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Quantifying T Cell Response in 3D Tumor Spheroids Using Advanced Flow Cytometry Workflows

Kirsty Mcbain, Minnie Oliver, Nicola Bevan and Clare Szybut
Sartorius UK Ltd., Units 2 & 3 The Quadrant, Newark Close, Royston Hertfordshire SG8 5HL UK

Correspondence
Email: Kirsty.Mcbain@sartorius.com

Introduction

Discovery of novel immunotherapies that specifically target and enhance the T cell response against cancer, for example CAR-T cells, bispecific antibodies, and checkpoint inhibitors, is a rapidly expanding research area. Robust and relevant *in vitro* models for evaluation of these immunotherapies is essential throughout their development. In the past, these models have often relied on the use of suspension cells which has limited studies to liquid tumors, such as blood cancers. Others have used 2D cell monolayers to explore drug effects on solid tumors, but these models lack many of the key features of the *in vivo* tumor microenvironment.

Recent evidence supports the use of 3D advanced tumor models, such as multicellular spheroids or organoid cultures, as opposed to the 2D monolayer.[1] These bring enhanced biological relevance to the model, with a closer resemblance to the *in vivo* clinical scenario. For example, a spheroid model has more complex cell-cell interactions; optional extracellular matrix proteins and a layered structure, with zones undergoing proliferation and quiescence, as well as an inner necrotic core.[2,3] These structural features have a multitude of effects on cells, including graduated distribution of nutrients and gases alongside the added requirement for immune cells to penetrate the tumor during killing.[2,3]

Each immunotherapeutic targeting the T cell pathway is designed to bind to a specific partner protein on the T cell or antigen-presenting cell (e.g., tumor cell) which in turn triggers an effect on the T cell function. For example, checkpoint inhibitor drugs bind to immune checkpoints, such as PD-1 or CTLA-4, and act to 'take the brake off' T cells. This leads to an increase in their activation and enhancement of their immune cell killing (ICK) capacity, which can manifest through several downstream effects, such as upregulated production and excretion of cytotoxic protease granzyme B. Other elements of the T cell response become particularly important to control during the production of immunotherapies such as CAR-T cells. For example, it is important to produce cells with minimized sensitivity to T cell exhaustion, which can occur during repeated antigen exposure. Exhaustion is hallmarked by reduced killing potential, a reduction in cytokine production and increased expression of markers such as LAG-3 and TIM-3. It is also critical that CAR-T cells can differentiate to form memory T cells, as these provide the essential prolonged, antigen-specific protection from tumor cells, long after the initial infection has been cleared.

Another important consideration in immunotherapeutic development is the effect a therapy might have on T cell penetration into the tumor. Immune cells must maintain the ability to survive, proliferate, and kill the cancerous cells once within the tumor microenvironment if effective treatment is to occur. Increased numbers of tumor infiltrating lymphocytes (TILs) within the 3D tumor are associated with improved response in neoadjuvant chemotherapy and with better overall pathological outcomes.[4]

Traditional methods for measuring T cell response in 3D models, such as imaging-based techniques, are often limited because they:

- Provide bulk measurement of infiltration or killing without a deeper investigation into effects on cell marker expression or cytokine release
- Require multiple workflows for quantification of different parameters, often using multiple instruments
- Involve lengthy, time-consuming workflows, which require multiple rounds of protocol optimization, fixation, and repetitive washes
- Necessitate correlation of data from several different assays for each treatment condition, increasing the risk of data variability

In this application note, we provide two advanced flow cytometry-based solutions to address these problems. The first is a TILs assay to measure the degree of T cell infiltration into spheroids and to determine the phenotype of infiltrated compared to non-infiltrated cells. The second is a spheroid ICK workflow, which uses subset analysis and cytokine quantification to examine the activation, killing, exhaustion, and memory profiles of T cells during co-culture with spheroids. Both workflows utilize the iQue® Advanced High Throughput Flow Cytometer, associated suite of T cell characterization reagent kits, and validated spheroid washing and dissociation protocols to provide an end-to-end solution for the evaluation of T cell response in advanced 3D tumor models.

