A Brand New CAR:
Moving CAR Constructs into Macrophages

By Yenny Feng

Chimeric antigen receptor (CAR) T cells have revolutionized cancer therapy, yet the challenges of off-target effects and excessive cytokine release persist.[1] The single-chain variable fragment (sFv) plays a critical role for receptor expression and antigen recognition, with framework regions, complementarity-determining regions, gene locus, and construct type all significantly influencing CAR T cell performance.[2] In their 2020 Nature Biotechnology article, Klichinsky *et al*. explore strategies to optimize CAR T cells for enhanced efficacy and precision by applying sFv optimization to CAR macrophages (CAR-M), for improved tumor infiltration and phagocytosis of solid tumors.[3]

Klichinsky *et al.* developed CAR-Ms by engineering human macrophages with CARs targeting Human Epidermal Growth Factor Receptor 2 (HER2), using an sFv linked to a CD3ζ signaling domain. The sFv was optimized for stability, affinity, and to ensure robust expression and specific antigen binding. *In vitro*, CAR-Ms exhibited enhanced phagocytosis of HER2-positive tumor cells compared to un-transduced macrophages. *In vivo*, mouse models of ovarian and lung cancer demonstrated significant tumor reduction and prolonged survival after a single CAR-M infusion. Optimized sFv affinity reduced off-target effects and excessive immune activation, addressing key CAR T cell limitations.[1] By leveraging macrophages' ability to infiltrate solid tumors, CAR-Ms offer a novel platform for sFv-based immunotherapy.[4]

CAR T cell therapy has undergone continuous enhancement across multiple generations, as extensively reviewed by Tomasik *et al.*[5] First-generation CARs, which included only the CD3ζ signaling domain, demonstrated limited persistence and clinical efficacy.[6] Second-generation CARs improved outcomes in hematologic malignancies by incorporating a costimulatory domain—such as CD28, 4-1BB, or OX40—to enhance T cell activation and survival.[7,8] Despite these advances, their effectiveness remained largely confined to hematologic, or "liquid," tumors. These cancers produce abundant circulating malignant cells but do not form dense, solid tumor masses, which are often protected by immunosuppressive barriers within the tumor microenvironment (TME).[4]

Third-generation CARs addressed this by combining multiple costimulatory domains (*e.g.*, CD3ζ-CD28-OX40 or CD3ζ-CD28-4-1BB), resulting in enhanced T cell proliferation and persistence.[9] Fourth-generation CARs, known as TRUCKs (T cells Redirected for Universal Cytokine Killing), further advanced the technology by delivering cytokines like IL-12 to modulate the TME and improve antitumor activity.[1] Armored CARs added yet another layer by including a second receptor to disrupt inhibitory pathways in T cells, such as those mediated by CTLA-4 or PD-1. Finally, fifth-generation CARs integrated these elements with the chemokine receptor CCR2b, enhancing cellular migration and achieving improved tumor control over fourth-generation constructs. Despite these innovations, challenges such as tumor heterogeneity and immunosuppressive TMEs continue to impede success in solid tumors.[4]

Klichinsky *et al.* introduced CAR-Ms (chimeric antigen receptor macrophages) as a complementary approach to traditional CAR T cell therapies, incorporating sFv optimization to improve tumor targeting and control. Unlike T cells, macrophages are naturally adept at infiltrating solid tumors, helping to overcome one of the major limitations of early anti-CD19 CARs.[4,10] CAR-Ms were generated using chimeric Ad5f35 adenoviral vectors to transduce primary human macrophages with CAR constructs. This strategy enhances innate immune responses, and the scalability of the vector system suggests potential for both autologous and allogeneic applications—though the complexity of production introduces regulatory challenges.

Further, CAR-Ms uniquely cross-present tumor antigens to $CD4^+$ and $CD8^+$ T cells, a capability not shared by CAR T cells, which primarily mediate cytotoxicity.[4] As antigen-presenting cells, macrophages phagocytize tumor antigens and present them via both MHC class I or II.[10] Klichinsky *et al.* showed that CAR-Ms traffic to tumor sites, upregulate MHC genes in other antigen presenting cells, and recruit T cells in humanized mouse models, enhancing tumor control. This cooperative mechanism amplifies adaptive immunity, potentially leading to sustained anti-tumor responses. This feature positions CAR-M as a bridge between innate and adaptive immunity, critical for solid tumor therapies.[4]

