Investigating the Effectiveness of Organoids-Based Chimeric Antigen Receptor Macrophage Immunotherapy against Hepatocellular Carcinoma

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Research Colloquium for Science Undergraduate Students 2022-23

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Hepatocellular carcinoma (HCC) is a deadly type of cancer that leads to a mortality rate worldwide. Previous research and pharmaceutical efforts have highlighted the potential of immunotherapy in treating HCC. Immunotherapy enhances or rectify the immune system's ability to precisely kill the tumor cells. One of the most prominent cell-adoptive immunotherapies is Chimeric Antigen Receptor (CAR) T cell, which has clinically cured some hematopoietic cancers. However, the nature of T cells renders the therapy ineffective against solid tumors with an immunosuppressive tumor microenvironment (TME). Attempts to overcome the challenge include the co-delivery of immune checkpoint blockers, such as programmed cell death protein 1 (PD-1) antibodies. Among the immunocytes armory, macrophages are equipped with a natural tumor-killing ability, either by phagocytosis or cytokine release. Due to the macrophages' ability to remodel and penetrate the extracellular matrix, they are also the most persistent immunocytes in solid tumors. Their antigen-presenting ability can cause epitope spread and the recruitment of effector T cells, making them ideal platforms for cell therapy. However, while tumor-associated macrophages (TAMs) may persist and influence the TME, TAMs also express the PD-1 receptor and are subjected to less effective phenotype change.
The research develops a novel design of CAR structure that targets HCC and secretes PD-1 antibody fragments. The CAR structure is introduced into extended pluripotent stem cells (EPSCs) through the PiggyBac transposon system for macrophage generation. Phagocytosis assay, phenotype change assay and peripheral blood mononuclear cells (PBMCs) exhaustion-preventing assay were performed to assess the effectiveness of such CAR macrophages. It is hoped that the EPSC-derived novel CAR macrophage will harbor stronger anti-tumor activities.

Introduction:

1. **Hepatocellular carcinoma:** Hepatocellular carcinoma (HCC) is a significant health problem worldwide (Luo et al., 2022). Current treatment options for liver cancer include surgery, radiation, targeted therapy and so on (Bai et al., 2023). Phosphatidylinositol proteoglycan 3 (GPC3) is a key regulator of cell growth and differentiation (Amer et al., 2022). Its expression in tumor tissues highlights it as a potential therapeutic target.
2. **Chimeric Antigen Receptor (CAR) Technique:** CAR is a newly developed fusion protein that can be expressed on the surface of T cells. CAR T cells showed profound effectiveness in treating leukaemia, lymphoma, and myeloma (June & Sadelain, 2018). However, traditional CAR-T therapy has several limitations, restricting its safety profile and application in solid tumors (Zhang et al., 2023). In view of the unmet needs, one approach is to upgraded CAR contracts to adapt to the safety concerns and solid tumor challenges, see Figure 1.
3. **CAR-Macrophage:** Compared to T cells, macrophages have some natural advantages in immunotherapy:
   - Professional antigen presenting cells to induce epitope spreading
   - Active transportation to tumor sites
   - Penetrate into solid tumor via extracellular matrix modelling
   - High persistence within TME
   - Improved safety profile
4. **EPSC:** Extended pluripotent stem cells (EPSCs) are a more stable cell line with an expanded developmental potential to form both embryonic and extra-embryonic tissues (Gao et al., 2019). EPSCs also excel in genetic engineering stability. Indeed, the two major populations of macrophage have distinct embryonic and extra-embryonic origins (Kenneth & Weaver, 2016). Hence, EPSCs harbor the potential to generate macrophages that resemble their heterogeneity and is thus used in this research.