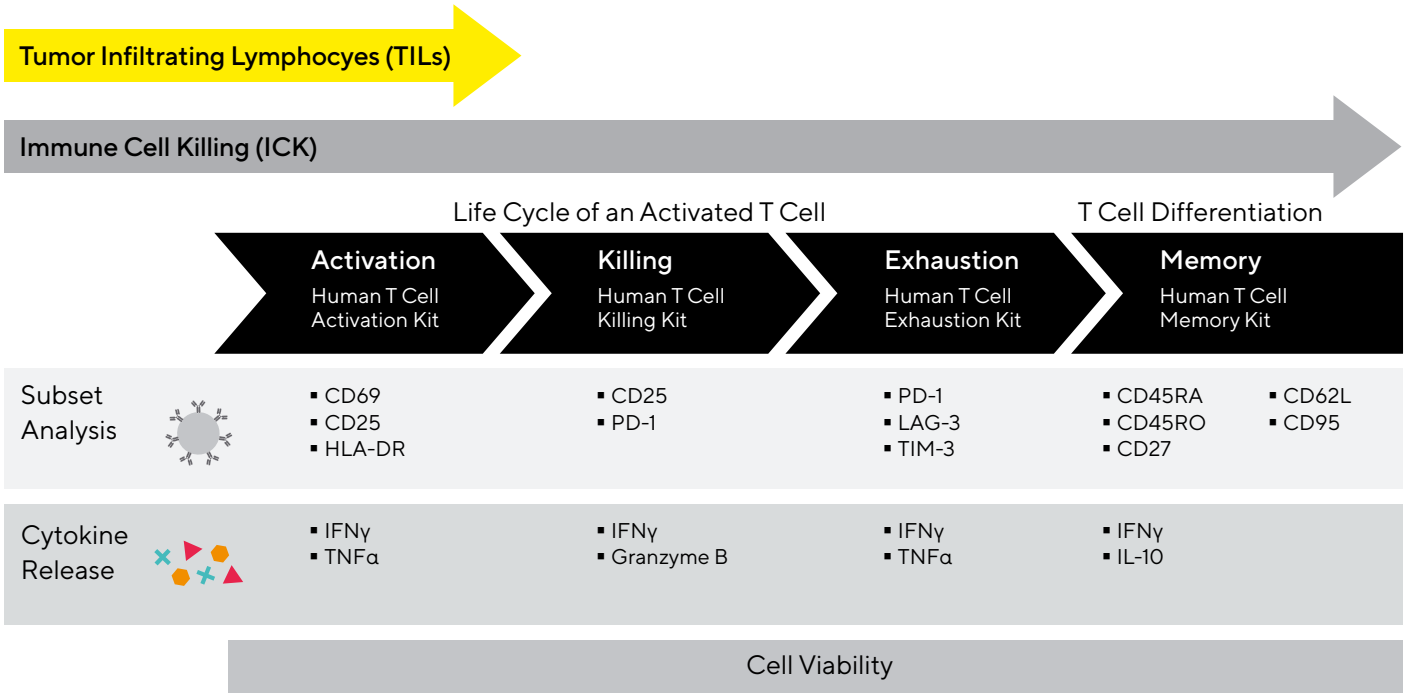
Assay Principles

For both the TILs and ICK assays, cells are seeded in 96-well Ultra-Low Attachment (ULA) plates and incubated for 72 hours to promote formation of spheroids before the PBMCs and activators are added. For the TILs assay, the spheroids are formed from wild-type tumor cells and the Incucyte® Cytolight Rapid Green reagent is used to label the PBMCs before seeding. This allows us to distinguish the tumor cells from the immune cells during analysis and gives the option of following infiltration over time by monitoring the increase in green fluorescence as PBMCs penetrate the spheroid. Conversely, the ICK assay uses Incucyte® Nuclight Green labeled tumor cells and unlabeled PBMCs, which enables spheroid green area or fluorescence intensity to be quantified over time to measure the rate of ICK.

At TILs assay endpoint (up to 48 hours), non-infiltrated immune cells are washed off before the spheroids and TILs are dissociated. T cell subsets are then analyzed using the iQue® Human T Cell Activation (TCA) Kit (Figure 1). At ICK assay endpoint, spheroids and immune cells are dissociated and labeled using either the iQue® TCA, iQue® Human T Cell Killing (TCK) Kit, iQue® Human T Cell Exhaustion (TCE) Kit or iQue® Human T Cell Memory (TCM) Kit, depending on the desired outputs. Supernatant samples (10 µL) are also taken for cytokine analysis using iQue Qbeads®. Each T cell characterization kit contains a pre-set gating template which is imported into the iQue Forecyt® software to facilitate instantaneous data readouts, including pharmacological readouts, such as EC_{50} values.

Figure 1

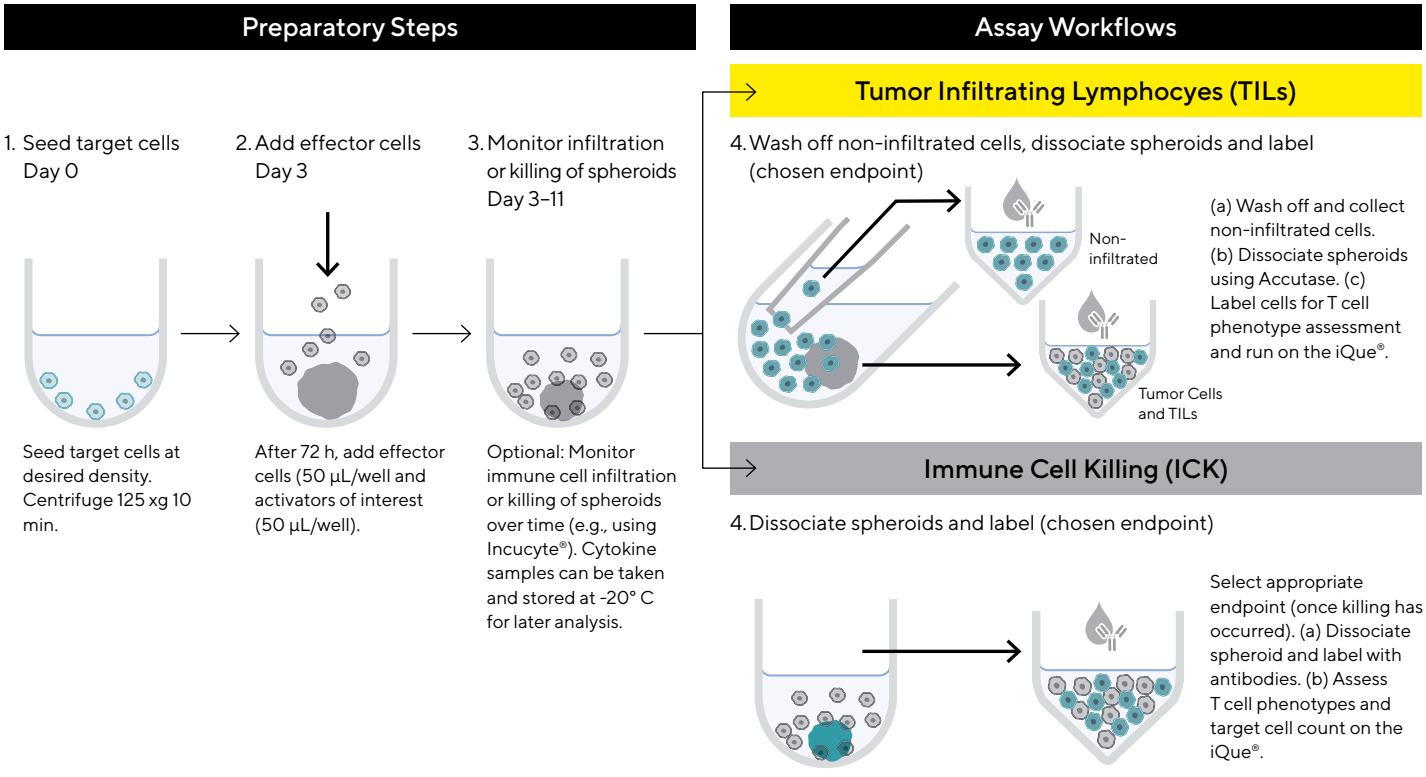
Illustration of the iQue® Spheroid TILs and ICK Assay Principles



