Chimeric Antigen Receptors Incorporating Novel (non-scFv) Binding Domains Targeting CD123 Direct Potent Antitumor Activity of T Cells: Correlation Between Affinity and Activity

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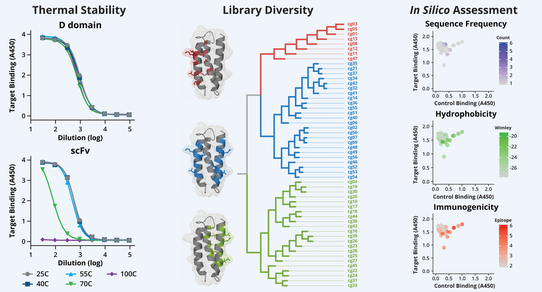
Abstract 3243

Introduction

D Domains Constitute a Robust Scaffold for Rapid Development of Targeting Domains



- Conventional chimeric antigen receptors utilize an antibody-derived scFv as the targeting domain
- D domains are 73 aa in length (approximately 1/3 the size of a scFv) and contain no disulfide bonds or native glycosylation[1]
- Rapid folding of the scaffold[2] imparts exceptional thermal stability **(left)** while simultaneously accommodating a high number of residue substitutions
- Multiple library designs of the triple alpha helical structure, translate into a high degree of paratope and sequence diversity **(center)**
- Combined *in vitro* and *in silico* screens of phage library clones afford predictive assessments of D domain target specificity, sequence diversity, expression and immunogenicity **(right)**
- D domains are selected for low potential immunogenicity and are ultimately engineered to produce an Abzena EpiScreen™ score of zero[3]

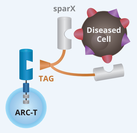
The ARC-sparX Platform

Conventional CAR-T cell therapies often target tumors through a mono-specific receptor that is constitutively expressed. To improve the flexibility and control of T-cell response, we developed the ARC-sparX platform, which separates the antigen recognition and killing functions of the conventional CAR-T cell.

The ARC-sparX platform is comprised of two key components:

1. sparX (**s**oluble **p**rotein **a**ntigen-**r**eceptor **X**-linker) protein: binds specific antigens on diseased cells and flags those cells for destruction.
2. ARC-T Cells (**A**ntigen **R**eceptor **C**omplex **T** Cells): bind sparX proteins and kill flagged cells.

Tri-complex (ARC-T + sparX + target cell) must be formed to activate cytolytic killing.



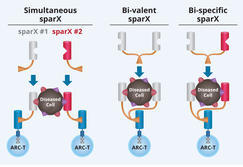
sparX protein

- No inherent activity
- Mono-valent affinity comparable to that of scFv
- Engineered to minimize immunogenic potential
- **"TAG"** is a fragment of human alpha fetoprotein (AFP)

ARC-T Cell

- Conventional CAR architecture incorporating an anti-TAG D domain
- Receptor for **"TAG"** has affinity in low nanomolar range
- Only activated upon formation of tri-complex (ARC-T + sparX + antigen-expressing target cell)
- Same viral vector regardless of antigen target

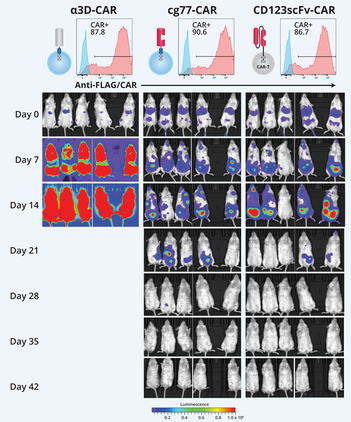
sparX Can Be Mono-valent, Multi-valent, or Multi-specific



- Can be given in combination or in sequence
- Addresses low density or heterogeneous expression of antigen
- Bi-specific sparX proteins support "AND-gated" as well as "OR-gated" targeting of diseased cells

