FCs and CAR Ts:

Teamwork Makes the Dream work

By Yusef Elghzali

Chimeric Antigen Receptor (CAR) T-cell therapy uses genetically engineered receptors to redirect T cells toward tumor-associated antigens (TAAs). These synthetic receptors typically include a single-chain variable fragment (sFv) linked to a CD3ζ signaling domain, enabling precise recognition and killing of cancer cells.

Gross and Eshhar first demonstrated immunoglobulin-based chimeric molecules as functional receptors for T cells in 1989[1]; since then, CAR T-cell therapy has rapidly developed, resulting in seven FDA-approved therapies to date which target B-cell antigens such as CD19 or BCMA.[2,*] In a 2023 Cancer Immunology and Immunotherapy article, Sun et al., investigated whether combining a dendritic cell/tumor fusion cell (FC) vaccine with EGFRvIII-specific nanobody-based CAR T cells could enhance anti-tumor efficacy against solid glioblastoma tumors.[14-16]

Without any modifications, these early CARs (now referred to as 'first-generation') bearing only CD3ζ signaling regions were limited by poor persistence, as T-cell activation requires both a primary and a co-stimulatory signal for intracellular signaling. Successive generations of receptors implemented revisions and additions to address activation, persistence, and efficacy of these cells.

- First-generation CARs: CD3ζ only; limited persistence
- Second-generation: CD3ζ + CD28 or 4-1BB; improved persistence and activation[3]
- Third-generation: CD3ζ + two costimulatory domains (CD28 + 4-1BB)[4]
- Next-gen modifications: IL-12 secretion, armored CARs, etc., aim to overcome tumor microenvironment suppression.[5]

The clinical efficacy of CAR T cells was demonstrated in 2013, when Brentjens and colleagues showed remission in adults with B-cell tumors after second generation CAR T-cell treatment.[6] Since then, many studies have shown clinical efficacy of CAR T cells when treating cancer.

Despite their success, CAR T cells are still limited in their ability to treat solid tumors. This is likely due to poor infiltration and persistence within the complex tumor microenvironment. Solid tumors are characterized by complex tumor microenvironments that include conditions like high oxidative stress, acidic pH, hypoxia and presence of suppressive immune cells/cytokines.[7]

Nanobody-based CAR T cells (Nb-CAR T Cells) offer a promising solution. Nanobodies, derived from camelid heavy-chain antibodies are smaller and have less steric hindrance than traditional sFv domains, increasing the CAR's ability to access hidden epitopes, thereby enhancing tumor recognition. Because of the inherent simplicity of their structure, Nb-CAR T Cells are less likely to aggregate due to their single-domain structure, nanobodies eliminate the need for heavy/light chain pairing, reducing misfolding and aggregation risk.

Lastly, due to their camelid origin, they are more stable in the complex tumor microenvironment leading to decreased T cell exhaustion.[8] Overall, nanobody-based CAR T cells have demonstrated improved tumor regression compared to traditional sFv-based CARs. [9-12] (and Mustafa *et al*.)

Researchers generated three CAR T-cell types:

- EGFRvIII-specific Nb-CAR T Cells
- Irrelevant-CAR T Cells (expressing a non-specific Nb-CAR)
- Mock CAR T cells (expressing no CAR).

EGFRvIII-specific Nb-CAR T cells showed enhanced proliferation, increased expression of activation markers (CD25, CD69), and elevated cytokine production (TNF-α, IL-2, IFN-γ), outperforming irrelevant and mock CAR T cells. Cytotoxicity assays revealed higher lysis of $EGFRvIII^+$ tumor cells by EGFRvIII Nb-CAR T Cells, with minimal activity against EGFRvIII-negative cells, demonstrating antigen-specific cytotoxicity.

In vivo, tumor-bearing mice treated with EGFRvIII Nb-CAR Ts exhibited increased survival and reduced tumor growth compared to controls. No significant systemic side effects were observed. Flow cytometry showed higher $CD3^+$ T-cell infiltration in tumors and spleens, and greater CAR T-cell persistence in blood.

