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CAR-NK's balancing act: when scFv affinity is not too tight, not too loose… but just right?

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ABSTRACT

Chimeric antigen receptor (CAR) therapies have revolutionized cancer treatment by enabling immune cells to target tumor cells with high specificity. While extensive research has focused on optimizing single-chain variable fragment (scFv) affinity in CAR-T cells, its impact on CAR-natural killer (NK) cell function remains less understood. A recent study by Rahnama *et al*, published in the *Journal for ImmunoTherapy of Cancer*, addresses this gap by investigating how fine-tuning scFv affinity influences CAR-NK efficacy against acute myeloid leukemia. The study demonstrates that lower-affinity 7G3-based CAR-NK cells exhibit superior antigen discrimination, prolonged persistence, and enhanced tumor control compared with their high-affinity counterparts. However, findings with 26292-based CAR-NK cells reveal a more complex, context-dependent relationship between scFv affinity and cytotoxic function. These results highlight the need for individualized optimization of CAR designs, considering factors such as epitope accessibility, ligand-binding kinetics, and cellular context. Future studies incorporating real-time kinetic analyses and tumor microenvironment modeling will be crucial for refining CAR-NK therapies. Striking the right balance between binding affinity, dwell time, and serial killing capacity could enhance CAR-NK therapeutic potential while minimizing toxicity risks.

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INTRODUCTION

Chimeric antigen receptors (CARs) have revolutionized immunotherapy by enabling immune cells, such as T cells and natural killer (NK) cells, to recognize and attack target cells independently of major histocompatibility complex (MHC) recognition. At the core of this innovative technology is the single-chain variable fragment (scFv), an engineered antibody fragment derived from the variable regions of the heavy and light chains of an antibody. The scFv serves as the antigen-binding domain of the CAR, conferring high specificity for target cells and facilitating immune-mediated cytotoxicity.

Despite the success of CAR therapies, early Food and Drug Administration-approved CAR T-cell therapies relied on high-affinity scFvs originally developed for conventional antibody-based treatments. While these high-affinity constructs demonstrated strong antitumor responses, they often led to excessive T-cell activation and exhaustion, reduced CAR T-cell persistence, and serious on-target off-tumor toxicities. In contrast, clinical trials have shown that CAR T-cells with moderate-affinity ScFvs (dissociation constant K_D of 20–200 nM) achieved superior response rates in solid tumor treatment compared with high-affinity CARs (K_D<20 nM).[1] Similarly, in hematological malignancies, the lower-affinity CAR T-cells, such as a CD19-targeting CAR with~40-fold lower affinity than the original high-affinity (K_D of 0.3 nM) FMC63 CAR, exhibited enhanced CAR T cell expansion, prolonged persistence, and durable responses in patients with relapsed/refractory acute lymphoblastic leukemia.[2 3]

While extensive efforts have been made to optimize scFv binding affinity in CAR T-cell therapies, its impact on CAR-NK cell function remains less explored. A recent study published in the *Journal for ImmunoTherapy of Cancer* by Rahnama *et al* addresses this gap, demonstrating how fine-tuning the affinity of scFv targeting the CD123 epitopes of acute myeloid leukemia (AML) cells can enhance the efficacy and persistence of CAR-NK cells—shedding light on key challenges in the field.[4]

Why do we still need CAR-NK cells?

NK cells are innate immune cells that recognize stressed or transformed cells without prior antigen presentation and eliminate tumor cells through multiple mechanisms, including direct cytotoxicity, death receptor-mediated apoptosis, antibody-dependent cellular cytotoxicity and cytokine secretion. Engineering CARs onto NK further provides antigen-specific targeting and enhances cytotoxicity while retaining innate tumor surveillance capabilities.[5 6] Compared with CAR T-cells, CAR-NK cells have a faster onset of action, a lower risk of cytokine release syndrome and graft-versus-host disease, and

