One CAR to Rule Them all - the Exemplary Fitness of CAR-CD19 T Cells and Their Repurposing for the Eradication of Solid Tumors

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Paul Rennert, Lan Wu, Fay Dufort, Tom Sanford, Lihe Su, Alyssa Birt, Roy Lobb, Christine Ambrose
Aleta Biotherapeutics, Natick, MA USA

Introduction

The successful development of CAR-T cells for the treatment of solid tumors requires that key hurdles be overcome.

1) CAR T cells must expand substantially and then persist in the patient.
2) the CAR T cell population must retain cytotoxic activity and resist exhaustion and immunosuppression, a quality we can define as 'fitness'
3) 3) CAR T cell therapy must completely eliminate tumors in order to avoid relapses; in many indications this requires multi-antigen targeting
4) 4) CAR T cells must be able to attack tumor antigens safely, without causing toxicity

We built a novel platform for repurposing CAR T cells that target CD19 (CAR-CD19 T cells). We create bridging proteins that encode redirect the CAR-CD19 domain to any antigen of interest: each bridging protein contains the CD19 extracellular domain linked to one or more anti-tumor antigen binders such as scFvs or VHHs:

Figure 1. Technology Overview

Building a CAR-T engager

- **Design & characterize bridging proteins built from modules: the CD19 extracellular domain (ECD) and at least one antigen binding domain**
- **Bridging protein**

- **Clone the bridging protein downstream of a CAR-CD19 sequence in a lentiviral construct:**

- **Transduce primary T cells, monitor expression, secretion and activity:**

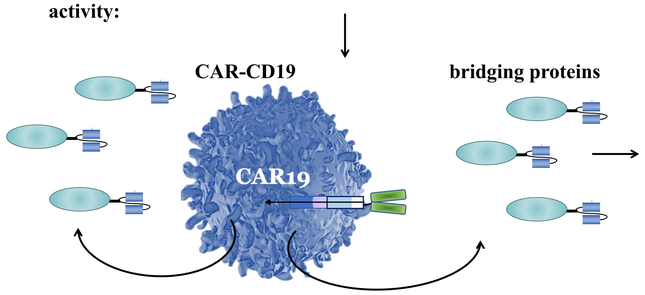


Table 1. The bridging protein serves as a highly selective and potent CAR-T-engager: both domains of an optimized anti-Her2 bridging protein have potent binding activity: the CD19 ECD binds the anti-CD19 scFv and the anti-Her2 scFv binds Her2.

A) Binding activity by ELISA and FACS

Bridging protein binding affinity (EC_{50}) in ELISA assays				
Capture	**Detection**	**CD19-anti-Her2**	**CD19 control**	**CD22-anti-Her2 control**
Anti-CD19	Her2-biotin	0.08 nM	-	-
Her2-Fc	Anti-CD19	0.16 nM	-	-
Bridging protein binding affinity (EC_{50}) in flow cytometric assays				
Cells	SKOV3 carcinoma	1.5 nM (anti-CD19)	-	1.5 nM (anti-CD22)
	BT474 carcinoma	0.2 nM (anti-CD19)	-	0.5 nM (anti-His)

B) Cytotoxic activity. using the anti-Her2 bridging protein to trigger a CAR-CD19 T cell and kill a Her2-positive tumor cell

Bridging protein mediated cytotoxicity (IC_{50})				
Donor	**Target cells**	**CD19-anti-Her2**	**CD19 control**	**CD22-anti-Her2 control**
54	SKOV3 carcinoma	2 pM	-	-
69	BT474 carcinoma	7.1 pM	-	-

Result - the bridging protein binds with low nM affinity to the anti-CD19 scFv (as used in the CAR-CD19 T cell) and to Her2. These binding characteristics allow the bridging protein to act as a CAR-T engager and mediate low pM cytotoxicity against a Her2-positive target cell.

Figure 2. The active bridging protein is secreted by the CAR-CD19 T cell: CAR-CD19 T cells that secrete the bridging protein are cytotoxic against CD19-positive Nalm6 cell and Her2-positive SOV3 cells

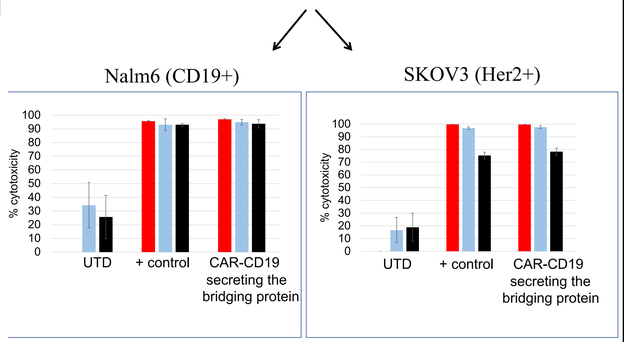
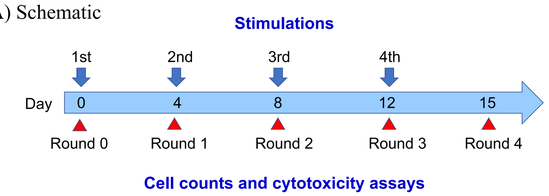
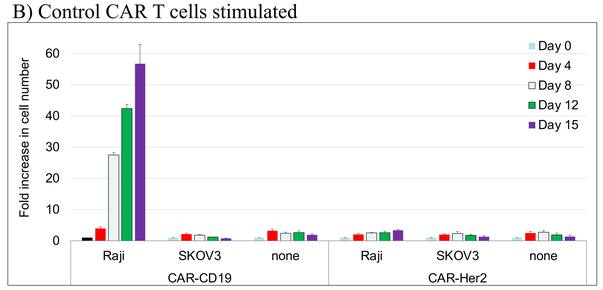


