Evaluation of CAR T cell function in advanced 3D heterospheroid models reveals monocyte-dependent protection against cancer cell killing

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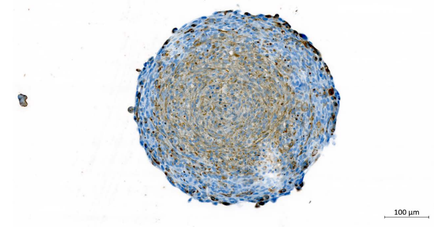

Introduction

CAR T cells are a type of adoptive cellular therapy that redirects T cells' functions toward target cells through a synthetic receptor[1]. Although CAR T cell therapy has shown remarkable success in treating hematologic malignancies, its effectiveness in solid tumors remains limited[2]. Clinical translation has been hindered by immune escape mechanisms and the immunosuppressive tumor microenvironment, which restrict CAR T cell infiltration and function[3]. These complexities are poorly mimicked in traditional preclinical models, thereby limiting their predictive value for clinical outcomes.

Advanced 3D heterospheroid models, incorporating multiple cell types such as fibroblasts and monocytes, provide a more physiologically relevant platform for evaluating CAR T cell therapy and other immunotherapies. Here, we assess EGFR-CAR T cell function across different *in vitro* models, demonstrating reduced efficacy in 3D compared to 2D, underscoring the importance of using 3D models to better mimic solid tumor complexity. Additionally, we show that monocytes within the tumor microenvironment can impair CAR T cell-mediated killing, highlighting their immunosuppressive role. Finally, we demonstrate that exhausted CAR T cells exhibit impaired function, which can be significantly enhanced with an α-LAG-3 antibody. Overall, these findings highlight the value of advanced 3D heterospheroid models for immunotherapy evaluation.

CAR T Cell Killing 2D vs 3D Models

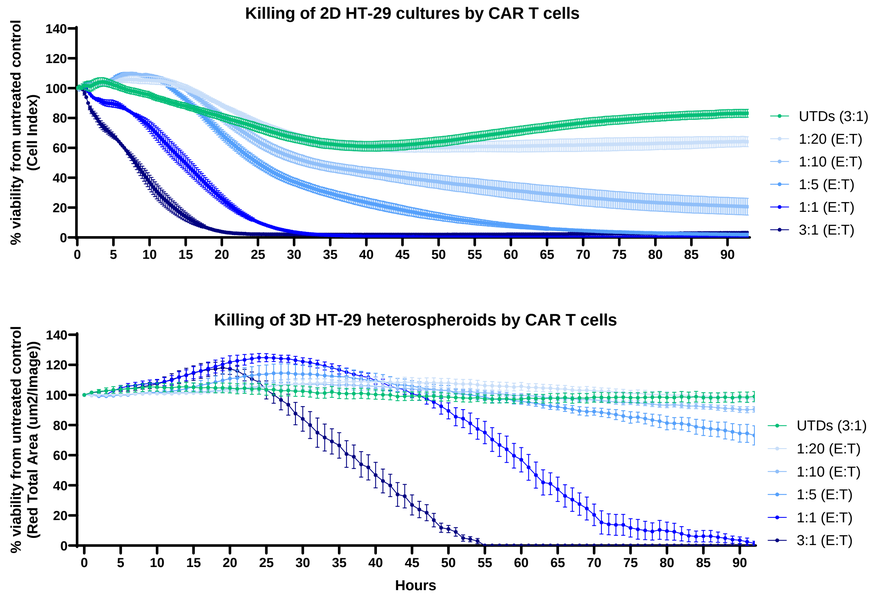

Figure 1. Real-time cytotoxicity of CAR T cells against HT-29 cancer cells in traditional 2D cultures vs 3D heterospheroid models. EGFR-CAR T cells or untransduced T cells were added to cancer cells at different Effector:Target (E:T) ratios. Killing was measured by a decrease in Cell Index (2D) or Total Red Area (3D) using xCELLigence (Agilent). Note the enhanced killing in the 2D setting, which diminishes in the 3D heterospheroid model mimicking the structural complexity of solid tumors.

