Unlocking the potential of cancer immunotherapy: Overcoming the challenges of 3D immune cell killing assays

Immunotherapy harnesses the innate capabilities of the body's immune system to prevent, control, and eliminate cancer. This transformative approach to cancer treatment encompasses an array of strategies, including chimeric antigen receptor (CAR) T-cell therapy, immune checkpoint inhibitors (ICIs), monoclonal antibodies, and adoptive cell transfer.

CAR T-cell therapy involves genetically modifying T cells to recognize specific antigens expressed by tumor cells, effectively activating the patient's immune system to target cancerous cells. CAR T-cell therapies have shown impressive results in treating hematological malignancies, such as leukemia and lymphoma, with the FDA already approving six CAR T-cell therapies. But developing specialized therapies for the treatment of solid tumors has progressed at a much slower pace.

Obstacles include the challenge of identifying tumor-specific antigens due to tumor heterogeneity, the limited ability of CAR T-cells to reach and infiltrate tumor tissue, and the risk of off-target toxicity. Additionally, most solid tumors are embedded in a hostile microenvironment, with poor blood supply, inadequate oxygen, and nutrient delivery, an acidic pH, and a dense extracellular matrix. These factors can hinder the effector function of CAR T-cells and inhibit their clinical efficacy. To overcome these challenges, researchers are attempting to model the tumor environment *in vitro*, which is crucial for making informed decisions about the effectiveness of CAR T-cell and other immunotherapies.

Immune cell killing assays are critical to these pursuits as they are used to gain insights into the cytotoxic functions of different immune cell types and evaluate the efficacy of potential treatments. Traditionally, cells are grown in two dimensions on a flat surface of a culture dish. More recently, three-dimensional (3D) cell cultures, which more closely mimic the complexities of the *in vivo* microenvironment, are being used to gain more physiologically relevant data compared to 2D cell culture assays. Various technologies are employed for 3D immune cell killing assays, each offering unique advantages and insights into the cytotoxic functions of immune cells and the evaluation of treatment efficacy. Analysis approaches include flow cytometry, high-content analysis, live-cell imaging and *in vivo* bioluminescence imaging in small animal models. Although 3D immune cell killing assays yield more physiologically relevant results, they come with various technical and logistical difficulties. Some of the challenges include:

- Difficulties growing consistent and reproducible 3D cultures
- Reliable quantification of target cell killing in 3D cultures over time
- Reliable quantification of immune cell infiltration into 3D cultures over time
- Distinguishing between different cell types, such as differentiating dead cancer cells from dead immune cells
- Obtaining kinetic information over a sufficient time period to assess immune cell persistence
- Identifying suitable fluorescent dyes that can penetrate the 3D cell model, do not impair immune cell function, and sufficiently stain over the whole time course.

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To address these challenges, this paper presents several successful assay setups that utilize various analysis technologies to study the antitumor functions of immunotherapies in a 3D environment. We also provide a technology comparison to help researchers understand the benefits and challenges associated with these different approaches.

High-content analysis

Modified CAR T-cells targeting membrane-proximal epitope of mesothelin

Developing high-efficiency CAR T-cells requires appropriate antigen selection to eliminate tumor cells with minimal toxicity. A promising antigen for targeted immunotherapy is mesothelin (MSLN), a cell surface glycoprotein with low expression in normal tissues and high expression in various solid tumors. In a recent study, Zhang *et al.* engineered two types of CARs targeting either a membrane-distal (meso1 CAR T-cells) or membrane-proximal (meso3 CAR T-cells) epitope of MSLN using a modified piggyBac transposon system.[1] The researchers utilized 3D cancer spheroid models to assess the killing activity of the modified T cells.

Gastric and ovarian cancer cells were stained with Hoechst and cultured in wells for 48 hours to generate 3D spheroid cancer cells. Calcein-AM stained CAR T-cells were then added to the wells at the effector/target (E:T) ratio of 2:1, along with propidium iodide. To determine the death ratio of tumor cells the researchers used the Opera Phenix™ high-content screening system to analyze the fluorescence values at 0, 4, 6, and 24 hours after co-culturation.

