Preclinical development of MSLN-targeting CAR T cells for enhanced long-term in vivo efficacy

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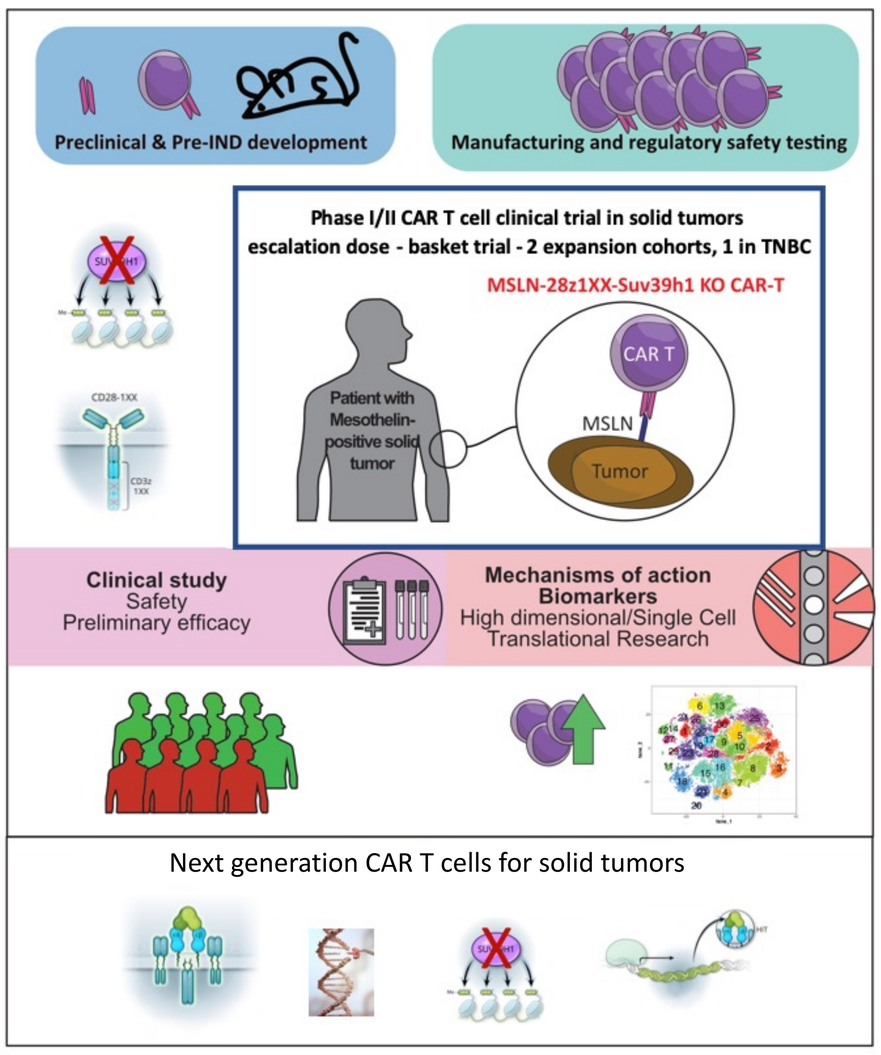


ABSTRACT

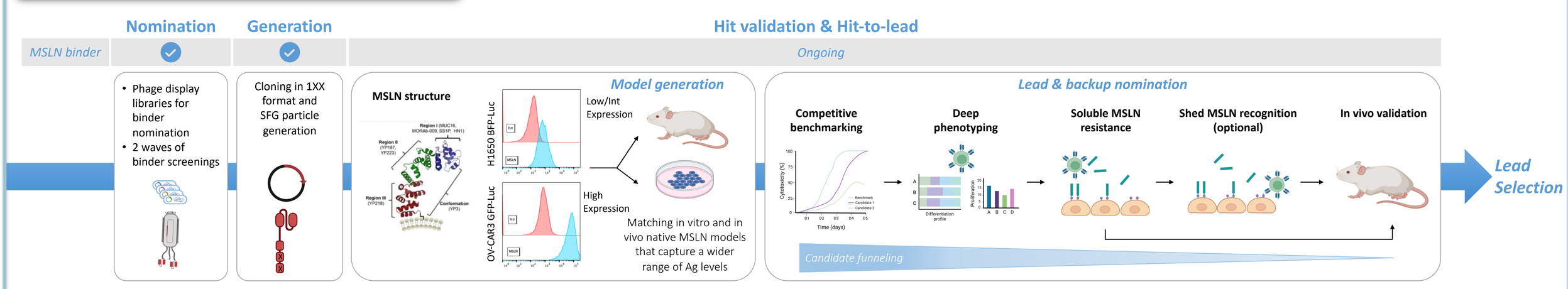
Mesothelin (MSLN) is a tumor differentiation antigen that is highly and recurrently expressed on a variety of human solid tumors with limited expression in healthy tissues and good clinical tolerability. However, clinical trials with MSLN-targeting chimeric antigen receptor (CAR) T cells have failed to show significant benefit due to limited T cell response and persistence in patients.