Figure 3. The CAR-CD19 T cells that secrete the bridging protein were assessed using *in vitro* restimulation assays. Stimulation was provided every 4 days using quiescent (mitomycin-C treated) Raji cells or SKOV3 cells (B). After each round T cells were counted and evaluated for cytotoxic activity (C):

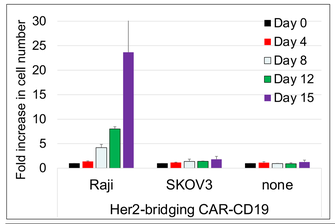
A) Schematic



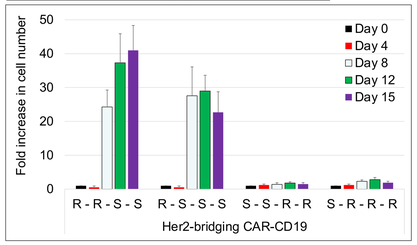
B) Control CAR T cells stimulated



C) Her2-bridging CAR-CD19 T cells stimulated



- 4 rounds of Raji stimulation induces robust CAR-CD19 and Her2-bridging CAR-CD19 proliferation.
- SKOV3 re-stimulation fails to provide robust stimulation
- Why not?



- Stimulation with Raji (R) "licenses" subsequent proliferation in the presence of SKOV3 cells (S)
- Why?

Figure 4. The functional consequence is subtle (note E:T is 10:1 though): Her2-bridging CAR-CD19 T cell cytotoxic activity following stimulations 1 and 3

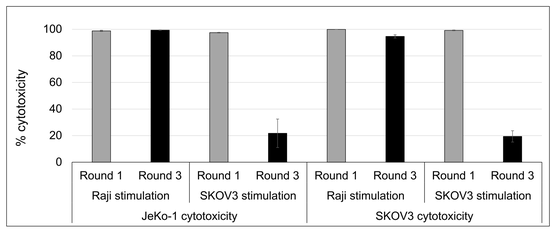
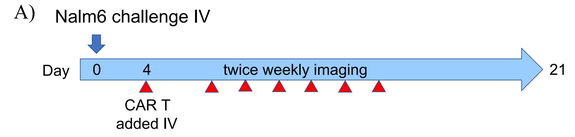


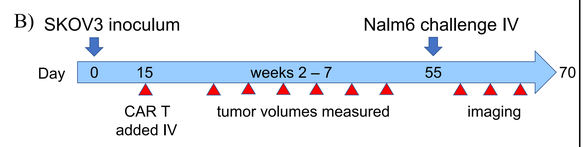
Figure 5. *In vivo* modeling: control of CD19-positive Nalm6 leukemia by Her2-bridging CAR-CD19 T cells (A), of SKOV3 cells and both in sequence (C)

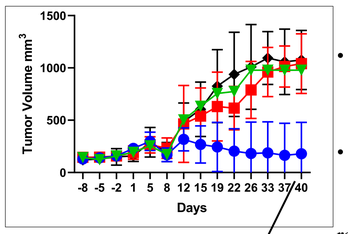
A) Nalm6 challenge IV



- Control T cells
- CAR-CD19 and Her2-bridging CAR-CD19

B) SKOV3 inoculum — Nalm6 challenge IV

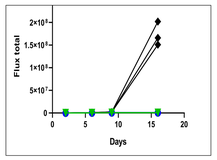




- Control T cells, CAR-CD19 T cells
- Her2-bridging CAR-CD19

rechallenge with Nalm6

C)



- Naïve mice (positive controls)
- rechallenged Her2-bridging CAR-CD19 treated mice

Results – Her2 bridging CAR-CD19 T cells are capable of cytotoxic activity against CD19-positive cells and SKOV3 positive cells *in vitro* (Figure 2) and *in vivo* (Figure 5A,B). The possess serial killing capability against both antigens *in vitro* (Figures 3, 4) and *in vivo* (Figure 5C). Serial exposure to B cells greatly enhances CAR T cell proliferation; serial exposure to a Her2-positive tumor cell does not (Figure 3C).

Current modeling focuses on the mechanisms by which B cell stimulation is beneficial to the CAR *(in vitro*) and the consequence of stimulating with B cells prior to solid tumor challenge (*in vivo*)