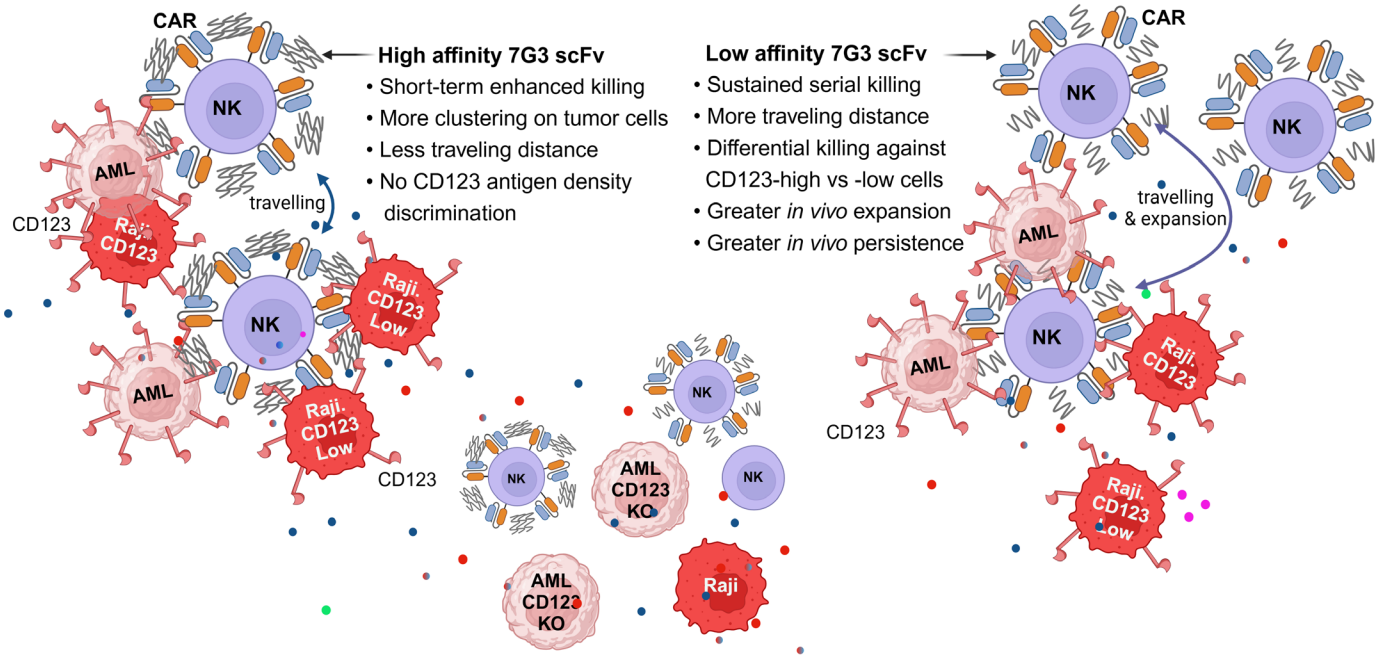

Figure 1 Impact of scFv affinity on CAR-NK cell function and tumor targeting: a comparison of CAR-NK cells engineered with high-affinity (left) and low-affinity (right) 7G3 scFv targeting CD123. AML, acute myeloid leukemia; CAR, chimeric antigen receptor; KO, knockout; NK, natural killer; Raji, a B-lymphoblastoid cell line, CD123, the alpha chain of interleukin 3 receptor, highly expressed on AML cells and engineered to be expressed on Raji cells at high and low levels; scFv, single-chain variable fragment.

can be manufactured as off-the-shelf products, making them a powerful alternative or complementary approach to existing T-cell therapies.[7 8]

scFv affinity tuning: a key modulator of CAR-NK efficacy

Rahnama *et al* engineered NK cells to express CARs with a range of high-affinity and low-affinity scFv variants targeting two CD123 epitopes (7G3-based and 26292-based variants)[9 10] and compared their anti-AML functionalities.[4] CD123, the alpha chain of interleukin-3 receptor, is highly expressed on leukemia stem and progenitor cells and differentiated blast cells. However, it is also present at low levels in healthy hematopoietic and endothelial cells. Therefore, fine-tuning the anti-CD123 CAR to distinguish antigen density is crucial for selectively targeting AML while sparing normal cells, thereby minimizing the risk of on-target, off-tumor toxicity in CAR immunotherapies.

The 7G3 and 26292 scFv wide-type and variants were cloned into the same CAR backbone, transduced into the immortalized NK-92 cell line and primary NK cells, and screened for antigen-driven CAR-NK activation, and CAR-mediated, antigen-specific cytotoxicity. This screening was performed using AML cells with high CD123 expressions, CD123 knockout AML cells, CD123-negative lymphoma cell line (Raji), and Raji cells engineered to express CD123 at the surface densities comparable to healthy hematopoietic progenitors (Raji.CD123Low) and AML cells (Raji.CD123) (summarized graphically in figure 1).

Both 7G3 and 26292-based CAR NK cells exhibited enhanced anti-AML effects compared with unmodified NK cells. Notably, the lower affinity 7G3 (7G3L; K_D of 20 nM) CAR-NK cells demonstrated more effective killing of CD123-expressing tumor cells, compared with higher-affinity variant (K_D of 5 nM) and WT (K_D of 3 nM), and exhibited the best antigen density discrimination, with significantly higher cytotoxicity against CD123-high tumor cells than CD123-low targets (figure 1). Immunological synapse analysis revealed that in the span of 30 min, the higher-affinity 7G3WT CAR-NK cells aggregated more readily around tumor cells and traveled less distance at slower speed, leading to increased short-term killing than 7G3L CAR-NK cells. However, in the long-term assays with serial target stimulation, lower-affinity 7G3L CAR-NK cells maintained more sustained killing activities. The lower-affinity 7G3L CAR-NK cells also showed greater expansion and anti-tumor efficacy in two xenograft models.

