Tailoring Cytolytic Activity, Proliferation and Cytokine Release via CD3 Engineering of DART® Molecules for Redirected T-cell Killing

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*** Equal contribution**

Abstract

Introduction: CD3-engaging bispecific molecules mediate potent redirected T-cell killing. As with CAR-T cells, the potent killing mediated by these molecules is associated with cytokine release. While severe cytokine release effects can be mitigated through dosing strategies and anti-cytokine antibodies, an expansion of the therapeutic window of these molecules is desirable. Several novel CD3-targeted bispecific DART candidates are currently undergoing clinical testing, with promising initial evidence of anti-cancer activity. Considering that the strength of T-cell receptor signaling, naturally regulated by the affinity of the peptide/MHC complex for its cognate receptor, can induce both quantitative and qualitative changes in responder cells, we explored signal modulation through modification of the anti-CD3 arm of the DART molecule to differentiate cytolytic activity and proliferation from cytokine release.

Methods: A panel of anti-CD3 variants spanning a wide range of affinities was generated, converted to DART formats with three different tumor targeting arms and benchmarked against the parental wild-type CD3 arm DART molecule.

Results: In general, decreasing the affinity for CD3 decreased the DART molecules' cytolytic activity. Analysis of a subset of representative DART molecules across the affinity range showed variants with a much larger decrease in target-induced cytokines (particularly IL-2 and IFN-γ) than in cytolytic or proliferative activity. Furthermore, the relationship between killing activity and affinity was not absolute, as selected variants with nearly identical affinities showed contrasting properties for cytolysis and cytokine release. A variant with reduced cytolytic potency but comparable maximum killing as the parental molecule was active in vivo in PBMC-reconstituted tumor-bearing mice, but showed only modest target-induced cytokine release in vitro and in vivo. In conclusion, modulating the binding properties of the CD3 arm can tailor the pharmacological profile of T-cell engaging DART molecules to retain maximum killing while minimizing the potential for untoward effects via cytokine release.

Introduction

- **Redirected T-cell killing via bispecific molecules delivers potent cytolytic activity against liquid and solid tumors**
  - **Cytokine release, however, may limit the therapeutic window**
- **Can cytolysis be uncoupled from cytokine release?**
  - **Different T-cell responses can be triggered by changing the strength of T-cell receptor signaling**
  - **Furthermore, CD3-engaging bispecific DART proteins mediate cytolysis with greater potency than cytokine release**

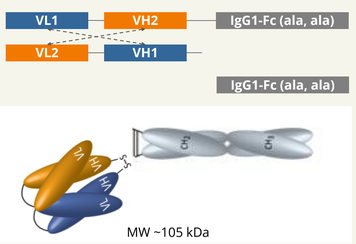
Hypothesis:

- **Broaden the therapeutic window by modifying the T-cell receptor's signaling strength via modification of the anti-CD3 arm of the DART molecule**

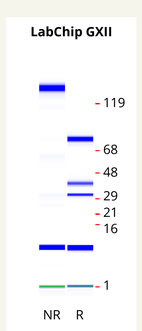
Approach:

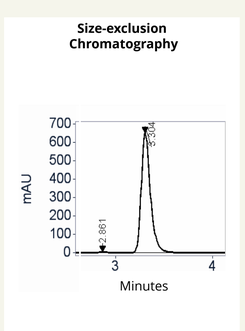
- **Generation of affinity variants of the CD3-binding arm with different kinetic properties**
- **Incorporation into Fc-bearing DART molecules directed against multiple tumor-associated antigens**

Fc-bearing DART Molecules



- **Bispecific diabody**
- **Covalently linked via disulfide bond at carboxyl end**
- **E/K coil to promote heterodimerization**
- **Linked to huIgG1 (234A,235A) Fc domain**