Functional Differences in CAR T Cells Between 2D and 3D Models

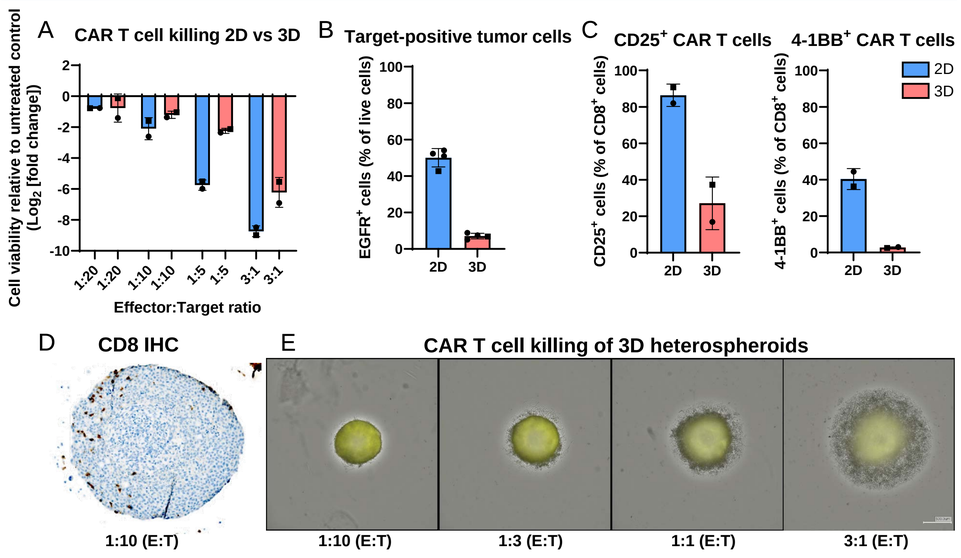

Figure 2. Impact of 3D tumor structure on CAR T cell killing, target expression, and activation. **(A)** CAR T cells were added to HT-29 cultures at various Effector:Target (E:T) ratios. Killing was measured by a decrease in live luciferase+ HT-29 cells after 96h. **(B)** Percentage of HT-29 cells expressing the target antigen (EGFR) in 2D and 3D cultures, showing higher target expression in 2D. **(C)** Expression of the activation markers CD25 and 4-1BB on CAR T cells added to 2D or 3D HT-29 cultures after 96h, demonstrating higher activation in 2D. **(D)** CAR T cell infiltration in 3D heterospheroids after 24h, assessed by CD8 immunohistochemistry. **(E)** Fluorescence images of HT-29 heterospheroids show reduced mCherry fluorescence with increasing CAR T cell ratios.

References:
1. Sadelain M, Brentjens R, Rivière I. The Basic Principles of Chimeric Antigen Receptor Design. Cancer Discov. 2013;3(4):388-98.
2. Ai K, Liu B, Chen X, Huang C, Yang I, Zhang W, et al. Optimizing CAR-T cell therapy for solid tumors: current challenges and potential strategies. J Hematol Oncol. 2024;17(1):105
3. Secondino S, Canino C, Alaimo D, Muzzana M, Galli G, Borgetto S, et al. Clinical Trials of Cellular Therapies in Solid Tumors. Cancers (Basel). 2023;15(14).

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Evaluating CAR T Cells in Advanced 3D Models: Monocyte-Dependent Protection of Killing

