COMMENTARY

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# Next-generation macrophages: repolarizing CAR-macrophages against cancer

**Sima Ebrahimabadi[a], Dan S. Kaufman[b,*]**

*[a]Center for Cell-Based Therapy - CTC, Regional Blood Center of Ribeirão Preto, University of São Paulo - USP, Ribeirão Preto, São Paulo, Brazil; [b]Division of Regenerative Medicine, Department of Medicine and Sanford Stem Cell Institute, University of California - San Diego, La Jolla, CA, USA*

Chimeric antigen receptor (CAR) T-cell therapy represents a groundbreaking advancement in cancer treatment, as evidenced by the 6 food and drug administration (FDA)-approved products currently available. Despite its success in treating certain types of hematological cancers, the therapy is associated with known limitations that include potentially significant side effects, the inability of T cells to penetrate solid tumors, and cell exhaustion and loss of antitumor activity due to the inhibitory tumor microenvironment (TME).[1]

As part of the innate immune system, macrophages participate in diverse activities that include maintaining tissue balance, defending against pathogens, regulating cancer progression, and mediating crosstalk between the adaptive and innate immune systems. Tumor-associated macrophages are abundant in the TME, and their response to cancer is significantly influenced by the surrounding environment. These stimuli lead to both antitumor and tumor-promoting effects. Due to the unique potential of macrophages to phagocytize tumor cells, mediate antibody-dependent cellular cytotoxicity, and provoke vascular damage and tumor necrosis, macrophages are promising candidates for cell therapy, including the potential to develop CAR-expressing macrophages.[2]

In recently published studies, Shen et al[3] describe the development of CAR-expressing macrophages derived from both human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs). The use of either hESCs or iPSCs provides a stable starting cell platform for gene editing and cell production. Most previous studies of CAR macrophages have used primary peripheral blood (PB) monocytes as the starting cell source. These are typically more challenging to gene modification and cell production may be limited compared to the essentially unlimited cell numbers that can be produced from hESCs/iPSCs.[4]

Studies in this paper use a monolayer culture system and sequential differentiation of the hESCs/iPSCs toward functional macrophages over a 3-week period.[3] The differentiated macrophages are phenotypically, functionally, and transcriptionally similar to PB macrophages. These studies show that second-generation CARs containing co-stimulatory domains such as 41BB, FcRγ, and TLR4, along with CD3ζ do not demonstrate higher antitumor activity than the first-generation CAR only encoding CD3ζ (CD19-CD3ζ). CD3ζ shares homology with the Fc common-γ chain, FcεRI-γ, involved in antibody-dependent cellular phagocytosis (ADCP) in macrophages and can direct antitumor phagocytic activity.[5] Notably, in a similar study by our group, an anti-mesothelin (Meso)-Bai1-iPSC-CAR-Macrophages demonstrated significantly improved phagocytic activity compared to Meso-CD3ζ-iPSC-CAR-Ms.[6] Whether this difference is due to the CAR targets, other aspects of the CAR construct, or other cellular differences remains to be determined. Notably, in these studies by Shen et al,[3] the expression of the anti-CD19-CD3ζ CAR does not interfere with the differentiation or the expression of macrophage and M1-related markers and interestingly leads to a decrease in M2-related markers.

Further studies show that on stimulation with CD19$^+$ Raji, the CD3ζ domain mediates potent phosphorylated activation of Syk (p-Syk), Erk 1/2 (p-Erk1/2), and CD3ζ. Accordingly, in vitro studies demonstrate the potent functional activity of hESC/iPSC-CAR-macrophages with antigen-dependent phagocytosis and killing of CD19$^+$ target cells. The activated cells also produce proinflammatory cytokines upon tumor cell co-culture. Additional studies demonstrate the tumoricidal activity of hESC/iPSC-CAR-macrophages against solid tumors by using the human breast cancer and ovarian cancer cell lines that over-express CD19 to allow the same anti-CD19 CAR to be used.[3]

Antitumor activity was then tested in vivo using a standard Burkitt lymphoma (Raji cell) xenograft model. These studies demonstrate that the hESC-CAR-Ms persist in vivo in immunodeficient mice for at least 61 days, yet the CAR-macrophages alone proved only modest antitumor activity.[3] However, hESC-CAR-macrophage stimulated in vivo with interferon gamma (IFN-γ) and monophosphoryl lipid A (MPLA) combined with the stem cell factor (SCF), interleukin-3 (IL-3), and granulocyte-macrophage colony-stimulating factor (GM-CSF) cytokines (CAR-M++ treatment group) demonstrated significantly improved antitumor activity and prolonged survival of mice in this treatment group. RNA-seq analysis demonstrated that IFN-γ + MPLA stimulation results in the activation of NOD/Shi-scid IL2rgamma(null) (NOG)-like receptor signaling, the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling, as well as chemokine, and interferon

*Address correspondence: Dan S. Kaufman, Division of Regenerative Medicine, Department of Medicine and Sanford Stem Cell Institute, 9500 Gilman Drive, MC#0695, La Jolla, CA 92093, USA. E-mail address: dskaufman@ucsd.edu (D.S. Kaufman).