Note. Spheroids are formed in round bottom ULA plates prior to the addition of immune cells (PBMCs or T cells). TILs assay analysis is performed using the iQue® Human TCA kit. In the ICK assay, cells and cytokines are analyzed using the iQue® T cell characterization kits (Activation, Killing, Exhaustion and Memory). Each kit contains a unique combination of antibodies, including basic T cell markers, for profiling cell surface marker expression, coupled with a viability dye and 2-plex iQue Qbeads® to quantify secreted cytokine concentration.

Figure 2

Schematic Outlining the Protocols for Analysis of TILs and ICK in 3D Tumor Spheroids Using the iQue® Platform



Assay Workflows

1 Assay Set Up

1.1 Seed target cells at an appropriate density into 96-well round bottom ULA plates and incubate for 72 hours to promote spheroid formation.

Note: For TILs assay seed wild-type cells and for ICK assay seed Incucyte® Nuclight Green labeled target cells.

Note: For the ICK assay, basement membrane extract, e.g. Matrigel® (typically1.5 – 2.5%) may be added to promote tight spheroid formation. Optimization is required depending on the target cell used.

1.2 Prepare desired concentrations of treatments | activators (e.g., Gibco™ Dynabeads™ Human T-Activator CD3/CD28) in cell culture media and add to the plate.

1.3 Add effector cells (e.g., T cells or PBMCs) at the desired effector-to-target ratios (e.g., 3:1, 5:1).

Note: For TILs assay, add Incucyte® Cytolight Rapid Green labeled effector cells and for ICK assay add unlabeled effectors.

1.4 Incubate for up to 2 days for TILs or for up to 8 days for ICK.

Note: Infiltration or killing can be monitored over time, for example using the Incucyte® Live-Cell Analysis Platform.

1.5 Optional: For the ICK assay, 10 µL supernatant samples can be taken at desired timepoints for kinetic cytokine analysis using iQue Qbeads® from the desired T cell characterization kit.

Note: Cytokine samples can be frozen at -20° C for later analysis.

2a TILs Endpoint Analysis

2a.1 Wash spheroids to carefully remove non-infiltrated cells and transfer them to a separate V-bottom plate for analysis, taking care not to remove spheroids from the ULA plate. Repeat wash step two further times.

Note: Use a microscope or scan the plate using the Incucyte® to check that spheroids have been washed effectively and that no non-infiltrated immune cells remain.

2a.2 Add Accutase to dissociate the spheroid and TILs and transfer to a separate V-bottom plate.

Note: Check that spheroids have broken down into a single cell suspension using a microscope.

2a.3 Label both infiltrated and non-infiltrated cells using antibody cocktail and viability dye from the iQue® TCA kit.

2a.4 Run on the iQue® and analyze using the gating template provided with the TCA kit.

2b ICK Endpoint Analysis

2b.1 Remove a final 10 µL supernatant sample and transfer to a V-bottom plate for cytokine analysis.

2b.2 Add Accutase to dissociate the spheroid and immune cells and transfer to a separate V-bottom plate.

Note: Check that spheroids have broken down into a single cell suspension using a microscope.

Note: If cells were seeded with Matrigel, incubation with Corning® Cell Recovery Solution is recommend prior to dissociation with Accutase

2b.3 Label cells using the antibody cocktail and viability dye from the iQue® TCA, TCK, TCE or TCM kit.

2b.4 Run on the iQue® platform and analyze using the gating template provided with the chosen kit.