Although both CAR T-cells were found to infiltrate into the tumor sphere, the team observed stronger killing activity against MSLN-expressing cancer cells from meso3 CAR T-cells in both cancer models (Figure 1). These findings showcase how 3D spheroid models and advanced screening systems can be used to gain valuable insights into the cytotoxicity of engineered CAR T-cells, while also underscoring the potential of targeting the membrane-proximal epitope of MSLN to treat MSLN-expressing tumors.

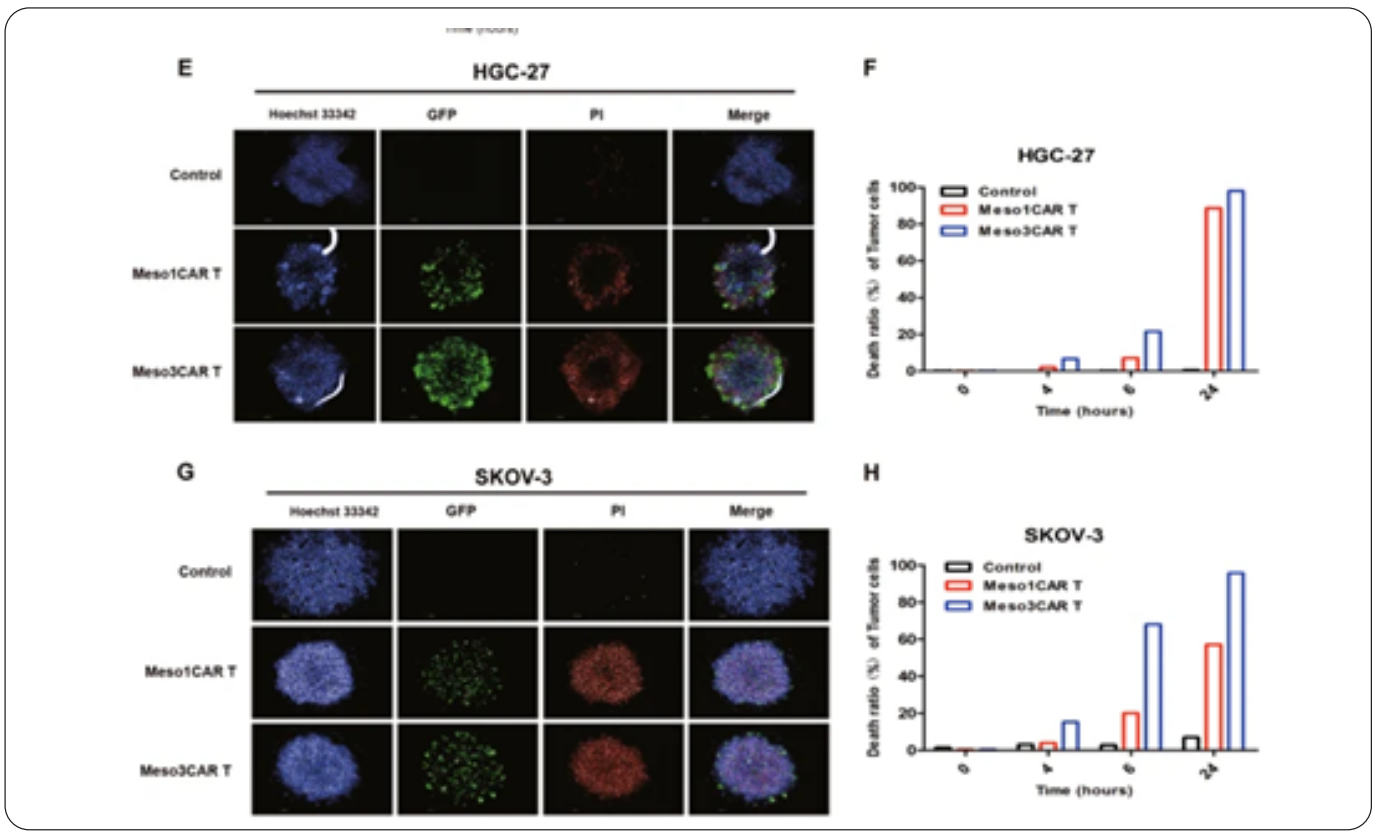

Figure 1: a) The killing activity of meso1 CAR and meso3 CAR T-cells was detected using the 3D cancer spheroid model in gastric cancer. b) The time effect for the death rate of tumor cells was shown by a histogram in gastric cancer. c) The killing activity of meso1 CAR and meso3 CAR T-cells was detected using the 3D cancer spheroid model in ovarian cancer. d) The time effect of the death rate of tumor cells was shown by the histogram in ovarian cancer. Image credit: Zhang *et al.*, 2019.[1]

Modeling immune cytotoxicity with tumor-derived organoids and effector T cells

ICIs have demonstrated remarkable efficacy in activating pre-existing anticancer T-cell responses among patient subsets affected by various advanced malignancies. However, it remains difficult to accurately predict which ICI will be effective for individual patients. Zhou and colleagues set out to establish a co-culture platform involving cholangiocarcinoma (CCA) organoids and immune cells that could potentially serve as an *in vitro* personalized model to evaluate the efficacy of ICIs.[2]

In the co-culture experiment, T cells were stained with CellTrace Far Red and combined with Hoechst-stained CCA organoids. To monitor cell death, a green caspase 3/7 detection reagent and Hoechst 33342 were also added to the medium. Confocal time-course imaging and quantitative assessments were performed on co-cultures every six hours for seven days. Time-lapse images presented at 0, 90, and 180 hours (Figure 2a) using the Opera Phenix high-content screening system indicate a noticeable increase in apoptosis in co-cultured organoids. Specifically, a higher number of CCA2 organoid cells were caspase 3/7 probe positive in the presence of T cells compared to the culture of only organoids. Quantitative image analysis confirmed that the apoptotic cell area increased about sevenfold in the organoid region after seven days of co-culture with T cells compared to the single culture of organoids (Figure 2b). As demonstrated in Figure 2c, the number of T cells was similar in the co-culture and single culture. These findings underscore the potential of this 3D co-culture system for quantifying patient-specific cytotoxic effects of immune cells in CCA organoids. The model could also be a useful tool for examining the efficacy of new ICIs and predicting which ICI will be effective for individual patients.