Is lower scFv affinity CAR always better? A context-dependent question!

While the 7G3 variant CAR NK cell results align with the previous CAR T cell studies—suggesting that lower scFv affinity enhances tumor antigen discrimination and antitumor efficacy—the results from the 26292 variants are more complex. Both high-affinity (K_D of 4.2 nM) and very low-affinity (K_D of 180 nM) 26292 CAR variants in NK-92 cell lines induced significantly enhanced killing of two AML cell lines compared with other scFvs. However, in primary NK cells, a very high affinity (K_D of 0.79) 26292 CAR induced significantly higher cytotoxicity than other variants. Additionally,

all 26292 CAR-NK cells exhibited significantly greater killing of antigen-dense Raji.CD123 cells compared with Raji.CD123Low cells, except for a low-affinity variant (K_D of 82 nM).

Therefore, these findings suggest that scFv affinity does not always dictate CAR function. Its impact appears to be context-dependent and influenced by factors such as timing, NK cell source and target epitope characteristics. Notably, both high-affinity and low-affinity 26292 CARs demonstrated similarly strong antitumor activity and antigen density discrimination, suggesting no direct correlation between 26292 scFv affinity and CAR NK function. The authors speculated that structural variables may have affected epitope accessibility and allostery. It is also important to note that K_D in this study was measured through on-cell binding assay, which assesses ligand occupancy at equilibrium. While this provides valuable insights into ligand-receptor interaction under physiological conditions, it does not capture the kinetic aspects of binding and dissociation. Indeed, evaluating association and dissociation rate constants (K_{on} and K_{off}, respectively) may be more beneficial than assessing kinetic K_D alone (K_{off}/K_{on}). A fast-on/fast-off "fly-kiss" mode of scFv engagement has been proposed for effective CAR immunotherapy.[1] Although a longer dwell time (higher $T_{1/2}$ and smaller K_{off}) of an scFv on its target prolongs receptor engagement, which may enhance CAR-mediated signaling and cytotoxicity, it could result in reduced serial killing capacity and chronic activation, thus exhaustion of CAR-T or NK cells. Prolonged scFv binding also increases the risk of on-target, off-tumor toxicity. Therefore, understanding all aspects of scFv binding is crucial in designing an optimal CAR with effective killing, persistence and reduced cytotoxicity.

CAR signaling activation is largely scFv affinity-independent

The authors demonstrated that on co-culture with CD123-positive tumor cells, all CAR-NK-92 cells and primary CAR-NK cells, regardless of scFv affinity, exhibited comparable activation of mammalian target of rapamycin (mTOR) signaling, a central downstream effector of CAR engagement. This activation, assessed by phosphorylation of mTOR and S6, was not observed following incubation with CD123-negative tumor cells. These results suggest that mTOR pathway activation in CAR-NK cells is antigen (CD123)-specific but largely independent of scFv affinity.

Notably, interferon gamma (IFNγ) secretion varied among different CAR-expressing NK cells. This variation may be influenced by several factors, including scFv affinity, epitope specificity, the source of NK cells (NK-92 vs primary cells), and the characteristics of the target cells. Future studies exploring the underlying cellular mechanisms, such as signaling pathways, gene expression, and metabolic profiles that mediate the difference in antitumor activity among affinity-tuned CARs would be of significant interest.

CONCLUSIONS

This study by Rahnama *et al* provides valuable insights into the role of scFv affinity tuning in optimizing CAR-NK cell function against AML. By demonstrating that lower-affinity 7G3 scFvs can enhance antigen discrimination and improve long-term persistence, the findings align with previous research in CAR-T cells while also introducing novel considerations for CAR-NK therapies. This study underscores the significance of CAR-NK cell affinity optimization for maximizing therapeutic efficacy and highlights the necessity of tailoring each scFv clone to its specific functional context.

Future studies incorporating real-time kinetic analysis and evaluating CAR-NK cellular mechanisms and function across different tumor microenvironments will be crucial to refining affinity-based CAR designs. Ultimately, striking the right balance between binding affinity, dwell time, and serial killing capacity will be key to enhancing the therapeutic potential of CAR-NK cells while mitigating toxicity risks. Lastly, similar future work on fine-tuning of CAR-NK against solid tumor antigens would be of great interest.

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