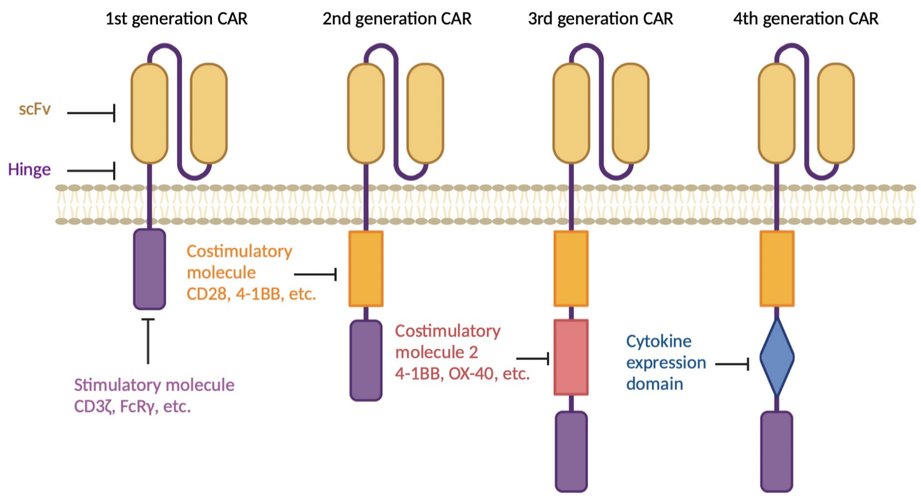

Figure 1. Current generations of CAR construct. 1st generation: ectodomain of single-chain variable fragment (scFv), hinge, stimulatory module. 2nd generation: one costimulatory module added. 3rd generation adds two costimulatory modules added. 4th generation: cytokine expression domain added.

Methods and Results:

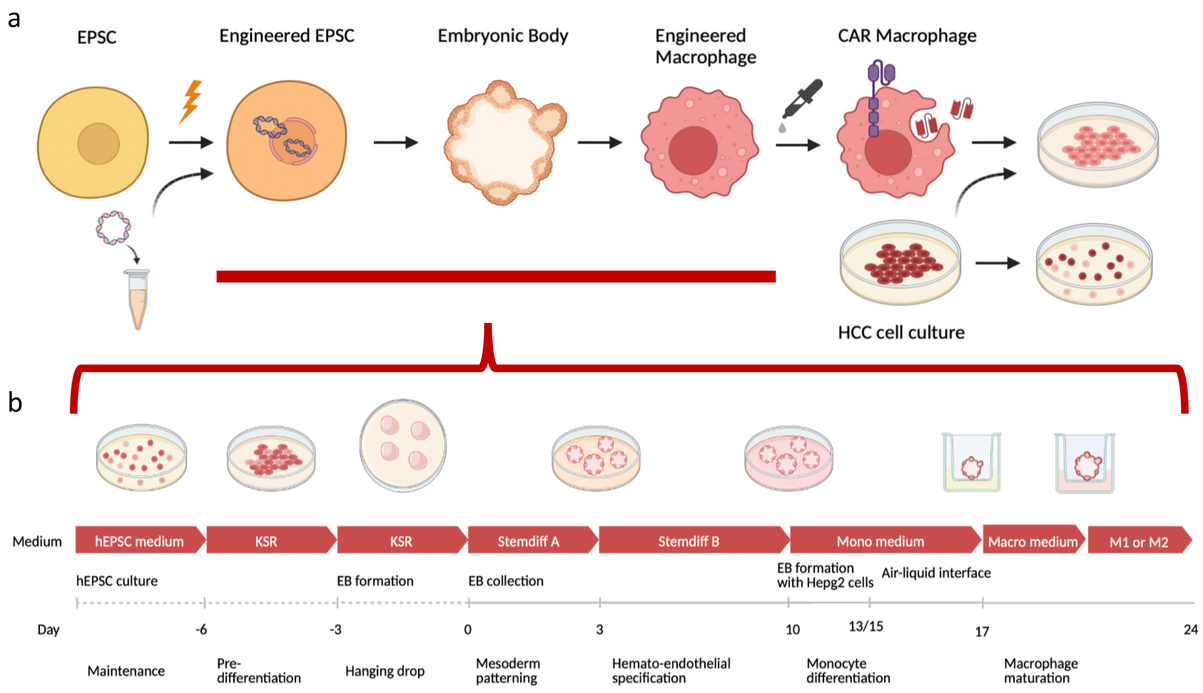

Figure 2. Experimental Design. a. An illustration of the experiment workflow. Human EPSCs are transfected with external plasmids by electroporation. The engineered EPSCs are then cultured into embryonic bodies (EBs). EBs would produce engineered macrophages, which would be induced by Doxycycline to express CAR. The group engineered with anti PD-1 scFv would secret the vectors. The matured macrophages were then harvested for various in vitro assays. b. The macrophage differentiation protocol. The engineered EPSCs undergo pre-differentiation, EB formation, mesoderm patterning, hepato-endothelial specification, monocytes differentiation and macrophage differentiation. All stages are induced by the indicated medium. Time points are specified.