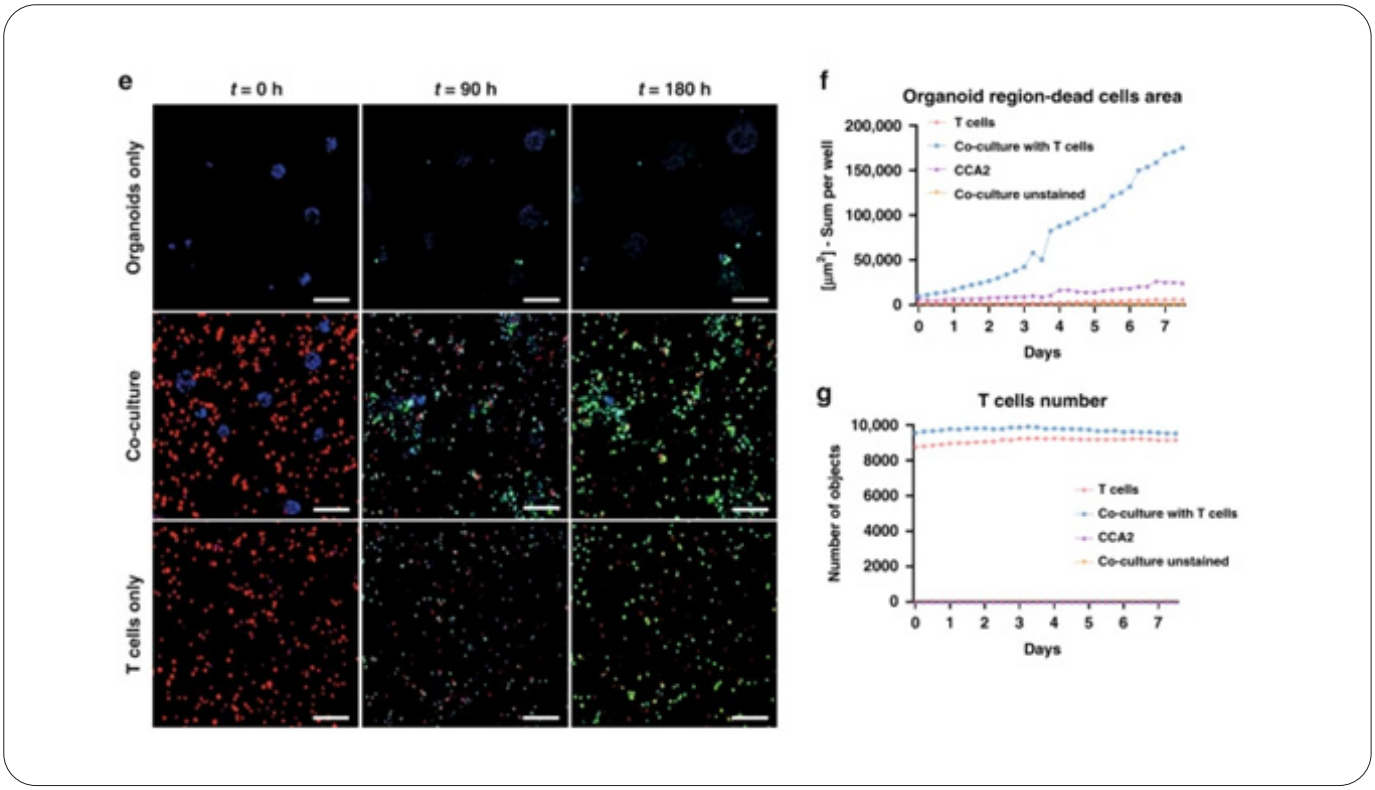

Figure 2: Representative confocal images of Cell Trace Far Red-stained T cells (red), Hoechst 33342-stained CCA2 organoids (blue), and co-cultures in the presence of a Caspase 3/7-probe (green) at timepoints 0, 90, and 180 h (a). Quantification of the dead cell surface (Caspase 3/7 probe-positivity) in the organoid area (Hoechst 33342 labeled) (b) and the number of T cells (c) in nine fields of view of confocal time-lapse imaging every 6 h for 180 h. Organoid death is higher in co-cultures with T cells compared to CCA2 organoids alone. The number of T cells (Cell Trace Far Red labeled) is comparable between solo culture and co-culture. Image credit: Zhou *et al.*, 2022.[2]

Image cytometry

A high-throughput method to analyze the cytotoxicity of CAR-modified immune cells in 3D tumor spheroid models using image cytometry

Image-based cytometry has emerged as a powerful technique for investigating and characterizing the functions of CAR-modified immune cells in a high-throughput manner. Plate-based image cytometry has shown its efficacy in analyzing various aspects of CAR T-cell therapy, including transduction efficiency, cell proliferation, and cytotoxicity. Recent advancements in 3D spheroid models have opened up new possibilities for image cytometry in the realm of CAR T-cell therapy, especially for treating solid tumors.

In a recent study conducted by Zurowski and colleagues, the potential of image cytometry was explored to characterize the cytotoxicity exhibited by CAR T-cells targeting the prostate-specific membrane antigen (PSMA) in a 3D tumor spheroid model.[3] PSMA is a transmembrane glycoprotein known for its expression in healthy prostate tissue and significant upregulation in cancerous tissue, making it an attractive target for CAR T-cell therapy.

