ORT_03 - The c-Myc-tag role in anti-CD19 scFv labeling and CAR's function alteration: an *in silico*, *in vitro* and *in vivo* study

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Introduction: Chimeric Antigen Receptors (CARs) are recombinant proteins expressed on T lymphocyte's surface to redirect these cells' action against antigens expressed on target cells, usually the CD19 protein. Protein epitopes, like the c-Myc-tag, can be used as markers to facilitate detection and purification experiments of the CAR. The c-Myc-tag can be added to the CAR antigen recognition domain, which is commonly composed of a single-chain variable antibody fragment (scFv) responsible for interacting with the target antigen. So, it is necessary to assess whether this tag addition affects antigen recognition by the scFv.

Objective: To evaluate the role of a c-Myc-tag in scFv as a CAR marker and its possible interference in antigen recognition and tumor elimination.

Methodology: *In silico*, *in vitro*, and *in vivo* techniques were used to understand the interaction between c-Myc- tag-scFv and CD19. *In silico* studies involve homology modeling techniques, molecular docking, and Molecular Dynamics (MD) simulation. *In vitro* studies included assessment of CAR+ cell expansion and phenotype along with target cell killing. NSG mice engrafted with human GFP+/Luciferase+/CD19+ Nalm-6 leukemia cells were treated with different doses of CAR-T cells with or without the c-Myc-based tag. Tumor load was evaluated by biolouminescence and survival curves were generated.

Results: Analyzing the MD trajectories and the calculated Intermolecular Interaction Potential, it was possible to infer that c-Myc-tag maintains the linear structural characteristic that facilitates its detection by antibodies. However, in an MD time fraction, c-Myc-tag positions itself in the scFv structure in such a way as to cause a steric impediment to bind with CD19. It is also possible to infer that after c-Myc-tag-scFv/CD19 binding, c-Myc- tag can stabilize the complex. *In vitro* data showed no significant difference between CAR-T lymphocytes with or without the c-Myc-tag regarding memory and exhaustion phenotype or expansion levels. Interestingly, CAR-T cells without the c-Myc-tag performed significantly better *in vivo* in extending mice survival and controlling leukemia load.

Conclusion: Our *in silico* results shows that the c-Myc-tag presence in the scFv could cause steric impediment that affect the interface formation and consequently the interaction with the CD19. Our *in vivo* data suggest that CAR-T cells without the c-Myc-tag show greater anti-tumor responses, with clear implications for adoptive immunotherapy.

Keywords: CAR-T; c-Myc-tag; CD19