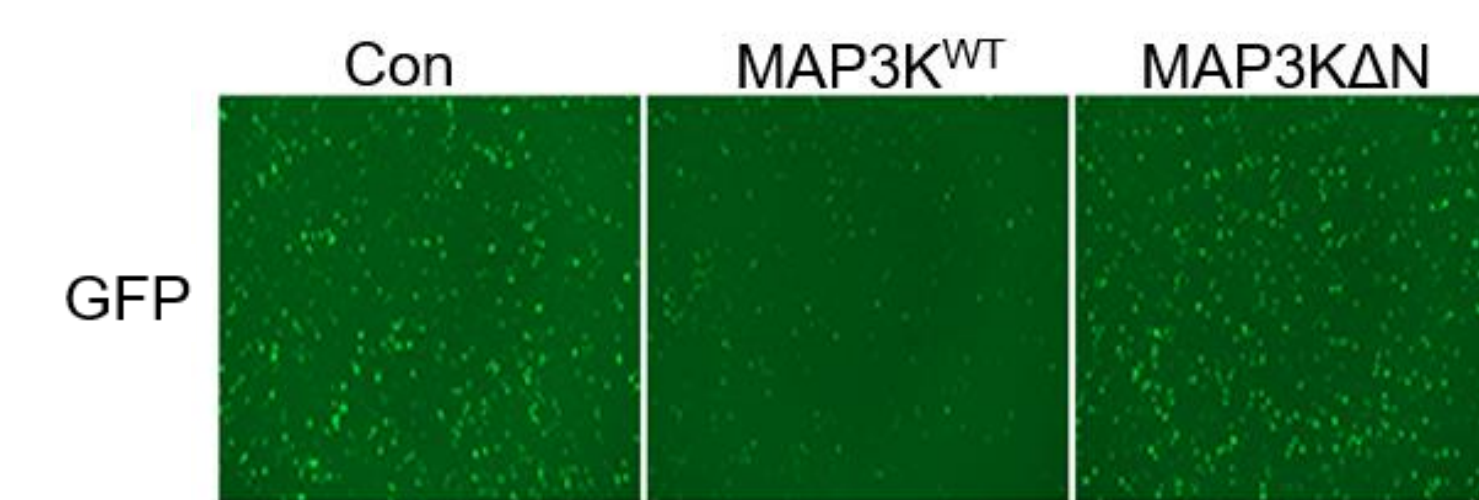


Figure 6. GFP expression in transduced OT1 mouse CD8⁺ T cells (method 2). CD8⁺ T cells from OT1 mice were isolated, activated, and transduced with the control CAR, MAP3K^{WT}-CAR and MAP3KΔN-CAR. The plasmids include a GFP construct and its expression allows to determine transduction efficacy. The three groups expressed GFP confirming adequate transduction.

MAP3KΔN Shows Similar Response to Control CAR, but MAP3K^{WT} Dramatically Reduces Tumor Burden

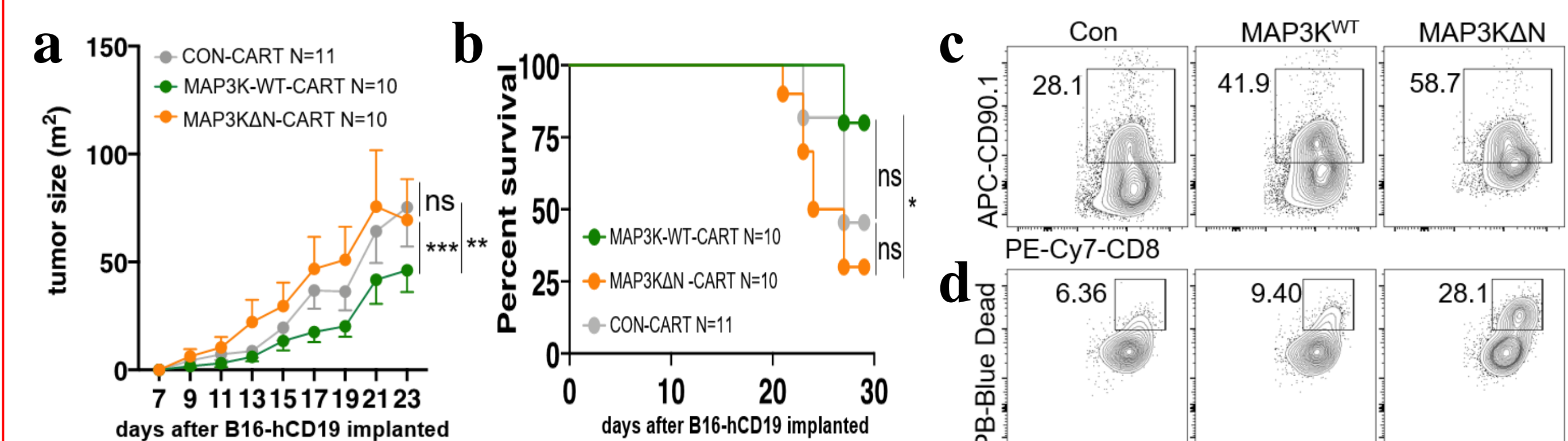


Figure 7. Expression of MAP3K^{WT} significantly slows down tumor progression and extends survival *in vivo* (method 3). C57BL/6 mice were subcutaneously injected with B16-hCD19 expressed cells (2×10^5 cells / mouse) on the ventral inguinal area and treated with MAP3K^{WT}, MAP3KΔN, and control CAR for the indicated time periods. **a.** Tumor growth curves highlight significant amelioration of tumor burden in B16 melanoma-bearing mice treated with MAP3K^{WT} compared to mice treated with MAP3KΔN. **b.** A survival graph underscores the success of MAP3K^{WT} to extend the lifespan of melanoma-bearing mice. **c.** Flow cytometric analysis after 3 days of *in vitro* culture of CAR-T cells using anti-CD90.1 monoclonal antibody (Thy-1.1), APC, to detect transduction efficiency. CD90.1 is a surface marker on the CAR-T cells that allows to distinguish them from B6 mice endogenous CD8⁺ T cells, which are CD90.2 positive. In this graph, MAP3KΔN shows the highest transduction efficacy with a 58.7% rate. **d.** Flow cytometric analysis after three days of *in vitro* culture of CAR-T cells using Blue Dead, PB, to identify dead cells in the samples. In this graph, MAP3KΔN displays a 28.1% of dead cells, which is much higher compared to the control CAR (6.36%) and MAP3K^{WT} (9.40%). **Surprisingly, these data aptly support MAP3K^{WT} as being a crucial promoter of CAR-T cell therapy compared to MAP3KΔN.** Summary data are mean \pm standard error of mean with P values being determined by two-way ANOVA with Bonferroni's post-test (a, b). * <0.05 , ** <0.01 , *** <0.001 , **** <0.0001 . Numbers in quadrants indicate percentage of cells (c, d).

Future directions

- Perform flow cytometric analysis of the tumor microenvironment after injection of MAP3KΔN-CAR-T, MAP3K^{WT}-CAR-T and control CAR-T.
- Analyze for potential cytokine storm or organ inflammation.
- Develop an inducible MAP3K^{WT} expression system to manipulate the metabolic activity and function of CAR-T cells based on temporary MAP3K^{WT} expression.
- Apply MAP3K^{WT} expression to tumor models beyond melanoma to test its universality.

Acknowledgements

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