Activation Marker Expression on TILs Is Greater Than on Non-Infiltrated T Cells

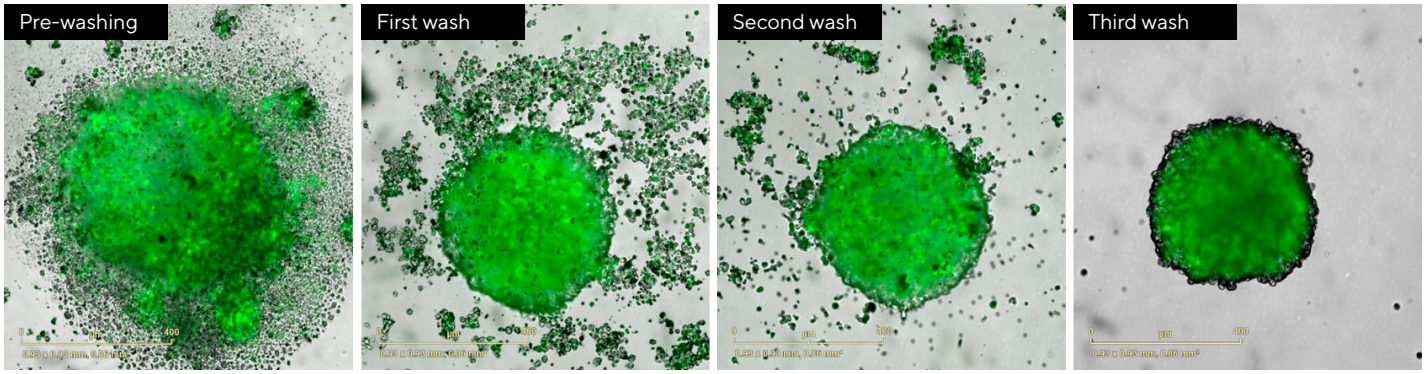
It is widely accepted that increased numbers of TILs within a tumor are often associated with improved clinical prognosis.[5] Evidence is now growing which suggests that the composition of the TILs, in terms of the cell subsets present and their activation status, is also highly important in denoting the quality of the anti-tumor response the TILs can exert.[5-7] Initial experiments aimed to investigate the phenotypic profile of these cells *in vitro* using the TILs assay model with wild type BT474 (breast cancer) spheroids and Incucyte® Cytolight Rapid Green labeled PBMCs, activated with a range of concentrations of CD3/CD28 Dynabeads™.

After 24 hours in co-culture, the non-infiltrated tumor cells were washed off and transferred into a separate plate, leaving only the spheroids and Green-labeled TILs in the assay plate. This three-step washing protocol was validated by taking images after each wash using the Incucyte® Live-Cell Imaging platform (Figure 3A). The images clearly show a reduction in the number of immune cells suspended around the spheroid with each progressive wash step, finally resulting in negligible remaining non-infiltrated cells after the third wash. Once the non-infiltrated cells had been separated, the spheroids and TILs were dissociated to create a single cell suspension suitable for analysis using the iQue® TCA kit.

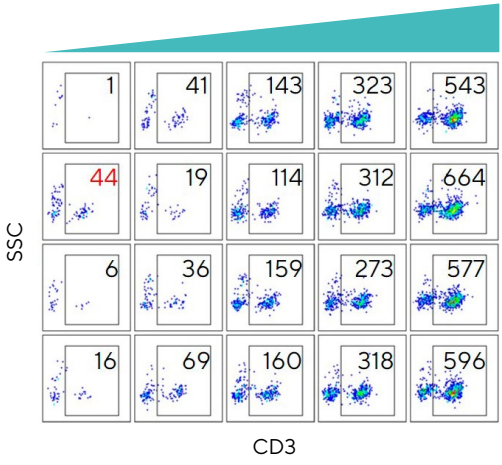
Figure 3

Comparison of the Activation Status of Infiltrated and Non-infiltrated T Cells

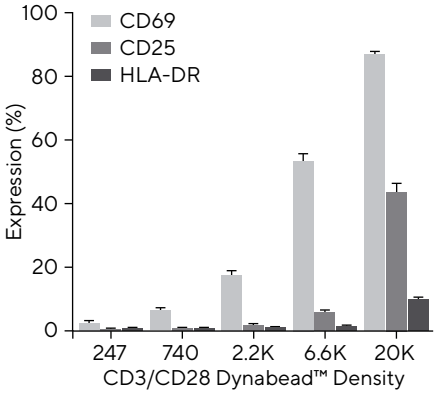
A.



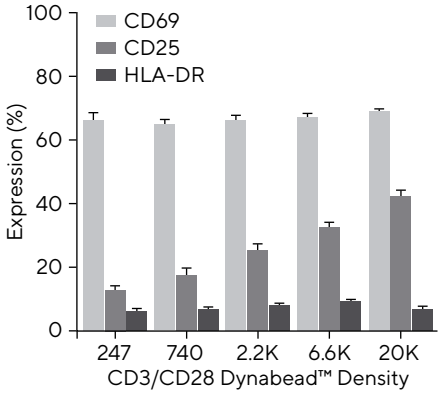
B. Dynabead™ Density



C. Non-Infiltrated



D. Infiltrated



Note. BT474 spheroids were formed in ULA plates for 72 hours before Incucyte® Cytolight Rapid Green labeled PBMCs (5:1 effector-to-target ratio (E:T)) and CD3/CD28 Dynabeads™ were added for 24 hours. (A) Incucyte® images used to validate the washing protocol for separation of non-infiltrated immune cells from the spheroid and TILs. (B) Spheroids and TILs were dissociated to a single cell suspension and analyzed using the iQue® Human T Cell Activation Kit. Plate view shows individual well plots of side scatter (SSC) vs. CD3 with gates highlighting the number of CD3 positive TILs per iQue® sip from each well. Each column represents a different Dynabead™ density (n = 4). An outlier (highlighted in red) was excluded from subsequent analyses. (C) and (D) Activation marker expression comparison between the non-infiltrated and infiltrated T cells.