3D tumor spheroids were formed by seeding antigen-expressing (PC3-PSMA$^+$ GFP$^+$) or negative control (MCF7-GFP$^+$) cells into Nexcelom^{3D} 96-well ultra-low attachment round-bottom plates. After two days, CAR T-cells and untransduced (UTD) T cells were introduced at various E:T ratios (10:1 , 5:1 and 1:1). The co-cultures were then imaged using a Celigo image cytometer at 0, 24, 48, and 72 hours. By evaluating the removal of GFP expression, the efficacy of the effector cells in inducing cytotoxic effects on the target cells was assessed.

The image analysis revealed that the CAR T-cells exhibited high cytotoxicity against antigen-expressing spheroids, while UTD T cells exhibited no noticeable effects. When the GFP fluorescent intensities were quantified over time, the researchers observed a time- and E:T ratio-dependent killing of the spheroids by the CAR T-cells. Notably, high levels of cytotoxicity were observed at all E:T ratios after 72 hours (10:1 ≅90%, 5:1 ≅89%, and 1:1 ≅78% reduction). In contrast, no observable cytotoxicity was seen with UTD T cells or non-antigen-expressing spheroids.

In another study, Sefan Grote and his team investigated the cytotoxic effects of CAR-modified effector cells that targeted the immune checkpoint molecule B7-H3 (CD276) in a 3D tumor spheroid model.[4] CD276 is frequently overexpressed in the majority of solid human tumors, while its expression is either weak or absent in normal tissues and cell types, making it an attractive target for CAR-mediated interventions. Instead of relying on the more conventional approach of employing T cells as effector cells, the researchers chose to use the natural killer (NK)-92 cell line in an attempt to overcome the challenges associated with producing autologous CAR T-cell products.

After demonstrating the ability of CD276-CAR NK-92 cells to eliminate NB cells in monolayer cultures, the team seeded GFP-positive neuroblastoma cells (NB) into Nexcelom 96-well low-attachment U-bottom plates, allowing them to form spheroids. The study used three high-grade NB cell lines: Kelly, LAN-1, and LS. After 72 hours, CD276-CAR NK-92 cells or parental NK-92 cells were introduced into the 3D cultures and fluorescence was measured using a Celigo image cytometer over a period of 96 hours. CAR-mediated cytotoxicity was calculated by anaylyzing the fluorescence intensity of NB spheroids.

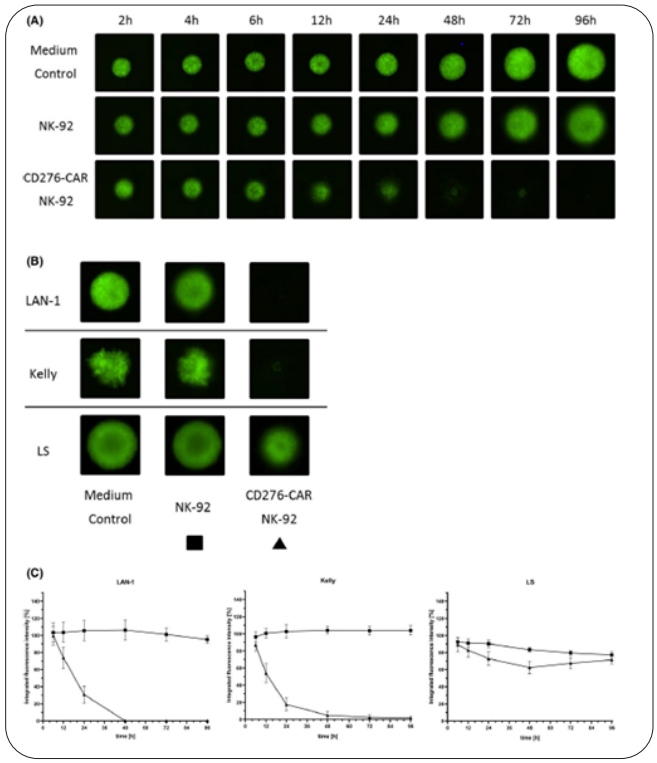

Figure 3: CD276-CAR NK-92-mediated lysis of 3D neuroblastoma spheroids. GFP-transduced neuroblastoma cell lines LAN-1, Kelly, and LS were grown as 3D spheroids and subsequently co-incubated with CD276-CAR NK-92 or parental NK-92 cells for 96 h in at least three individual experiments. Representative fluorescence images show LAN-1 spheroids (A). Integrated fluorescence intensity of neuroblastoma (NB) spheroids (LAN-1, Kelly, LS) was measured regularly using the Celigo S Imaging Cytometer (Nexcelom), representative fluorescence images of the NB spheroids are shown after co-incubation of 96 h (B, C). Image credit: Grote *et al.* 2020.[4]