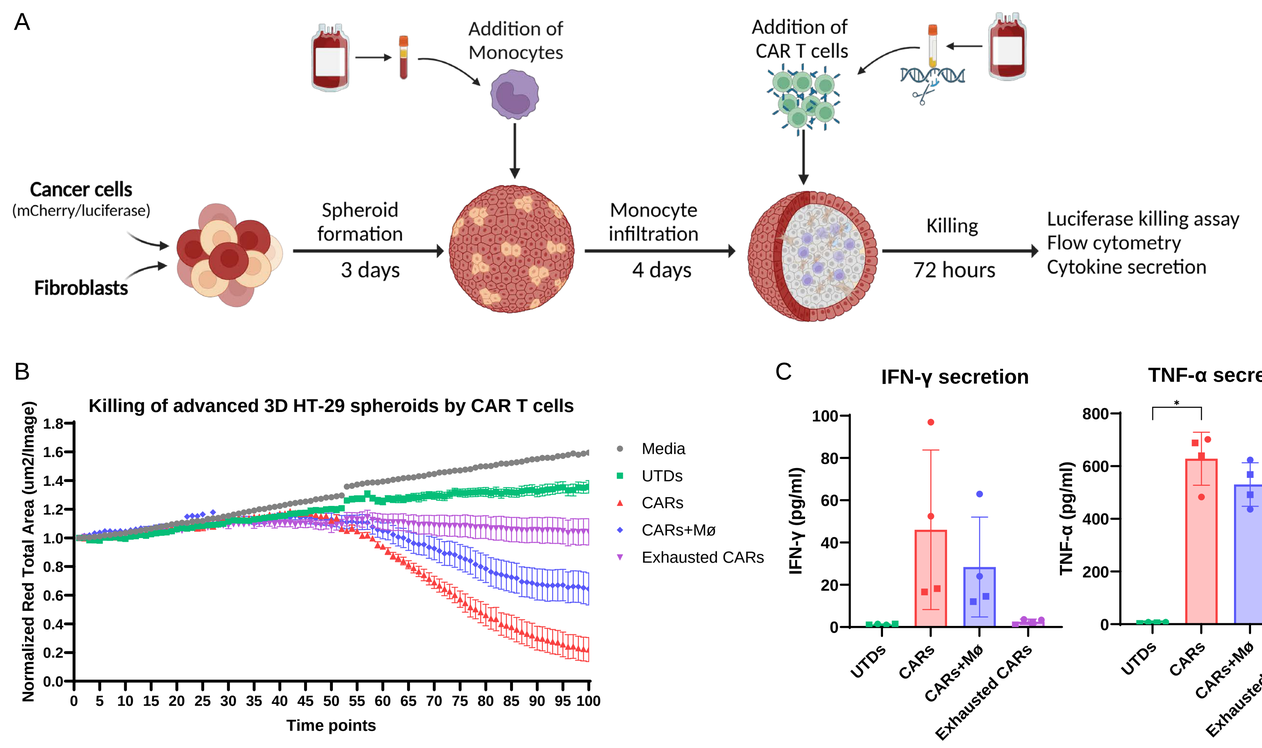

Figure 3. CAR T cell evaluation in advanced 3D models demonstrate monocyte-dependent protection against cancer cell killing. (A) Heterospheroids were generated from mCherry/luciferase reporter cancer cell lines and fibroblasts in ultra-low attachment plates. After 3 days, CD14+ monocytes from healthy donors were added and allowed to infiltrate before introducing EGFR-CAR T cells. **(B)** Real-time assessment of CAR T cell-mediated cytotoxicity against HT-29 spheroids without or including monocytes (+Mø). Untransduced T cells (UTDs) were included as control. Killing was measured by a decrease in Total Red Area using xCELLigence (Agilent), where a reduction in fluorescence indicates cancer cell death. Both functional and exhausted CAR T cells were evaluated. Note the impaired killing by exhausted CAR T cells and by functional CAR T cells when monocytes were present in the heterospheroids (+Mø). **(C)** IFN-γ and TNF-α secretion from CAR T cells in advanced 3D heterospheroids measured by Luminex. *: $p \leq 0,05$