The relative number of CD3 positive TILs per well is shown in a plate view diagram generated in iQue Forecyt® software (Figure 3B). This shows a Dynabead™ concentration-dependent increase in infiltration of T cells into the spheroid. Figures 3C and 3D compare the expression of early (CD69), mid (CD25) and late (HLA-DR) stage activation markers between the non-infiltrated and infiltrated T cells. At all but the highest concentration of Dynabeads™, the expression of the three activation markers was higher on the infiltrated T cells than on the non-infiltrated. On the infiltrated cells, high expression of CD69 was maintained, regardless of the density of Dynabeads™ present, with an average of 67.1 ± 0.6%. Non-infiltrated cells displayed increased sensitivity to changes in external stimuli with CD69 expression increasing in a Dynabead™ concentration dependent manner, from 2.3 ± 0.2% to 86.9 ± 0.7% when the Dynabead™ density was increased from 247 to 20K per well.

Tumor Cell Type Impacts the Number and Profile of TILs

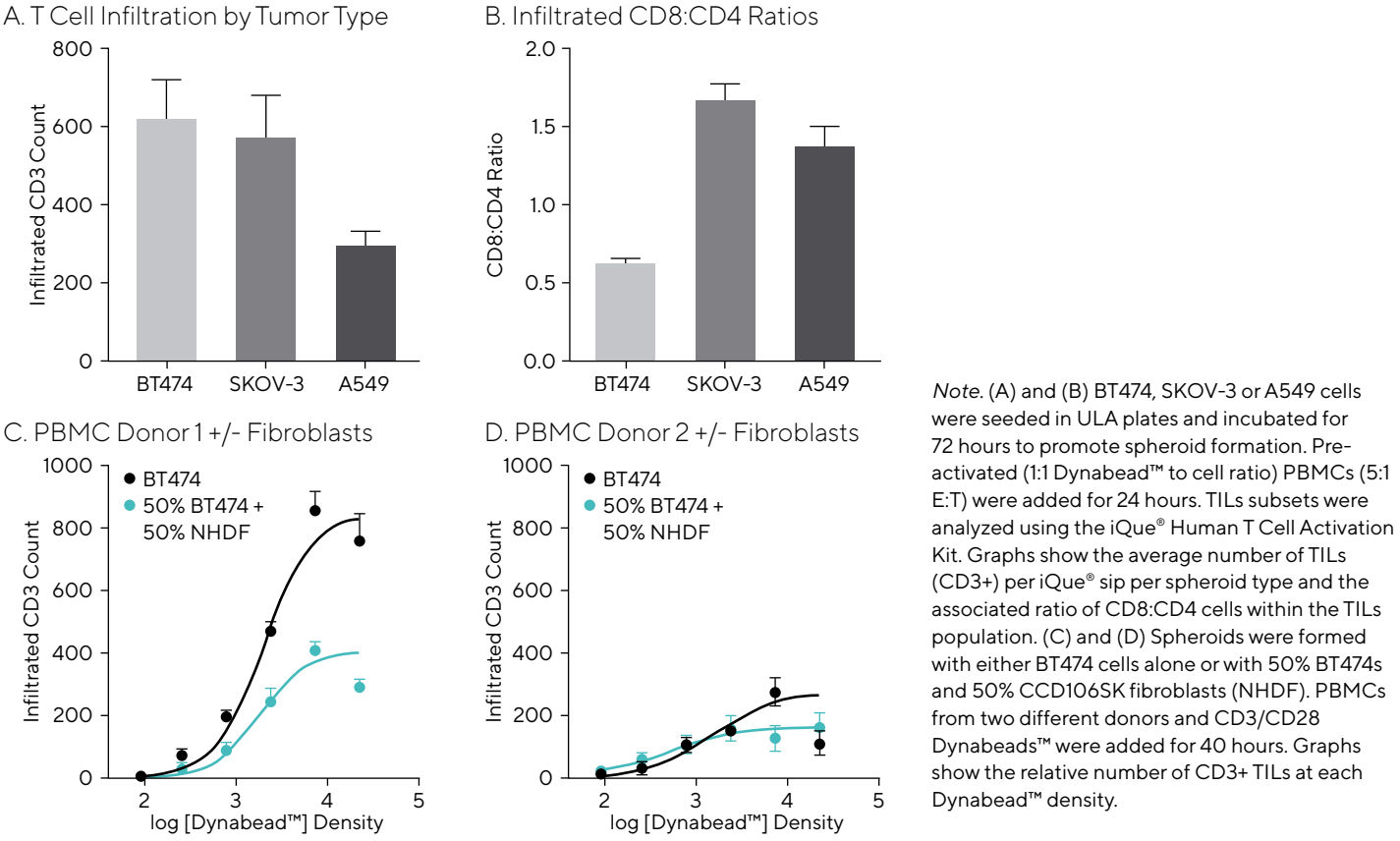
Studies that have measured rates of TILs across multiple tumor types have found differences between cancers in the likelihood of the presence of high density TILs and the significance of these in terms of prognostic outcomes.[8,9] To verify whether our model could be used to reveal differences in infiltration between different tumor types, we formed spheroids from breast (BT474), ovarian (SKOV-3) and lung (A549) cancer cell lines and analyzed the rate of infiltration and the phenotype of pre-activated PBMCs after 24 hours of co-culture using the iQue® TCA kit.