Figure 3. CAR expression in engineered human EPSCs and differentiation into monocytes. a. Representative Flow cytometry analysis plots of CAR transfected EPSC single colonies. The upper left panel shows the DAPI- population (alive) with a signal threshold of 103. The upper right and lower left panels are WT EPSC controls which set the threshold gating for positive CAR expression. The lower right panel showed a representative plot of a CAR positive colony. b. Images captured by the inverted microscope of EB-monocytes culture. The upper panel was captured under 10X objective and the lower panel 20X. The red frames highlights translucent cavities.

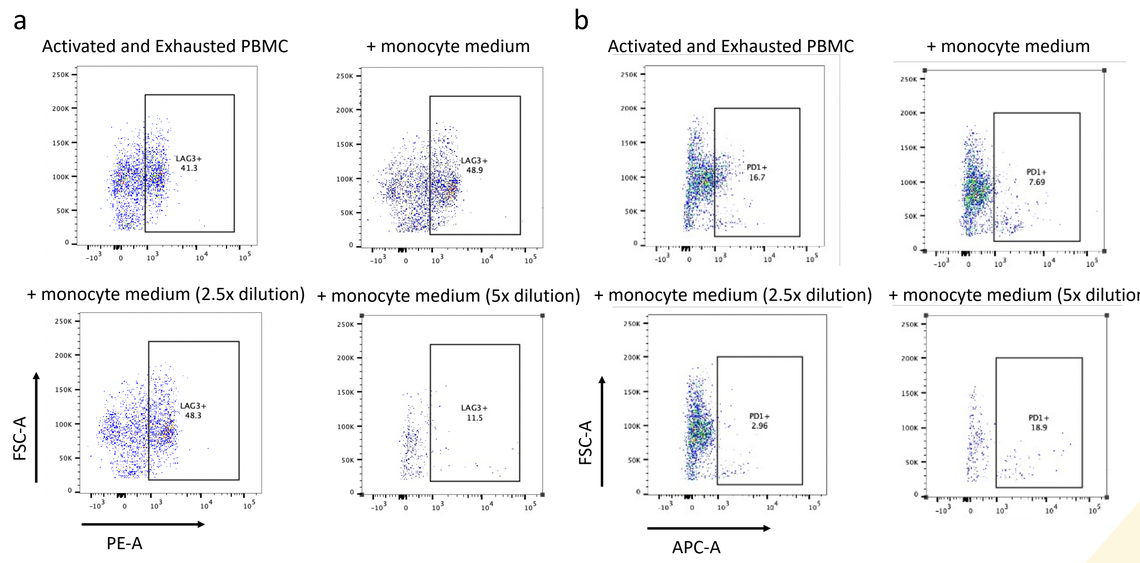

Figure 4. PBMC exhaustion rescuing assay. a. Representative Flow Cytometry analysis plots of LAG3 expression in the exhaustion preventing assay. The upper left panel shows the control group resulted from the PBMC exhaustion model. The upper right panel shows the experiment group added with monocyte medium. The lower left panel shows the experiment group with 2.5x diluted medium, while the lower right 5x. The LAG3 expression was reduced the most in the 5x diluted condition. b. Representative Flow Cytometry analysis plots of PD1 expression in the exhaustion preventing assay. The arrangement of panels follows that of 4b. The PD1 expression was reduced the most in the 2.5x diluted condition.

Future Directions:

After successful differentiation of macrophage:

1. Macrophage CAR expression assay
2. CAR Macrophage differential affinity assay
3. Macrophage phagocytosis assay
4. CAR Macrophage tumor specificity assay
5. Macrophage phenotyping after co-culture
6. Macrophage, PBMC coculture synergistic effects investigation
7. Future animal experiments with HCC model mice

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Acknowledgement:

Sincerely thanks to Dr. Rio Sugimura and my direct supervisor Dr. Hang Su for the guidance and support. Also give my gratitude to all BEL members for help and advice.