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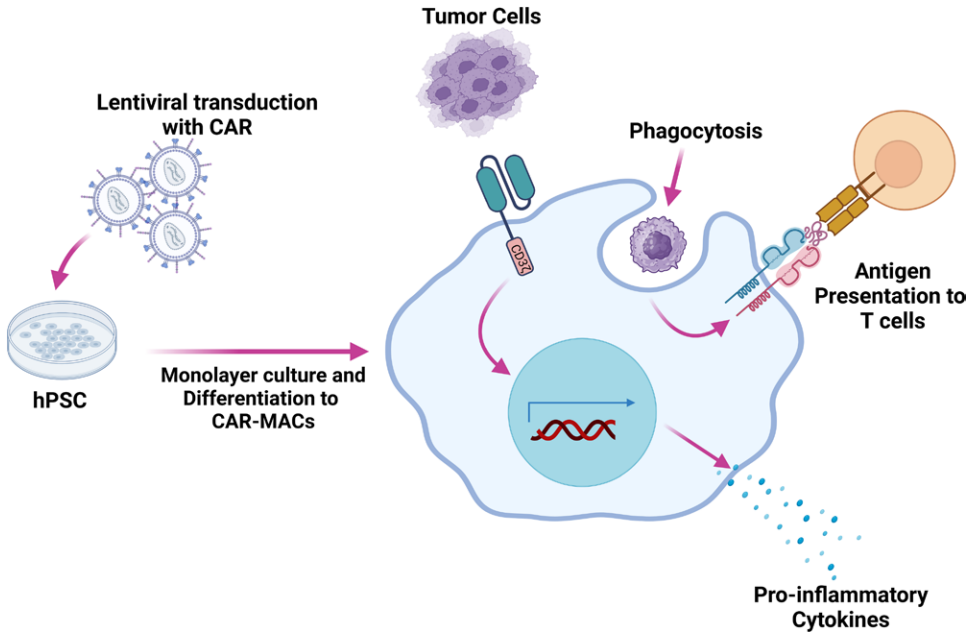


**Figure 1.** Differentiated hESC or iPSC-derived CAR macrophages can activate adaptive immunity. CAR engagement with a specific tumor antigen activates phagocytosis and increases the phosphorylation of signaling pathways including ERK1/2, which in turn activates the transcription factors such as NF-κB that leads to the release of inflammatory cytokines. As antigen-presenting cells, macrophages present the tumor antigen through HLA Class II that can further activate endogenous T cells. CAR = chimeric antigen receptor, ERK = extracellular signal-regulated kinase, hESC = human embryonic stem cell, HLA = human leukocyte antigen, hPSC = human pluripotent stem cell, iPSC = induced pluripotent stem cell, MAC = macrophage, NF-κB = nuclear factor-κB. Figure created with BioRender.com.

signaling pathways. Additionally, extracellular matrix organization and collagen formation signaling pathways were downregulated, all contributing to the proinflammatory phenotype.[3]

Macrophages, as specialized phagocytic cells, can also present peptide antigens via MHC class II to activate T helper cells.[7] Additional studies evaluated the ability of the hESC/iPSC-CAR-macrophages to present tumor-associated peptide antigens (**Fig. 1**). Here, the authors tested whether the simultaneous infusion of T cells with the hESC/iPSC-CAR-macrophages can further enhance the antitumor potential of the CAR-M++ treatment in tumor-bearing NOG mice. Indeed, these studies demonstrated the co-infused human T cells were further activated with increased expression of CD107a and cytolytic activity. A human ovarian cancer xenograft model was used to demonstrate that while CAR-M++ treatment provides significant antitumor activity in vivo, this activity was further improved with prolonged survival in mice treated with infusion of human T cells.[3]

Although these studies are novel and help demonstrate a potential benefit of hESC/iPSC-CAR-macrophages to mediate antitumor activity in suitable human xenograft models, one key concern is the dependence of these cells on exogenous stimulation with IFN-γ + MPLA for suitable in vivo activity. Indeed, these are potent immune-stimulating agents that likely would not be suitable for administration as part of a clinical therapy. Notably, the choice of the intracellular signaling domain(s) used in the CAR-macrophages may overcome the need for exogenous stimulation. In a study by Zhang et al,[8] the intracellular signaling domain of CD147, referred to as CAR-147, effectively inhibited tumor growth compared with the control macrophages without any prior stimulation. In another study by Lei et al,[9] human iPSC-derived CAR-macrophages with both CD3ζ and toll-like receptor (TIR) signaling domains plus anti-single-chain fragment variables (scFvs) against either epidermal growth factor receptor VIII (EGFRvIII) or Glypican-3 were pretreated with IFN-γ and lipopolysaccharide (LPS) in vitro to mediate antitumor activity and prolonged the survival in both hepatocellular carcinoma and glioblastoma xenograft models.

Additionally, these studies by Shen et al[3] also tested the combination of anti-CD47 treatment with the hESC/iPSC-CAR-Ms and demonstrated improved antitumor activity. Therefore, this approach may also provide another effective therapeutic option that is more clinically compatible. Alternatively, as signal regulatory protein-α (SIRPα) is the inhibitory receptor stimulated by CD47, other studies demonstrate deletion of SIRPα iPSCs-derived macrophages mediate improved phagocytic and killing activity in response to CD47-positive tumor cells.[10] Given these encouraging results, exploring the SIRPα knockout in the hESC/iPSC-CAR-Ms would be of great interest.

In summary, these studies demonstrated the utilization of an hESC/iPSC-based monolayer culture system and stepwise process to produce CAR-expressing macrophages with potent antitumor activity.[3] Notably, Shen et al[3] mention that this monolayer system increases the production of macrophages yet, the total number of cells produced is not clear. Indeed, the number of macrophages needed for clinical trials may prove challenging. For example, a clinical trial of anti-HER2-CAR-expressing macrophages engineered from autologous patient PB required 3 to 10 billion cells per dose (NCT06254807). As terminally differentiated macrophages have limited expansion potential, producing similar numbers from hESCs or iPSCs would likely require very large starting cell populations and large-scale bioreactors or similar technology. However, the hESC/iPSC provides a stable platform that enables incorporation of multiple gene edits to further enhance the antitumor activity of these cells. Therefore, clinical translation of iPSC-derived CAR macrophages will hopefully be advanced soon.

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