To address these issues, we are developing a cell therapy approach that combines a powerful CAR design, the 1XX-CAR, and epigenetic reprogramming towards enhanced T cell memory via inactivation of the histone methyltransferase SUV39H1. The 1XX-CAR was shown to promote strong antitumor response against liquid tumors both preclinically and in the clinic. We here show in a model system, that Crispr/Cas9-mediated inactivation of SUV39H1 enhances 1XX-CAR T cell functions. We used phage display library screenings and mouse immunizations to identify new and highly specific MSLN binders. These were then tested in the 1XX format in multiple functional assays, including an in vivo mouse model.

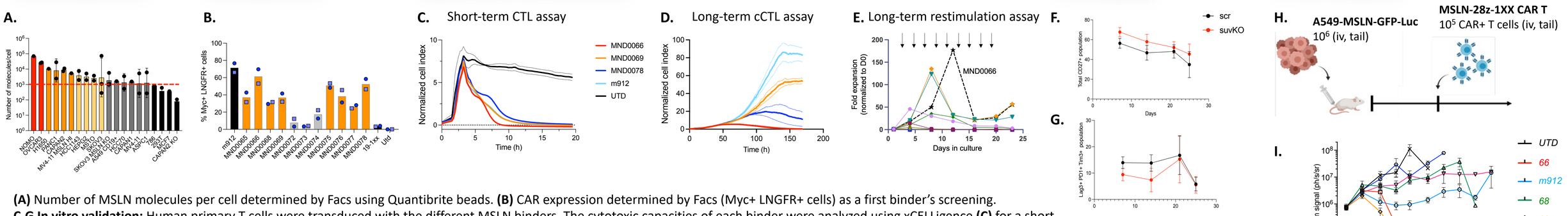
In parallel, we validated the safety of SUV39H1 inactivation by Crispr/Cas9, allowing further clinical development.

Our results suggest that MSLN-targeting 1XX-SUVKO CAR T cells have the potential to promote long-term tumor remissions in patients. Next steps will be to complete preclinical development, initiate clinical manufacturing, and finalize IND submission.

Mnemo's preclinical validation workflow



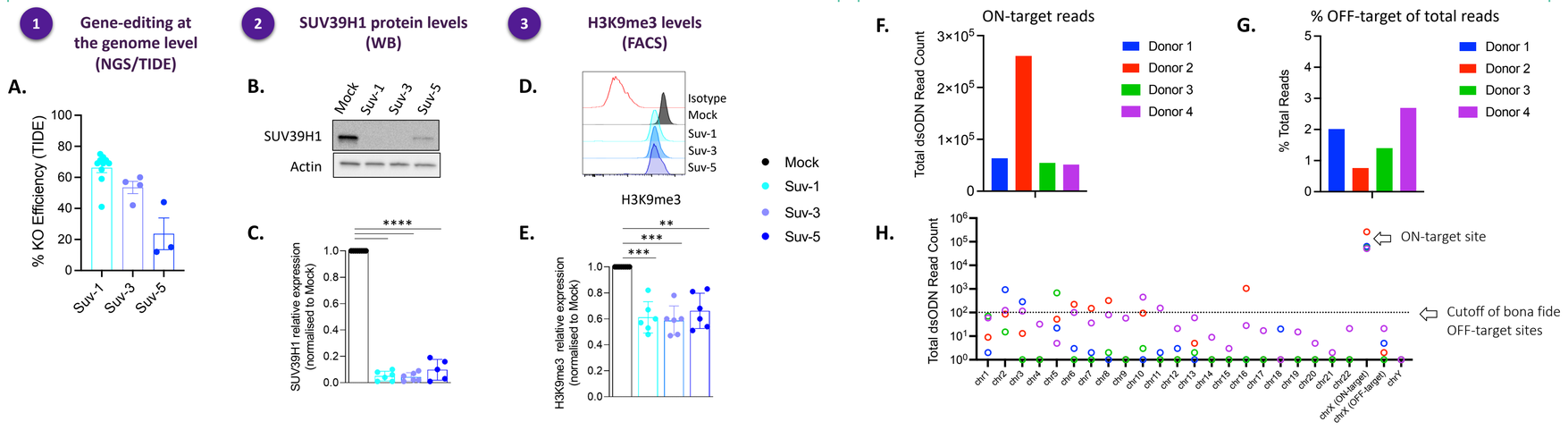
New and highly specific binders against MSLN can induce anti-tumor responses in vitro and in vivo (preliminary results)