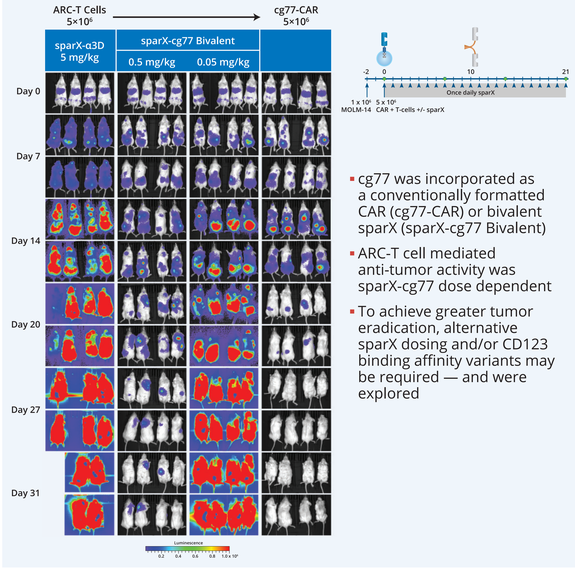
Results

CAR T Cells Incorporating D Domains Control AML Tumor Growth *In Vivo*



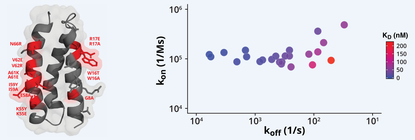
- A CD123-binding D domain was identified through phage display methods and further deimmunized to remove predicted T cell epitopes (cg77)[1]
- Detection of N-terminal Flag epitope by flow cytometry demonstrated comparable percent positive CAR expression on T cells between non-binding D domain CAR (a3D-CAR), cg77-CAR and a 32716-scFv-based CAR targeting CD123 (CD123scFv-CAR)
- AML-derived, CD123-positive MOLM14 tumor cells (3x10^6) were administered systemically 1 day prior to administration of 3x10^6 a3D-CAR, cg77-CAR and CD123scFv-CAR transduced primary human T cells
- Both cg77-CAR and CD123scFv-CAR eliminated detectible levels of tumor cells as measured by IVIS imaging

ARC-sparX Platform Incorporating D Domains Control AML Tumor Growth *In Vivo*



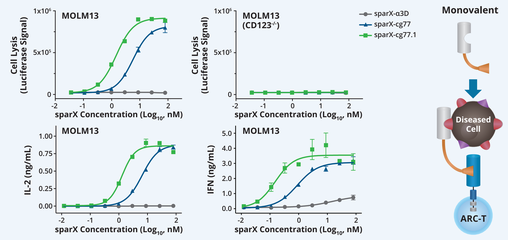
- cg77 was incorporated as a conventionally formatted CAR (cg77-CAR) or bivalent sparX (sparX-cg77 Bivalent)
- ARC-T cell mediated anti-tumor activity was sparX-cg77 dose dependent
- To achieve greater tumor eradication, alternative sparX dosing and/or CD123 binding affinity variants may be required — and were explored

D Domains are Affinity Matured Through Mutagenesis



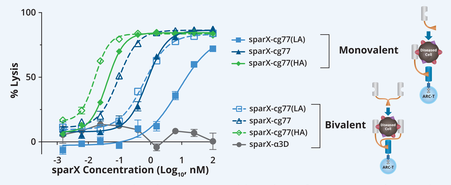
- A CD123-binding D domain was subjected to mutagenesis to identify point mutations that could either increase or decrease binding affinity
- A homology model of the scaffold illustrates the location of representative mutations in red **(left)**
- D domain mutants were expressed as monovalent MBP-fusions and binding kinetics were assessed by surface plasmon resonance **(right)**
- K_D values for binding of CD123-HIS to D domains spanned a 150-fold range (1.4 nM to 212 nM), with off-rate contributing more to the distribution than on-rate, suggesting these mutations impact stabilization of the complex, once formed

