401 HINGE LENGTH: A NOVEL METHOD OF PREDICTING CYTOTOXICITY OF CAR CONSTRUCTS AGAINST ANTIGEN-LOW LEUKEMIA

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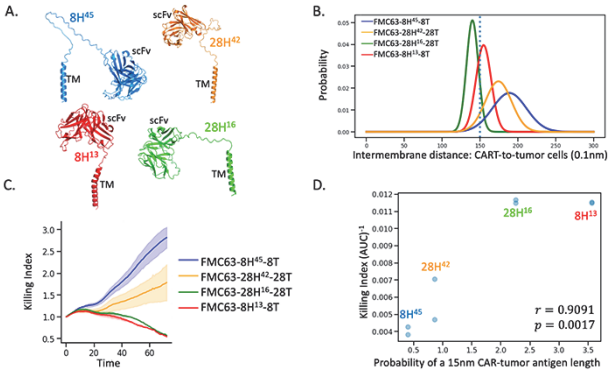
Background Genetic engineering of T-cells to target tumors through the expression of synthetic chimeric antigen receptors, or CARs, has led to a breakthrough in the treatment of relapsed/refractory B-cell leukemia. However, despite impressive initial clinical performance, 30-50% of patients eventually relapse, with the emergence of tumor cells expressing the targeted antigen at a level that is insufficient to induce CAR T responsiveness. We and others have shown that the hinge domain of CARs is critical in altering cytotoxic responsiveness of the CAR.[1,2] Thus, we evaluated whether optimal hinge length could be evaluated for a given scFv *in-silico*, given the epitope location, thereby accelerating optimal CAR design.

Methods Golden gate assembly was utilized to generate anti-CD19 (FMC63) and anti-CD22 (M971) CAR constructs. The lengths of the CD28 and CD8-alpha hinge domains were varied while intracellular 4-1BB and TCR-zeta signaling sequences remained constant. CAR structures were modeled with Alpha-Fold and intermembrane lengths modeled using Xplor-NIH. CAR function was evaluated by ex vivo cytotoxicity assays (Incucyte) against NALM6 cells engineered to express calibrated numbers of CD19 and CD22 molecules.

Results Through truncations and extensions of the CD28 and CD8-alpha hinge domains, we show that optimal hinge lengths for targeting CD19 and CD22 through their respective FMC63 and M971 scFvs are distinct and depend on the epitope location. Specifically, short and long hinges enhance cytotoxicity against membrane-distal epitope and membrane-proximal epitopes, respectively. Using Xplor-NIH and Alphafold (figure 1A-1B), we were able to model hinge dynamics. As signaling of the TCR by MHC-presented peptide antigen (pMHC) is tightly regulated by the dimensions of the TCR-pMHC interaction, with an optimal intermembrane distance of 15nm,[3] we assessed whether this distance would confer enhanced function to CAR T-cells following ligand encounter. Importantly, CAR constructs providing a predicted intermembrane CAR-ligand distance of 15nm exhibited enhanced cytotoxicity against antigen-low leukemic cells (figure 1C-1D).

Conclusions CAR responsiveness against a specific epitope can be modeled as a function of intermembrane distance, allowing a rapid optimization of CAR constructs by adjusting hinge length. The modeling presented here, based on epitope location and target protein dynamics, can be utilized to rapidly design CARs with optimized cytotoxic potential against a wide range of novel targets.



Abstract 401 Figure 1 Modeling of intermembrane CART-tumor cell distances promotes the generation of CAR constructs with increased cytotoxic potential. A: Alphafold-generated models of anti-CD19 (FMC63) CAR constructs reveal apparent differences in hinge length. CD8 hinge = 8H; CD28 hinge = 28H; TM=transmembrane domain. Superscripts refer to the number of amino acids within the hinge. B: A distance of 15nm has been shown to optimize signaling secondary to TCR-MHC interaction3. Distributions of the end-to-end distances of hinges of variable lengths were combined with the distribution of the CD19 target epitope to predict the probability that the combined distribution is 15nm, as evaluated by Xplor-NIH. C: Anti-CD19 CAR constructs with altered hinge lengths exhibit significant differences in ex vivo killing, as evaluated against a CD19low leukemia cell line. Killing index was evaluated in an Incucyte assay and is presented as a function of time (E:T ratio = 1:2). D: Killing indices of anti-CD19 CAR constructs correlate significantly with the modeling prediction of a 15nm optimum CAR-antigen intermembrane length. Data are from two replicates and the identity of the hinges are indicated.

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