Methods

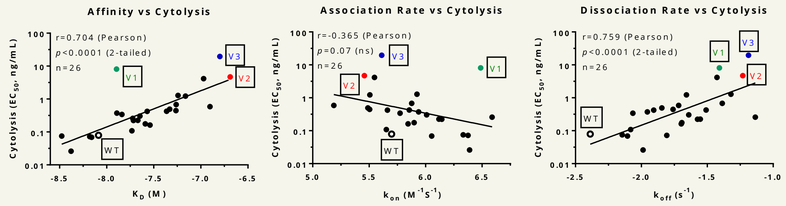
- A humanized CD3 mAb (hXR32) scFv saturation-mutant library at 29 CDR positions was expressed in E. coli (XL-1 Blue)
- A multi-well format was used to produce soluble scFv incorporating a C-terminal His-tag
- scFv in the supernatants were captured on immobilized anti-His and screened for binding to recombinant CD3 (ε/δ chain Fos/Jun heterodimer) using an Attana biosensor
- Twenty-six variants were converted to a basic (no Fc domain) CD123 x CD3 DART format and characterized for their CD3 binding kinetics, cytolytic activity and cytokine release
- A subset of variants were converted to Fc-bearing DART molecules with CD123- or 5T4-targeting arms and further characterized
- Purified DART molecules were assessed for homogeneity (SDS-PAGE and SEC, above)

Fc-bearing DART molecules (MW ~105kDa) : 1 nM = ~0.1 ng/mL; basic dart molecules (MW ~59 kDa): 1nM = 0.2 ng/mL

Results

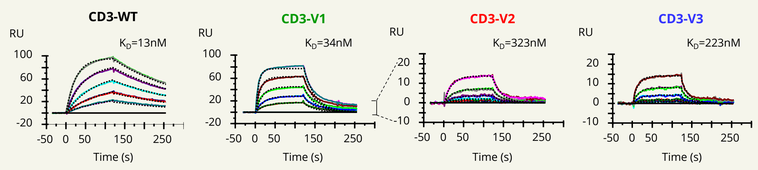
The affinity of the CD3-binding arm correlates with the cytolytic potency of DART molecules

Relationship primarily driven by the dissociation rate



Affinities for immobilized recombinant CD3 (ε/δ chain Fos/Jun heterodimer) of 26 CD3-binding arm variants in the DART format with a CD123-targeting arm were measured by BIAcore surface plasmon resonance and correlated with their activity in an 18-h LDH-release cytotoxicity assay with purified T cells as effectors and CD123-positive MOLM-13 as target cells (E:T = 5:1)

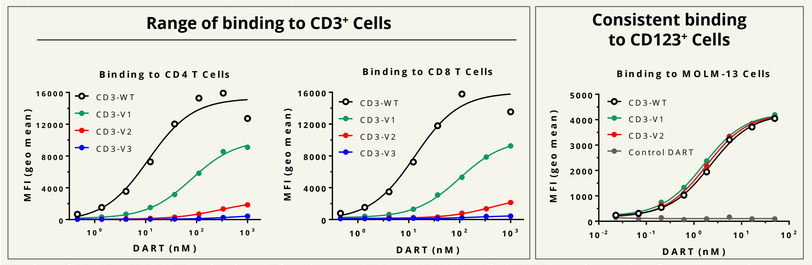
Three variants selected for further characterization: V1, fast k_{on}; V2 & V3, high K_D