(A) Number of MSLN molecules per cell determined by Facs using Quantibrite beads. **(B)** CAR expression determined by Facs (Myc+ LNGFR+ cells) as a first binder's screening.
C-G In vitro validation: Human primary T cells were transduced with the different MSLN binders. The cytotoxic capacities of each binder were analyzed using xCELLigence **(C)** for a short-term assay using MSLN overexpressing A549 cells (at 1:1 eff:target ratio), or **(D)** a long-term chronic CTL (cCTL) assay with OVCAR3 cells (at 1:20 eff:target ratio). **(E)** CAR T cell expansion was also measured by Facs in a long-term cytotoxic assay, in which effector cells were stimulated with Mv411 overexpressing MSLN tumor cells (at 1:2 eff:target ratio) and restimulated several times during the assay as soon as tumor cells were lysed. **F-G.** Phenotypic (**F**, CD27+ cells) and activation markers (**G**, Lag3+PD1+Tigit+) analyzed at different time during restimulation assay with MSLN over expressing Mv411 for MSLN-CAR T cells either treated with scrambled gRNA loaded (scr) or with SUV39H1 gene targeting gRNA loaded (suvKO) Cas9.
H-I. In vivo validation: (H) A549-MSLN-GFP-Luc tumor cells were injected iv into the tail vein (10^6/mouse). MSLN-28z-1xx CAR T cells were manufactured and frozen at Mnemo NYC. CAR-T were thawed and injected (10^5/mouse) by CRO 20 days after tumor cells injection and randomization of mice. **(I)** Tumor growth was followed in vivo by bioluminescence up to D90 post tumor injection.

Selection of SUV39H1-targeting gRNA for clinical development

In silico selection of top gRNAs → *In vitro validation of top gRNAs for KO efficiency* → *GUIDE-seq for top gRNAs for OFF-target assessment* → *Selection of top gRNAs for GMP synthesis*



Suv-1 gRNA consistently shows increased KO efficiency of SUV39H1 at the genomic and protein levels
Human primary T cells were isolated form PBMCs of 6 donors (3 males and 3 females), electroporated with Cas9 (Aldevron) and three different guide-RNAs targeting SUV39H1 (IDT).
Analysis of the KO efficiency of SUV39H1 was performed :
A. At the genome level by NGS/TIDE for the three gRNA.
B,C. At protein level by WB (**B**. one representative donor and, **C**. quantification for the 6 donors).
D,E. The downregulation of H3K9me3 was measured by Facs (**D**. one representative donor, **E**. quantification for the 6 donors).

Suv-1 gRNA results in high ON-target gene-editing with minimal OFF-target editing sites
Human primary T cells were isolated form PBMCs of 4 donors (2 males and 2 females), electroporated with GMP-grade Cas9 and the Suv-1 gRNA targeting SUV39H1, and a dsODN to perform GUIDE-seq.
Sequencing was performed by Creative Biogene to analyze ON- and OFF-target editing of the three different gRNA. The results for Suv-1 gRNA are shown
F. Total dsODN read count
G. Pourcentage OFF-target of total reads
H. Total dsODN read count

Conclusions

- A total of 64 MSLN binders have been generated (humanized scFvs and VHHs) and synthetized in a 28z-1xx CAR format and in a retroviral vector (SFG).
- Two models of tumor cell line naturally expressing MSLN at different levels are being developed for in vitro and in vivo analyses in order to measure efficiency of binders against a wide range of Ag levels, while different in vitro and in vivo assay have been setup to analyze CAR expression, cytotoxicity, memory phenotype and exhaustion of CAR+ T cells.
- Preliminary results show that our new MSLN binders show strong in vitro cytotoxicity, demonstrate enhanced memory phenotype with SUV39H1 inactivation, and can reject MSLN expressing tumor cells in vivo.
- Treatment with GMP-grade Cas9 and SUV39H1 gRNA shows high ON-target and low OFF-target gene-editing.

	Preclinical			*Lead Selection*	*Pre-IND*	*IND*
	Nomination	**Generation**	**Hit Validation**	**Hit-to-Lead & Optimisation**	**Safety**	**GMP manufacturing**
MSLN Binder	✓	✓	✓	*Ongoing*	*Pending*	*Depends on Lead Selection & Safety*
*Lentiviral Vector**	✓	*Ongoing*			✓	
*Crispr/Cas9**	✓	✓	✓	✓	✓	*sgRNA: Ongoing Cas9: Off-the-shelf*

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