To create the fusion vaccine, dendritic cells were fused with EGFRvIII-positive tumor cells using polyethylene glycol. The study then compared four groups: anti-EGFRvIII Nb-CAR T Cells alone, with live, non-fused dendritic cells, and with live FCs (dendritic cells fused with tumor cells), and FC alone. The -CAR T + FC group demonstrated the highest proliferation, activation marker expression, and central memory phenotype ($CD62L^+CD45RA^-$). Increased expression of LAG-3 and TIM-3 suggested early T-cell exhaustion, likely due to heightened activation. Additional cytotoxicity assays and in vivo experiments confirmed that the Nb-CAR T Cell + FC group had the greatest tumor cell lysis, persistence, longest survival, and lowest tumor burden.

While the study did not explicitly investigate bidirectional signaling between CAR T cells and FCs, it raises an interesting possibility: that cytokine secretion from CAR T cells—particularly IFN-γ—may counteract FC-induced regulatory T-cell differentiation, which may decrease potency of the vaccine in vivo. This hypothesis warrants further investigation.[17]

Pairing EGFRvIII-specific nanobody-based CAR T cells with a dendritic/tumor fusion vaccine significantly enhances T-cell activation, cytotoxicity, persistence, and anti-tumor efficacy in preclinical models. This combination outperformed CAR T cells alone and CAR T cells with non-fused dendritic cells, representing a promising advancement in CAR T-cell therapy for solid tumors. Overall, this study shows an innovative way to improve cancer treatment.

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Striking a Balance: scFv affinity modulates expression and activation in CAR T cells

By Carson Gray

Chimeric-antigen receptor (CAR) T cells represent a substantial advance in anti-tumoral therapies due, in large part, to their cell-specific toxicity and minimal off-target effects. These therapeutics are produced by transduction of a CAR construct containing, at least, an extracellular antigen-binding domain and a T cell activation domain in the form of CD3ζ into autologous T Cells from the patient.[1] Most commonly, the CAR will also include costimulatory motifs such as the signaling region of CD28.[2] Together, these allow the cell to bind targets in an antigen-dependent manner and mount a highly specific cytotoxic response. The antigen-binding domain of most CARs is a modified antibody variable fragment (Fv) known as a single-chain variable fragment (sFv).[1] This approach has yielded significant clinical successes, including some complete remissions in patients who otherwise had exhausted their therapeutic options.[3]

Since their inception in the 1980s, improvements have enhanced the durability and efficacy of CAR-based therapies. One area of insight has been in the avidity of CAR constructs. Avidity of a CAR is a combination of two important attributes: surface expression and antigen binding.[4] While it is clear that CARs must have a robust avidity to mount a response, excess signaling can also be problematic. Specifically, excessively potent CAR-mediated activation can result in T cell exhaustion, off-target tissue damage, and cytokine toxicity. An important advancement in this area was the use of CRISPR platforms to target CAR to the endogenous T cell receptor (TCR) promoter.[5] This placed CAR surface expression under the same regulation as the endogenous TCR, which is dynamically expressed in the presence of antigen. This has been beneficial in optimizing surface expression and avidity, but research into modulating antigen-affinity is lacking.

However, paralleling the challenges faced by natural lymphocytes during their development and antigen receptor randomization, basic questions concerning the physical expression, stability, and binding capacity of CAR sFv regions remained unanswered. To address these, Fujiwara et al., in the March 2020 issue of Biochemical and Biophysical Research Communications, examined sFv structural influences on antigen-binding and expression.1 They constructed four anti- Kinase insert Domain Receptor (KDR) CARs from the variable fragments of antibodies with a range of antigen affinities. Four distinct CAR T Cells were made from these constructs, which each included a GFP reporter to ensure equivalent transduction efficiencies. One construct exhibited significantly decreased surface expression efficiency, suggesting that it was intracellularly degraded. Two others had higher-than-expected molecular weights, suggesting post-translational modifications.

Only one construct bound KDR, as evidenced by specific lysis of a KDR-expressing B Cell line, L1.2. Immunofluorescent staining was done to observe the distribution of the CARs in each of the sFv types; it was found that CAR1-L3H was evenly distributed about the cell surface, while CAR2-L3H and CAR4-L3H formed clusters of aggregate. This provides further evidence for post-translational modifications that could be the cause of intermolecular adherence and aggregate formation.