sparX Incorporating High Affinity D Domain Enhances Potency of ARC-T Cells



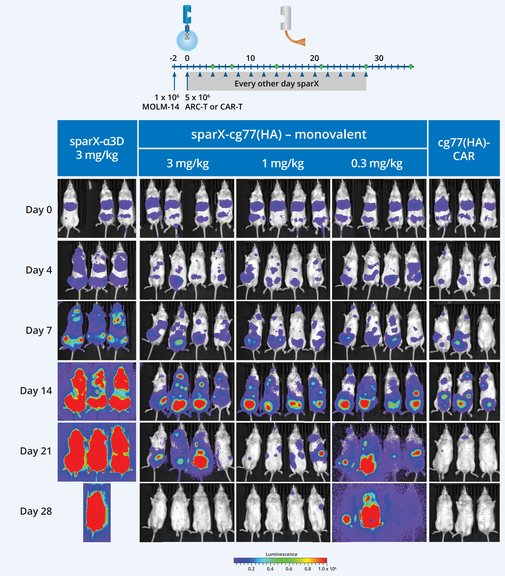
- Monovalent sparX were generated with the cg77 D domain or a high-affinity variant (sparX-cg77.1), with an approximately 10-fold lower K_D, as measured by SPR
- As measured by luciferase signal, ARC-T cell mediated lysis of AML-derived MOLM13/luciferase cells requires CD123-binding sparX **(top left)**, CD123 expression on target cells **(top right)** and ARC-T cells (not shown)
- ARC-T cell mediated target cell lysis **(top left)**, IL-2 (bottom left) release and IFN release **(bottom right)** are dose and affinity dependent
- As measured by EC50 and as compared to the parental sparX-cg77 the high-affinity variant (sparX-cg77.1) demonstrated enhanced potency for target cell lysis (4-fold increase), IL-2 release (5-fold increase) and IFN release (6-fold increase)

Combinations of Affinity and Valency of D Domain Can Be Used to Further Broaden Dynamic Range of sparX



- sparX were generated in both mono and bivalent formats, utilizing the parent cg77 D domain or variants incorporating high affinity (HA) or low affinity (LA) mutations
- ARC-T cell mediated lysis of CD123-expressing, NALM6 cells is modulated by CD123-binding sparX
- The extent of target cell lysis is dose-dependent for each sparX and collectively the EC$_{50}$s span 3 logs
- Low affinity sparX manifest greatest gain in potency when formatted as a bivalent
- The high affinity, monovalent sparX (sparX-cg77 [HA]) outperforms the bivalent sparX-cg77

Monovalent, High-Affinity CD123 sparX Eliminates Detectable AML Tumors *In Vivo*



- A high affinity CD123 D domain is incorporated into monovalent sparX (sparX-cg77[HA]) and a conventionally formatted CAR (cg77[HA]-CAR)
- *In vivo* anti-tumor activity of MOLM14 tumors by ARC-T is dependent on sparX dose
- Cohorts receiving 1 and 3 mg/kg sparX-cg77(HA), every other day, achieved comparable anti-tumor activity to that of the conventionally formatted CAR

Conclusions

- As exemplified by D domains discovered and optimized to bind CD123, D domains constitute a robust scaffold for development of therapeutic targeting domains
- CAR T cells comprised of D domains targeting CD123, direct potent *in vivo* anti-tumor activity of AML-derived tumors comparable to that of a conventional, scFv-based CAR
- The ARC-sparX platform affords the ability to modulate T-cell activity by controlling the specificity, dose, affinity and valency of the sparX protein
- sparX proteins targeting CD123, in combination with ARC-T cells, inhibit the growth of AML-derived tumors *in vivo* and may offer a novel therapeutic approach for treating AML
- More broadly, the ARC-sparX platform affords an opportunity for treating other challenging cell therapy indications where greater T-cell control may be warranted

References

1. Qin, Haiying, et al. "Chimeric antigen receptors incorporating D domains targeting CD123 direct potent mono- and bi- specific antitumor activity of T cells." *Molecular Therapy* 27.7 (2019): 1262-1274.
2. Zhu Y, Alonso DO, Maki K, et al. Ultrafast folding of alpha3D: a de novo designed three-helix bundle protein. *Proc Natl Acad Sci* U S A. 003;100(26):15486-15491. doi:10.1073/pnas.2136623100.
3. Baker MP, Jones TD. Identification and removal of immunogenicity in therapeutic proteins. *Curr Opin Drug Discov Devel.* 2007;10(2): 219-227.

Presented at the American Association for Cancer Research 2020 Virtual Annual Meeting II, June 22-24, 2020