The efficacy of CAR-Ms arises from multiple mechanisms. Direct phagocytosis of HER2-positive tumor cells, as demonstrated in vitro with SKOV3 cells, is a primary mechanism for removal of target cells. Antigen presentation and cross-presentation to $CD4^+$ and $CD8^+$ T cells, respectively, drives secondary T cell activation, boosting adaptive immunity. CAR-Ms also secrete pro-inflammatory cytokines, shifting M2 macrophages to pro-inflammatory M1 macrophages. The role of B cell activation or antibody-dependent cellular phagocytosis (ADCP) was not directly assessed, but plausible. Future studies in lymphocyte-depleted mouse models could clarify the specific cells involved in anti-tumor immunity. Klichinsky *et al.*'s innovation lies in using CD3ζ signaling, which is inherently T cell-specific, to activate macrophages. The CD3ζ domain's homology to the FcγR ITAM on macrophages enables phagocytosis, showing that a CD3ζ domain can drive innate immunity on these cells. This approach suggests CAR constructs could be adapted for a variety of innate cells, such as dendritic cells, NK cells, and others, expanding the potential for CAR-based cell therapy's reach.

The CAR-M strategy expands the potential of CAR-based therapies to include solid tumors, where TME barriers have historically limited CAR T cell efficacy.[4] However, the development of CAR-Ms does not resolve key challenges such as high production costs and regulatory complexity—especially when using healthy donor-derived monocytes for allogeneic applications. Optimizing scFv affinity remains essential for minimizing off-target effects and improving safety.[4] Like all cell-based therapies, CAR-Ms will require careful monitoring for adverse events, including cytokine release. Looking ahead, future strategies may combine CAR-Ms with advanced CAR T

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cells or CAR-NK cells to enhance antitumor responses.[4,9] Integration with mutation-specific T cell therapies could also support the development of highly personalized treatments.[11] Continued refinement of sFv design will be critical to increasing therapeutic precision and clinical impact.[4]

CAR-Ms interact with tumors directly through phagocytosis or indirectly by presenting and cross-presenting antigens to CD4+ and CD8+ T cells (See **Figure 1**). Direct phagocytosis offers rapid tumor clearance, while CD8+ T cell activation via cross-presentation drives robust cytotoxicity, while CD4+ T cell engagement enhances sustained immunity. Combining all interactions could maximize tumor control in vivo, engaging both native and CAR T cell types for long-term efficacy.

Klichinsky *et al*.'s sFv-optimized CAR-Ms advance immunotherapy for solid tumors, overcoming important T cell limitations. Enhanced phagocytosis, cross-presentation, and adaptive immunity distinguish CAR-Ms. Combining CAR-M with CAR T cells, CAR-T cells, or mutation-specific therapies could yield precise treatments. The novel CD3ζ signaling of these constructs in macrophages bridges innate and adaptive immunity, contributing to a new era of immunotherapy.

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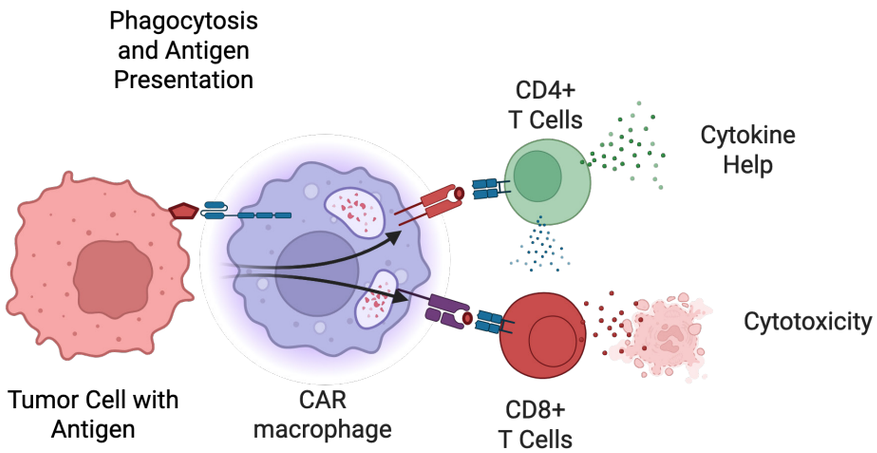

Figure 1 | CAR-M interactions with tumors and T cell activation lead to enhanced tumor control. CAR-M engages tumors directly via the sFv to mediate phagocytosis and subsequent presentation to CD4+ T and CD8+ T cells.