Both the BT474 and SKOV-3 spheroids displayed very similar levels of infiltration with an average of 617 ± 97 and 567 ± 105 CD3+ cells sampled per well, respectively, while average infiltration into the A549 cells was much lower at 282 ± 41 (Figure 4A). This indicated there may be a tumor-specific element to the degree of infiltration. The TILs from each of the different spheroid types were also analyzed for the ratio of CD8 to CD4 cells. A high proportion of cytotoxic CD8+ TILs is generally associated with more successful pathological complete response in cancer.[9] In the spheroid types we tested, both the SKOV-3 and A549 spheroids contained a high proportion of CD8+ TILs with average ratios of 1.65 and 1.35 CD8:CD4s, respectively (Figure 4B). Comparatively, the TILs in the BT474 spheroids had a much lower average CD8:CD4 ratio of 0.60. Further work is needed to investigate how this translates to the clinical scenario, but the ability to determine the CD8:CD4 ratio *in vitro* has potential to be a highly useful predictive tool.

The next experiments were designed to investigate the effect fibroblasts have on the degree of infiltration into a tumor. It has been shown previously that fibroblasts can lead to the formation of treatment-resistant tumors, which in turn results in poor clinical outcomes.[10] Our results demonstrated that, across both PBMC donors tested, there was a large reduction in infiltration when fibroblasts were included in the spheroid compared to tumor cells alone, with a 52% reduction in infiltration of Donor 1 and 55% of Donor 2 T cells with 25K Dynabeads™ per well (Figure 4C and D). Although the effects of fibroblasts were similar, the overall infiltration levels were strikingly different between the two donors, suggesting that immune cell donor-specific factors are impactful. It was noted that with both donors, at the top concentration of Dynabeads™, there was a decrease in the level of infiltration relative to the second highest density. It is likely that, after the 40-hour co-culture period, immune cell killing of the spheroid had started to occur, meaning that the spheroid broke up during the wash steps.

Figure 4

Infiltration Is Affected by Tumor Type, the Presence of Fibroblasts, and Immune Cell Donor



Note. (A) and (B) BT474, SKOV-3 or A549 cells were seeded in ULA plates and incubated for 72 hours to promote spheroid formation. Pre-activated (1:1 Dynabead™ to cell ratio) PBMCs (5:1 E:T) were added for 24 hours. TILs subsets were analyzed using the iQue® Human T Cell Activation Kit. Graphs show the average number of TILs (CD3+) per iQue® sip per spheroid type and the associated ratio of CD8:CD4 cells within the TILs population. (C) and (D) Spheroids were formed with either BT474 cells alone or with 50% BT474s and 50% CCD106SK fibroblasts (NHDF). PBMCs from two different donors and CD3/CD28 Dynabeads™ were added for 40 hours. Graphs show the relative number of CD3+ TILs at each Dynabead™ density.

Activation and Exhaustion Status of T Cells Changes Throughout Spheroid Killing

As was observed at the highest Dynabead™ concentration in Figures 4C and D, when the spheroid and activated immune cell co-culture is given strong activation and left for longer periods of time, we start to see a reduction in the number of target cells present in the spheroid, indicating that immune cell killing has started to occur. Over this longer time frame, we expect to see changes in immune cell phenotypic profiles, with increased expression of later-stage activation markers, such as CD25 and HLA-DR. It is also possible that immune cells start to express markers of exhaustion such as PD-1 due to the extended period of antigen exposure.

We aimed to explore these parameters by generating Incucyte® Nuclight Green labeled BT474 spheroids and co-culturing them with PBMCs and CD3/CD28 Dynabeads™. We created replicate plates which meant that on Days 1, 4 and 8, we could dissociate spheroids and analyze immune and target cell populations using the iQue® TCA and TCK kits. Prior to dissociation, 10 µL supernatant samples were taken from each plate for cytokine analysis using iQue Qbeads® from the chosen kits.