Exhaustion of CAR T Cells

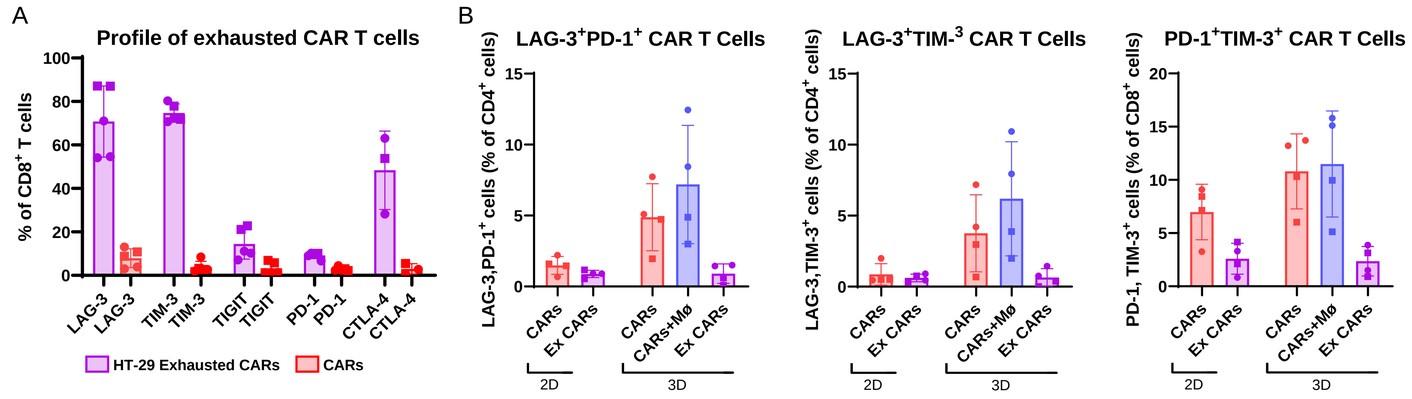

Figure 4. Exhaustion of CAR T cells in 2D and 3D cultures. **(A)** Exhausted CAR T cells (Ex CARs) were generated by repeated stimulation with 2D HT-29 cells over a period of 5 days, resulting in high expression of multiple checkpoint markers. **(B)** The percentage of CAR T cells expressing exhaustion markers LAG-3+PD-1+ (left), LAG-3+TIM-3+ (middle), and PD-1+TIM-3+ (right) was evaluated in 2D and 3D cultures after 72h of tumor cell killing. In 3D cultures, CAR T cells were analyzed in heterospheroids without or including monocytes (+Mø). Note that the frequency of CAR T cells double-positive for checkpoint markers was higher in 3D cultures, particular in the presence of monocytes (+Mø).

Enhanced CAR T Cell Killing and Activation in 3D Heterospheroids with α-LAG-3 Antibody

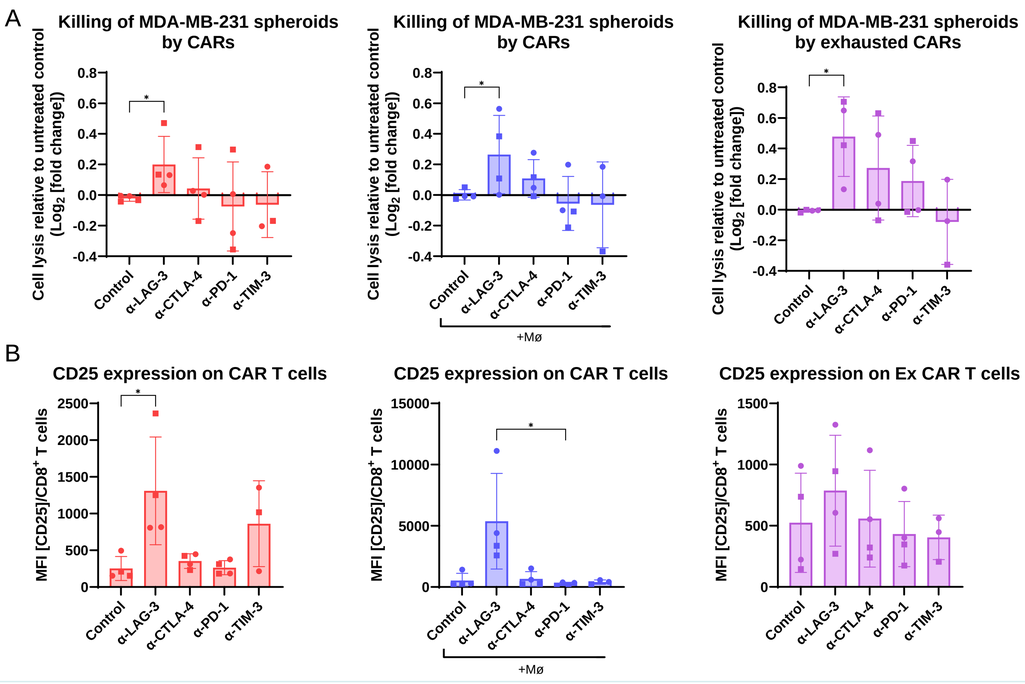

Figure 5: Enhanced CAR T cell killing of advanced 3D heterospheroids with α-LAG-3 antibody. CAR T cells or exhausted CAR T cells were added to 3D MDA-MB-231 heterospheroids, either without or with monocytes (+Mø), along with different immune checkpoint inhibitors. **(A)** Cancer cell lysis was assessed after 72h by measuring luciferase released from dying cancer cells. Higher luciferase levels indicate increased cell lysis. **(B)** Expression of the activation marker CD25 on CAR T cells was assessed after 72h killing of 3D heterospheroids and the presence of immune checkpoint inhibitors. Note the increased cancer cell lysis and CD25 expression upon treatment with α-LAG-3 antibody. *: $p \leq 0,05$

Conclusion

The 3D heterospheroid model alters CAR T cell responses compared to 2D cultures, resulting in reduced cytotoxicity, diminished antigen availability, and lower activation. Incorporating monocytes into the 3D model further decreases CAR T cell-mediated killing, reduces cytokine secretion, and increases checkpoint marker expression. These results underscore the impact of tumor architecture and the tumor microenvironment on the efficacy of CAR T cell therapy. By better mimicking solid tumors, 3D models improve preclinical immunotherapy evaluation and enhance translation to clinical outcomes.

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