BIAcore analysis was performed as described above

DART molecules with low-affinity anti-CD3 arms show limited binding to T cells

Opportunity for target-dependent, avidity-driven interaction

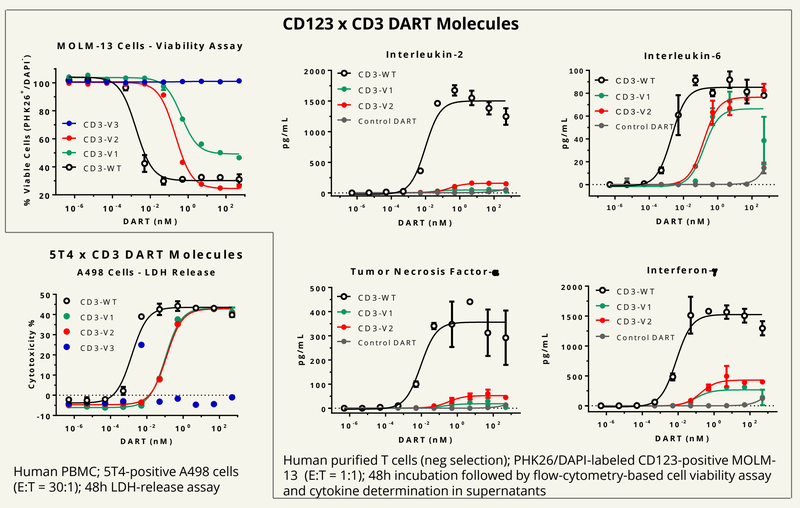


Binding of CD123 x CD3 DART molecules to T lymphocytes (CD3) or MOLM-13 cells (CD123) was detected via flow cytometry by using a DART molecule-specific biotinylated anti-EK-coil mAb followed by streptavidin-APC

A low-affinity CD3 DART variant retains full cytolytic potential with reduced cytokine release

Observation consistent across two independent targets (CD123 & 5T4)

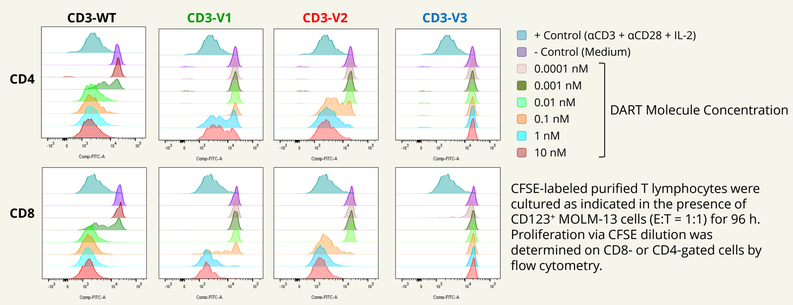
K_D relationship not absolute: Active (V2) & inactive (V3) variants with similar K_D



Human PBMC; 5T4-positive A498 cells (E:T = 30:1); 48h LDH-release assay

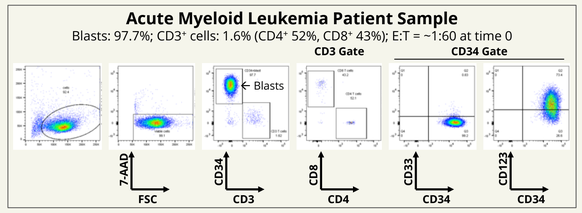
Human purified T cells (neg selection); PHK26/DAPI-labeled CD123-positive MOLM-13 (E:T = 1:1); 48h incubation followed by flow-cytometry-based cell viability assay and cytokine determination in supernatants

Active low-affinity CD3 DART molecules induce target-dependent T-cell expansion

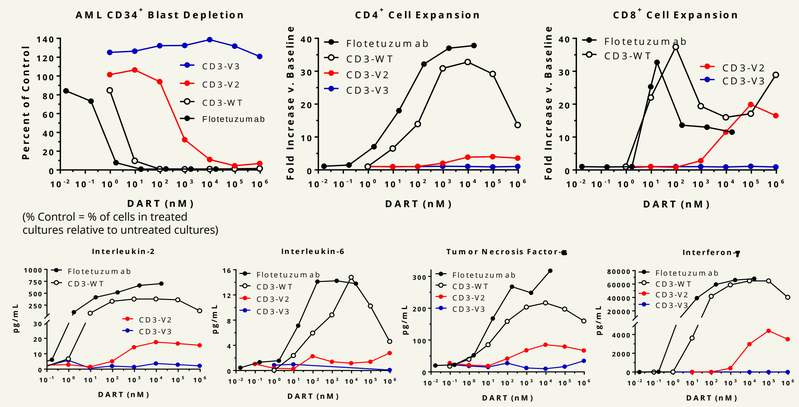


CFSE-labeled purified T lymphocytes were cultured as indicated in the presence of CD123+ MOLM-13 cells (E:T = 1:1) for 96 h. Proliferation via CFSE dilution was determined on CD8- or CD4-gated cells by flow cytometry.

