Rational design of pH-dependent MSLN-targeting VHHs for CAR-T therapy

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SUMMARY

CAR-T cell therapies have achieved tremendous progress in hematological tumors; however, limited efficacy was observed in solid tumors. One of the critical challenges in solid tumors was the risk of clinically on target off tumor (OTOT) toxicity due to the recognition of normal tissues expressing the target antigen[1]. The extracellular acidic characteristic of tumor tissues presents a novel mechanism to achieve tumor specificity[2].

In this study, we employed a structure-based computational approach to engineer anti-MSLN (mesothelin) VHHs with selective binding under acidic tumor microenvironment condition using methods developed as part of VHHMAb® platform. Through in silico dual-pH His/Asp/Glu-scanning mutagenesis of the complementarity-determining regions (CDRs) and paratopic amino acids, we optimized the VHH for acid pH selectivity.

Testing of 20 designed variants identified four variants with significant binding selectivity toward acidic pH. Notably, one variant (MT001) exhibited significant loss of binding at physiological pH, while retaining binding activity under acidic condition in protein binding assays such as SPR. Similar pH-dependent behavior was confirmed using FACS assays at the cellular level. Furthermore, when incorporated into a chimeric antigen receptor (CAR) construct, MT001 conferred pH-dependent cytotoxicity to CAR-T cells, with enhanced cell-killing efficiency at acidic pH compared to neutral pH. This pH dependence was also observed in other CAR-T activation measures, such as CAR-T cell expansion and cytokine release after co-culture with MSLN+ tumor cell lines.

This study demonstrates the feasibility of computational optimization of antibodies for selectively targeting the acidic tumor microenvironment, representing a potential approach for developing safer CAR-T therapeutics.

METHOD

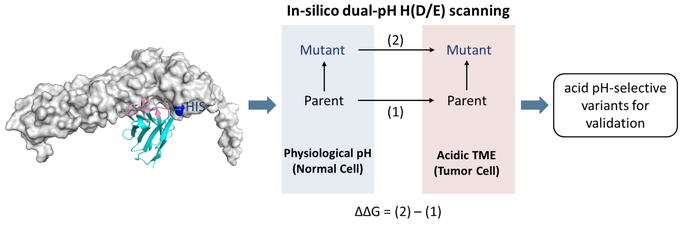
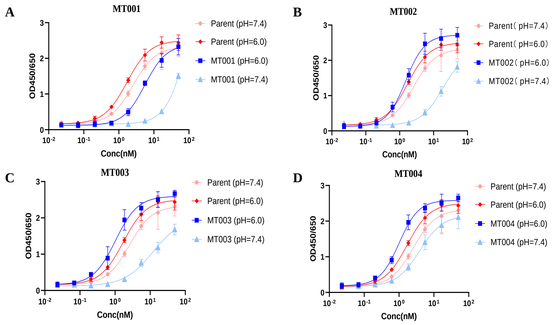


Fig.1 Strategies for engineering of acid pH selective variants. Based on MSLN-parent VHH complex structure, residues from CDRs and paratope were used for in-silico dual pH His(Asp/Glu) scanning.

RESULTS



E

Variant	pH6.0			pH7.4			pH7.4/pH6.0 ratio
	ka (1/Ms)	kd (1/s)	KD (M)	ka (1/Ms)	kd (1/s)	KD (M)	
Parent	4.57E+06	3.47E-03	7.59E-10	7.25E+05	7.62E-04	1.05E-09	1.3
MT001	8.35E+05	5.31E-03	6.36E-09	No binding			N.A.
MT002	5.75E+06	9.08E-03	1.58E-09	3.62E+06	2.66E-02	7.33E-09	4.6
MT003	8.10E+05	4.36E-03	5.37E-09	4.01E+05	3.82E-03	9.51E-09	1.8

Fig.2 Acid pH-selective binding of variants to MSLN protein using dual-pH ELISA and SPR. 4 variants showed differential binding activity at pH6.0 vs pH7.4. Notably, MT001 exhibited significant loss of binding at physiological pH. **A-D** Binding of purified VHHs using indirect ELISA at pH6.0 and pH7.4. Error bars represent standard deviations between 3 technical replicates. **E.** SPR data for MSLN parent VHH and its 3 variants under pH6.0 and pH7.4. VHH variants as the analytes over immobilized MSLN, data were analyzed using Langmuir binding models for fitting.