Analysis revealed that the CD276-CAR NK-92 cells were able to specifically target and eliminate NB cells in the spheroid model, as shown in Figure 3. Specifically, all LAN-1 speroids were completely eradicated in less than 48 hours of CAR exposure. Spheroids established from the Kelly cell line were almost entirely eliminated within 72 hours, whereas the LS cell line's spheroids exhibited partial lysis due to insufficient CAR-mediated cytotoxicity during the given timeframe. Interestingly, in prior investigations where the team had screened the cell lines for expression of known NK cell ligands using flow cytometry, they found complete absence of the inhibitory NK ligand HLA-ABC on Kelly cells, mediocre expression on LAN-1 cells, and elevated HLA-ABC expression on LS cells.

Altogether, these studies highlight the capacity of plate-based image cytometry as a high-throughput tool for characterizing CAR-based treatment within a 3D spheroid model. This approach opens opportunities to rapidly identify suitable CAR-modified immune cell candidates for subsequent downstream processes.

In vivo imaging

Evaluating *in vivo* CAR T-cell toxicity in a mouse model

One of the primary hurdles in CAR T-cell therapies is the risk of adverse effects caused by on-target, off-tumor toxicity. This is a concern in patients who exhibit target antigen expression on both the tumor and healthy tissues. Although preclinical animal studies serve as useful tools for testing the efficacy of therapeutic CARs, they are limited in their ability to accurately identify potential adverse events in humans, potentially leading to a false sense of safety.

To address this, Castellarin and collaborators established a mouse model expressing the human Her2 (hHer2) antigen on both tumor and normal tissue.[5] Her2 is an attractive target for CAR T-cell therapy since it can be overexpressed 40- to 100-fold in human tumors. They then assessed the antitumor efficacy of high and low-affinity Her2 CAR T-cells using *in vivo* imaging.

For the study, mice were engrafted with a tumor xenograft displaying high Her2 expression and injected with a low dose of Her2-AAV8 to produce a low Her2-expressing liver. The Her2$^+$ tumor cells were genetically modified to emit fluorescence via the IRFP720 fluorescent reporter, facilitating *in vivo* imaging. The mice were then infused with high or low-affinity CAR T-cells and the antitumor efficacy was assessed. (Figure 4A)