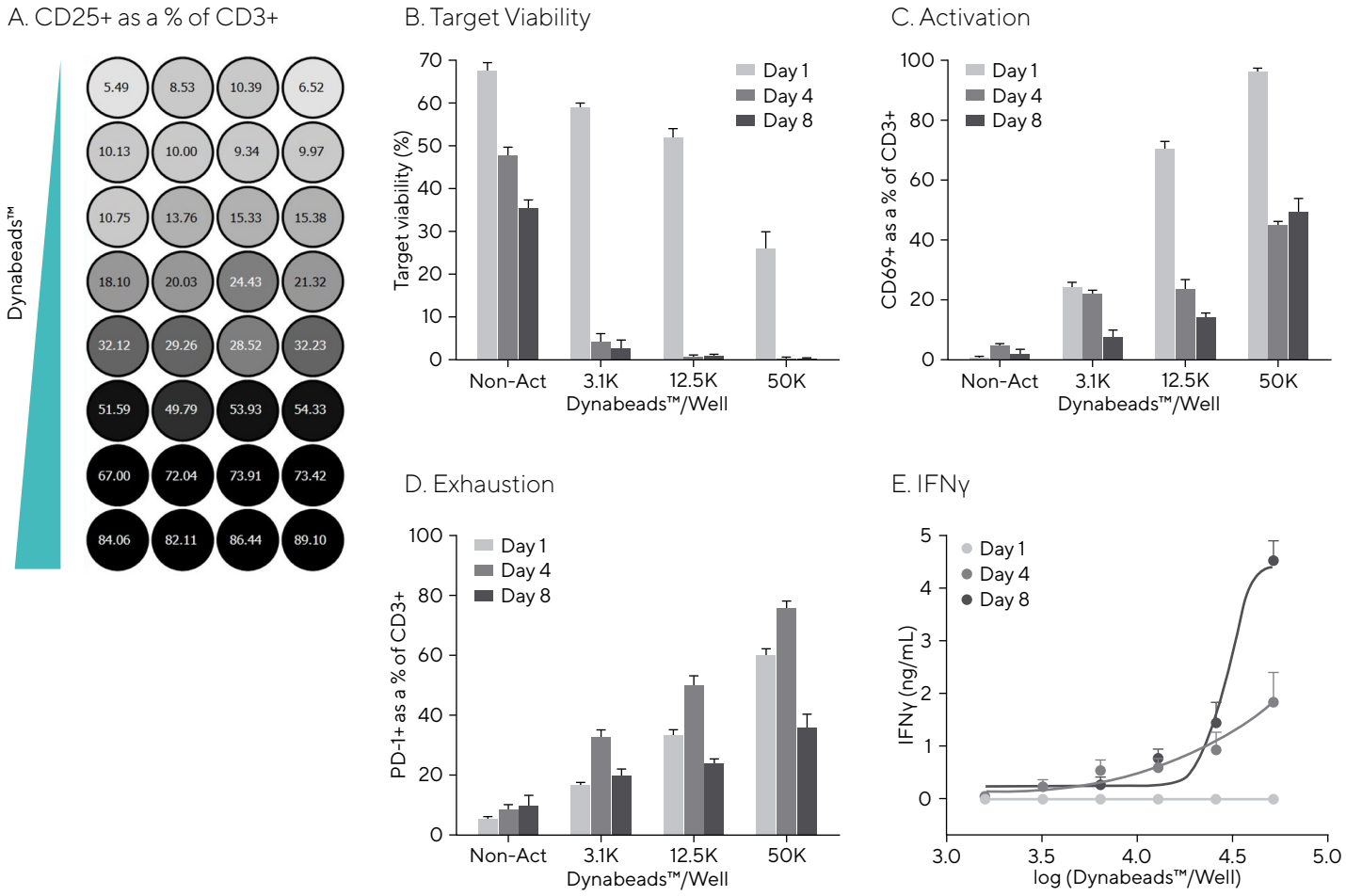
Monitoring the viability of the green target cells indicated that, even by Day 1, there had been a considerable level of immune cell killing at the top concentration (50K) of Dynabeads™, with only 26 ± 4% live target cells remaining compared to 68 ± 2% in the non-activated control (Figure 5B). By Day 4, there were very few live target cells remaining at any Dynabead™ density, suggesting the immune cells had largely acted to eliminate the spheroid. This increase in killing was supported by the granzyme B data (not shown) which increased to high levels over the time course, suggesting that the granzyme B and perforin cytolytic pathway was contributing to the killing of the spheroids.

Throughout the assay, we observed changes in the activation status of the immune cells, with consistent Dynabead™ concentration dependent increases in activation marker expression. As expected, expression of early activation marker, CD69, peaked on Day 1, with 98 ± 0.5% expression at the top Dynabead™ density (50K). CD69 expression then decreased by Day 4 and 8 (Figure 5C). Expression of mid-stage activation marker CD25 peaked on Day 4 (98 ± 0.7%, 50K Dynabeads™) and remained high on Day 8, while late-stage activation marker HLA-DR expression hit a peak on Day 8, with 77 ± 2% expression with 50K Dynabeads™ (data not shown). These data fit the expected temporal shift between the early, mid and later stage activation markers throughout immune cell killing and highlight the importance of monitoring the progression through these phenotypic changes in immune cells over time in order to build up a fuller picture of their killing profile and how it is affected by immunotherapeutics.

PD-1 expression increased from Day 1 to 4, peaking at 76 ± 3 % with 50K Dynabeads™, then decreased again by Day 8 to 36 ± 5% (Figure 5D). This, perhaps, suggests that during the peak of killing through Days 1 to 4 the cells began to display early indicators of exhaustion, but that after spheroids were eliminated, some T cells were 'rested' and retrieved from the exhaustion pathway by Day 8.[11]

Figure 5

During Spheroid Killing T Cell Cytokine Release and Expression of Markers of Activation and Exhaustion Change Over Time



Note. Incucyte® Nuclight Green labeled spheroids were incubated with unlabeled PBMCs (5:1 E:T) and Dynabeads™. On Days 1, 4, and 8, 10 µL supernatant samples were removed for cytokine analysis before spheroids were dissociated and immune cell subsets analyzed using the iQue® Human T Cell Killing, Activation and Exhaustion kits. (A) Heat map showing changes in % of CD25 expression with Dynabead™ density on Day 1. (B) Temporal changes in target cell viability as measured by the TCK kit. (C) Early activation marker CD69 expression changes over time. (D) Expression of PD-1 as an indicator of early-stage exhaustion. (E) Concentration response curves showing temporal changes in the cytokine IFNγ.