The CD123 x CD3-V2 DART variant mediates acute myeloid leukemia blast depletion *in vitro*



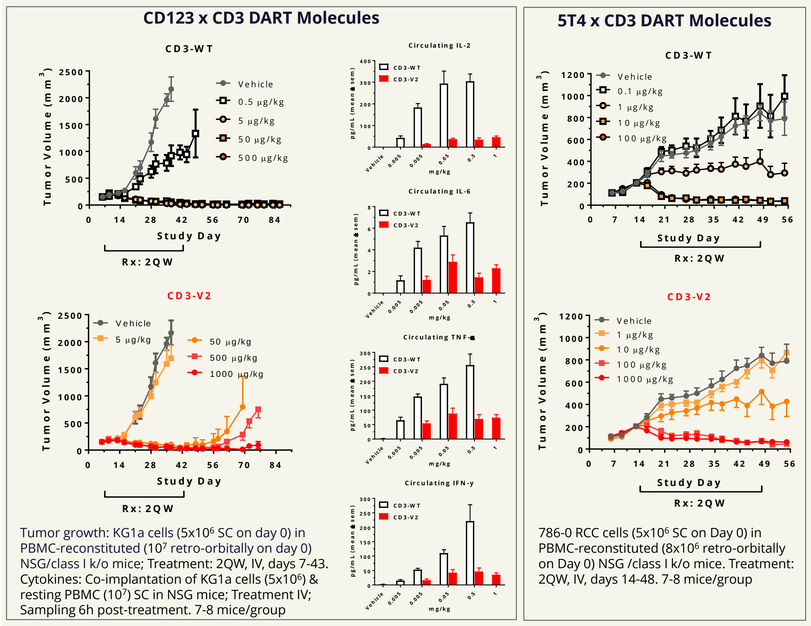
Peripheral blood cells from an AML patient were incubated in supplemented medium in the presence of the indicated DART molecules. Cellularity (CD34+ blasts, CD3+ and CD8+ T cells) was analyzed by flow cytometry at time 0 and on day 6. Cytokine levels were analyzed by cytokine-bead array (BD) on supernatants harvested on day 4 of incubation. Flotetuzumab: a clinical stage, CD123 x CD3-WT DART molecule (no Fc domain, MW ~59 kDa).



(% Control = % of cells in treated cultures relative to untreated cultures)

CD3-V2 DART variants demonstrate anti-tumor activity in human PBMC-reconstituted mice

Activity observed against two independent targets



Tumor growth: KG1a cells (5x10^6 SC on day 0) in PBMC-reconstituted (10^7 retro-orbitally on day 0) NSG/class I k/o mice; Treatment: 2QW, IV, days 7-43. Cytokines: Co-implantation of KG1a cells (5x10^6) & resting PBMC (10^7) SC in NSG mice; Treatment IV; Sampling 6h post-treatment. 7-8 mice/group

786-0 RCC cells (5x10^6 SC on Day 0) in PBMC-reconstituted (8x10^6 retro-orbitally on Day 0) NSG /class I k/o mice. Treatment: 2QW, IV, days 14-48. 7-8 mice/group

The CD123 x CD3-V2 DART variant is well tolerated in cynomolgus monkeys

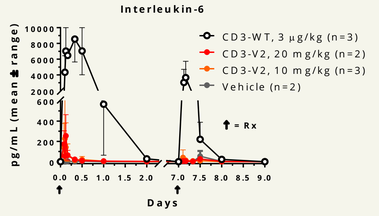
Minimal, transient elevations in IL-2, IL-6 (shown), TNF-α & IFN-γ

Up to 20 mg/kg, QW x2

- No mortality or body weight loss
- No adverse observations or hematology, clinical & anatomic pathology changes

CD3-WT (3 µg/kg):

- Not tolerated, 1/3 mortality
- Cytokine-release syndrome



(CD3 & CD123 affinities similar to human targets)

Conclusions

- **A low-affinity CD3-binding DART molecule was identified that retained maximum killing potential with limited induction of cytokine release *in vitro* and *in vivo***
- **The low-affinity CD3 DART variant was well tolerated in cynomolgus monkeys with minimal cytokine induction at doses exceeding projected therapeutic levels**

Affinity modulated CD3-engaging DART molecules may expand the therapeutic index in redirected T-cell killing applications

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