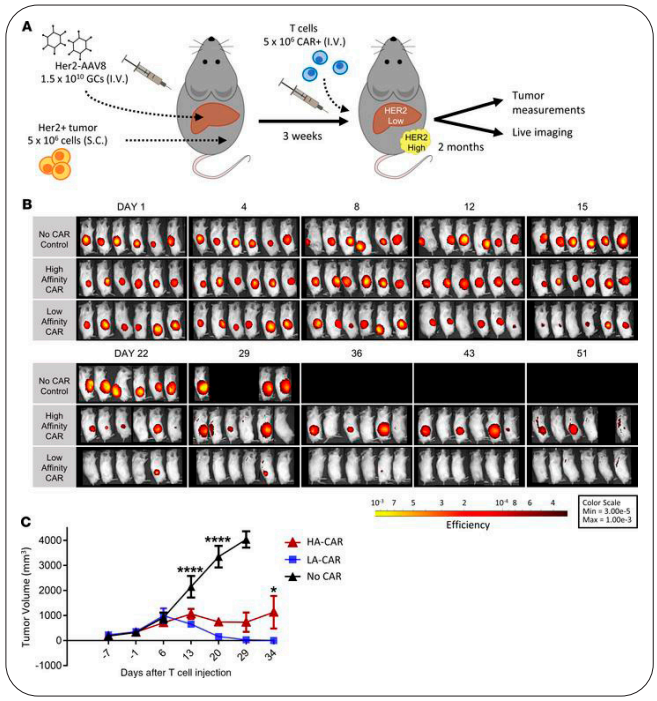

Figure 4: The low-affinity CAR has better tumor control than the high-affinity CAR when antigen is also expressed in normal tissue. (A) Overview of the experimental design for comparing Her2$^+$ tumor control between affinity-tuned Her2 CAR T-cells. All mice received 1.5 × 10^{10} GCs of Her2-AAV8 and were implanted with 5 × 10^6 Her2$^+$ SKOV3 tumor cells. Then, three groups were injected with either 5 × 10^6 high-affinity (HA) or low-affinity (LA) Her2-CARTs or no CAR control T cells. (B) The Her2$^+$ tumor cells, SKOV3, were genetically modified to express the fluorescent reporter, IRFP720, for *in vivo* imaging. Tumor xenograft fluorescence is shown in a yellow-to-red spectrum. Lateral views of fluorescent tumor imaging. (C) Mean tumor volume ± SEM measured by calipers in n = 6 mice per group. A 2-way repeated measures ANOVA with Bonferroni's multiple comparisons test was used for statistical analysis. Statistical significance is denoted as $^*P < 0.5$ and $^{****}P < 0.0001$. Image credit: Castellarin *et al.*, 2020.[5]

Utilizing the IVIS® Spectrum *in vivo* imaging system, the researchers found that mice treated with low-affinity CAR T-cells exhibited significantly better antitumor efficacy compared to those treated with high-affinity CAR T-cells (Figure 4B and 4C). To investigate whether differences in tumor control between the two groups were due to differences in T-cell abundance and/or trafficking, the team used *in vivo* bioluminescent imaging (BLI) to track the bioluminescence emitted by T cells expressing the CBR luciferase reporter gene 10 minutes post injection of IVISbrite™ D-luciferin, using the same IVIS Spectrum. While there were initial discrepancies in abundance between the

high- and low-affinity CAR T-cells, they converged after the first week and became comparable throughout the rest of the experiment (Figure 5). Importantly, the low-affinity CAR T-cells migrated out of the liver to the tumor site faster than their high-affinity counterparts. This suggests that low-affinity CAR T-cells exhibit an enhanced ability to differentiate between low-antigen healthy tissue and high-antigen tumor tissue, resulting in a better therapeutic outcome. The study demonstrates the efficacy of an off-tumor model for evaluating the recognition of tumors by CAR T-cells. This approach holds promise for predicting the therapeutic effectiveness of CAR T-cells compared to tumor xenografts alone.