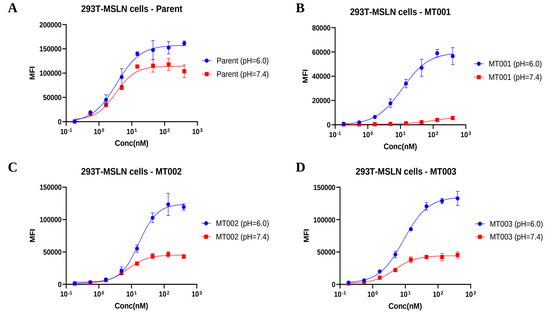


Fig.3 Acid pH-selective binding of variants to MSLN-expressing 293T cells. MT001 exhibited significant loss of binding at physiological pH. **A-D** Binding of purified VHHs using flow cytometry. VHHs were tested for the ability to bind MSLN transfected cell line (293T-MSLN) at different pH. Error bars represent standard deviations between 3 technical replicates.

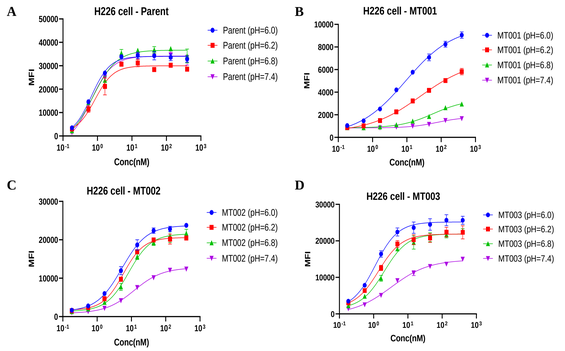


Fig.4 Acid pH-selective binding of variants to H226 cells. MT001 exhibited significant loss of binding at physiological pH. **A-D** Binding of parent and mutant VHHs to H226 cell line at different pH conditions by flow cytometry. Error bars represent standard deviations between 3 technical replicates.

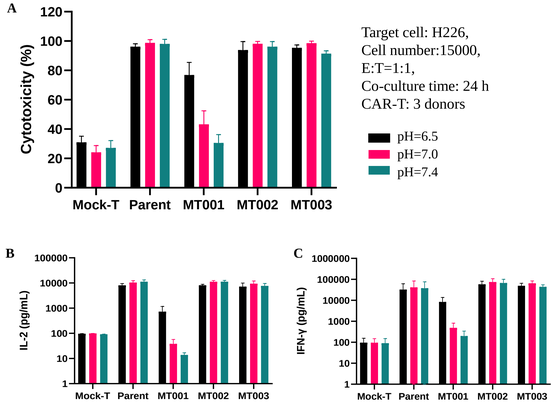


Fig.5 Acid pH-selective cytotoxicity and activation of CAR-T with MT001. A. MT001 CAR-T cells conferred significant pH-dependent cytotoxicity at 3 different pH conditions when targeting H226 cells. It can kill target cells efficiently only at acidic pH (pH 6.5), but not physiological pH. **B-C.** Similar trend was observed for cytokine release after co-culture with H226 cells. Error bars represent standard deviations between 3 donors.

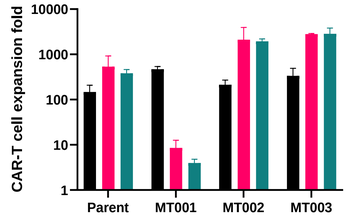


Fig.6 MT001 conferred acid pH-selective proliferation to CAR-T cells. CAR-T cells were co-cultured with H226-PDL1 cell at 1:1 E:T ratio, and target cells were replenished every 5 days. CAR-T cell number was detected after each round of stimulation, and accumulated CAR-T cell expansion after 4 rounds was shown. MT001 showed very low level of expansion at physiological pH but high expansion at acidic pH, which is consistent with its pH-dependent binding affinity. Other groups showed lower expansion at acidic pH, probably due to the inhibition effect of acidic pH on T cell proliferation. Error bars represent standard deviations between 3 donors.

CONCLUSION

1. Four out of 20 designed MSLN VHH variants demonstrate acid pH-sensitive targeting of MSLN, showcasing the high effectiveness of our structure-based computational design approach.

2. MT001 exhibits strong binding selectivity in acidic conditions and loss of binding at physiological pH.

3. Enhanced CAR-T efficacy: MT001-based CAR-T cells show improved cytotoxicity, activation and proliferation in acidic conditions compared to physiological pH.

REFERENCE

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