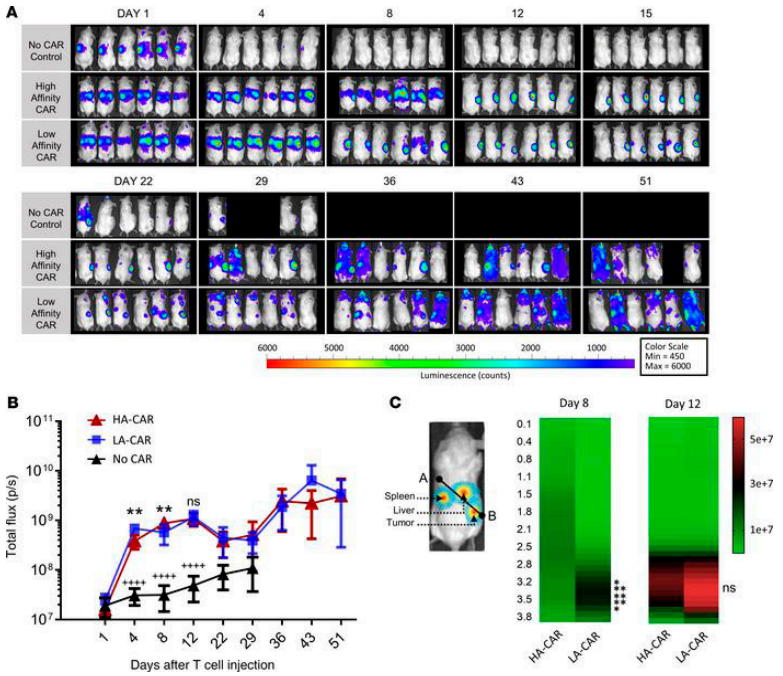

Figure 5: Low-affinity CAR T-cells spend less time off-tumor than high-affinity CAR T-cells. *In vivo*, CAR T-cell kinetics were captured using IVIS imaging for n = 6 mice per group. (A) T cells were engineered to express a luciferase gene for *in vivo* luminescent imaging. The dorsal views of the mice were kept in the same order as in Figure 4B, and luminescence intensity is shown in a blue-to-red spectrum. In addition to luciferase expression, the T cells contained either no CAR expression (negative control) or were engineered to express a high-affinity (HA) or low-affinity (LA) Her2 CAR. (B) Whole body bioluminescent imaging (BLI) of T cell luciferase. Statistical significance for HA-CAR versus LA-CAR (*) or HA-CAR vs. No CAR (+) was compared by 2-way repeated measures ANOVA with a Tukey's multiple comparison test. (C) Spatial luciferase expression was measured along a line that starts in the upper left thorax (point A) and ends in the lower right abdomen (point B). Luminescence from the spleen, liver, and tumor appears at the beginning (~0–1.5 cm), middle (~1–3 cm), and end (~2.5–4 cm) of the line, respectively. Mean luminescence along the line was compared between groups by 2-way repeated measures ANOVA with Bonferroni's multiple comparisons test. Statistical significance is denoted as $^{**}P < 0.01$ and $^{++++}P < 0.0001$. Image credit: Castellarin *et al.*, 2020.[5]

Table 1. Technology comparison for 3D immune cell killing assays.

Detection Method	Description	Benefits	Challenges
Image cytometry	Quantification of spheroid size and killing *in vitro*	▪ Live cell kinetic assay ▪ Provides morphology information ▪ Simple and robust method	▪ Need GFP recombinant cancer cell line or appropriate live-cell dyes
High-content analysis	Quantification of spheroid size, killing, and infiltration *in vitro*	▪ Live cell kinetic assay ▪ Multiplex cell type analysis ▪ Provides morphology and infiltration information ▪ Creates movies	▪ Need appropriate live-cell dyes ▪ Data volumes ▪ Imaging depth is limited in large 3D models without tissue clearing
Flow cytometry	Digest tumor samples and measure the number of viable and apoptotic cells in the sample	▪ Quantification of a large number of cells ▪ Multiplex cell type analysis ▪ Tumor infiltration information available	▪ No kinetic information ▪ No morphology or spatial information
In vivo imaging	Quantification of tumor size *in vivo* in mouse models	▪ Non-invasively quantify tumor size and metastasis ▪ Analyze on-target cell activity ▪ Analyze toxicity to organs/off-target effects	▪ Mouse models have limited physiological relevance ▪ Xenograft models cannot recapitulate toxicity sufficiently ▪ Costly models

Conclusion

Immune cell killing assays have emerged as pivotal tools in immunotherapy research for investigating immune cell functions and evaluating treatment efficacy. These assays are not without their challenges, especially when run in 3D. Technical and logistical hurdles include consistent and reproducible 3D culture establishment, accurate quantification of target cell elimination, reliable measurement of immune cell infiltration, identification of various cell types, acquisition of kinetic data, and identification of suitable fluorescent dyes.

These difficulties demand innovative solutions, and this paper presents a range of successful assay setups that leverage diverse analytical technologies, including high-content analysis, flow cytometry, image cytometry, and *in vivo* imaging to gain valuable insights into the complex interplay of immune cells, target tissues, and therapeutic interventions (Table 1). These innovative techniques open new avenues for precise assessment, informed therapeutic design, and the advancement of cancer immunotherapy.

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