Stimulated T Cells Shift from Early to Late Stage Memory Cell Development

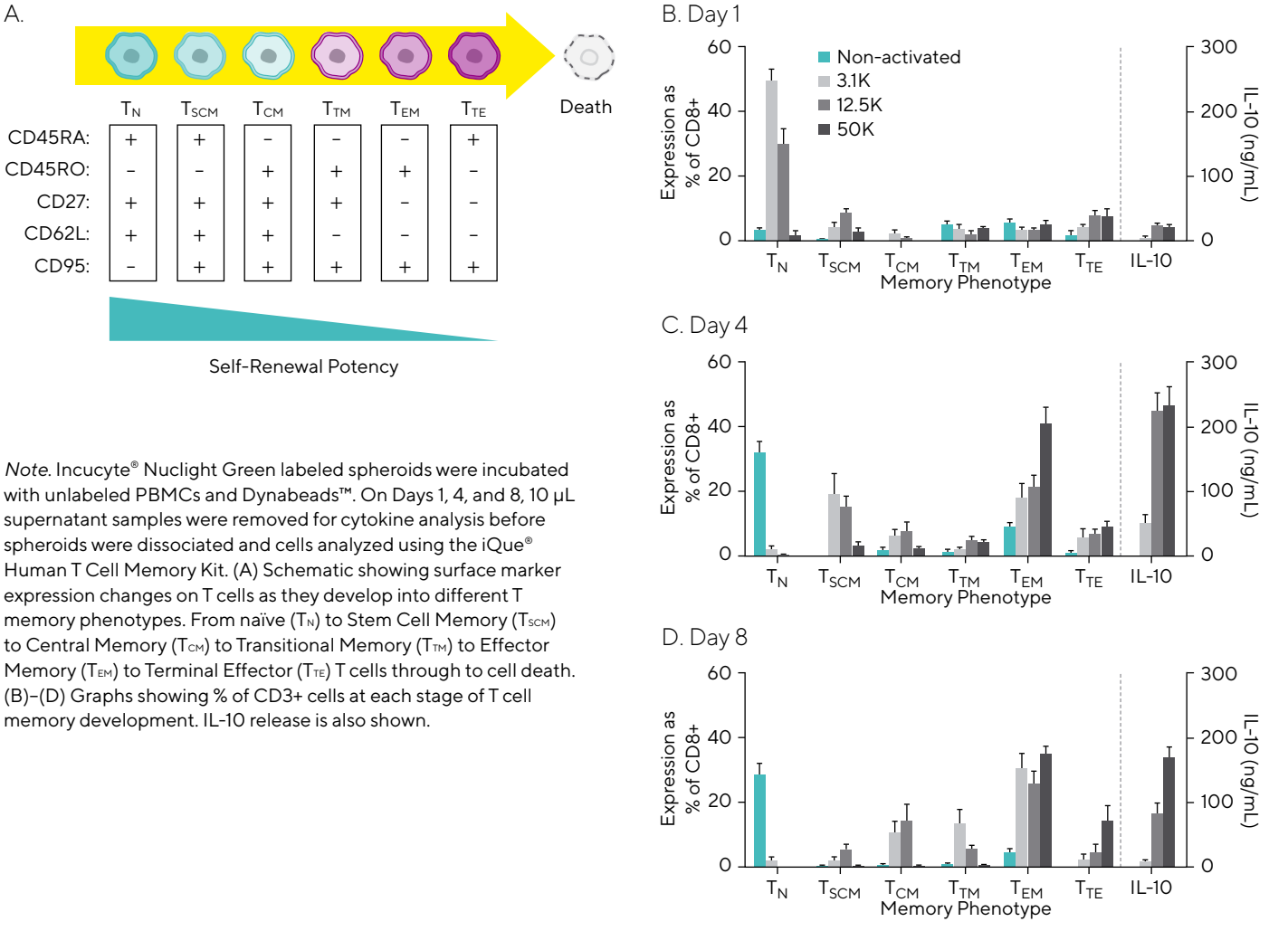
In vitro analysis of T memory cell populations is critical during vaccine development and in production of adoptive cell therapies, such as CAR-T cells. The bracket 'T memory cell' envelops multiple, functionally distinct subsets each of which are characterized by differences in expression of key surface markers (Figure 6A). As T cells progress through the T memory development pathway, they gain effector function but lose the ability to proliferate. For applications such as adoptive cell therapies, earlier phenotypes such as the stem-cell memory (T_{SCM}) and central memory (T_{CM}) are critical for persistent, long-term therapeutic effectiveness, while effector phenotypes such as Effector Memory (T_{EM}) and Terminal Effector (T_{TE}) cells are likely to result in a transient response.

With replicate plates to those used in the experiments in Figure 5, we also looked at the changes in CD8+ memory T cell development during Dynabead™ activated killing of spheroids (Figure 6B-D). There was a general shift across the killing time course from the earlier stage memory phenotypes such as T naïve (T_N) on Day 1 to the later stage phenotypes such as T_{EM} and T_{TE} by Days 4 and 8. Production of IL-10 increased from Day 1 to 4 and remained high through to Day 8. Both the progression through to the later stages of development and the production of IL-10 were enhanced by Dynabeads™ in a concentration-dependent manner.

Unexpectedly, the proportion of T_N cells in the non-activated PBMC control on Day 1 was very low (3 ± 0.4%) compared to at the lowest Dynabead™ density (50 ± 4%), however, these levels appeared to recover by Day 4 and 8 which saw 32 ± 4% and 29 ± 3% T_N cells in the non-activated control. Despite the very high level of killing by the immune cells (Figure 5B), only a very small proportion of them progressed to the final stage of the differentiation pathway (T_{TE}), suggesting that many of the cells may have maintained their self-renewal potency, despite their high activity.

Figure 6

T Cells Progression Through T Cell Memory Development During Spheroid Killing



Note. Incucyte® Nuclight Green labeled spheroids were incubated with unlabeled PBMCs and Dynabeads™. On Days 1, 4, and 8, 10 µL supernatant samples were removed for cytokine analysis before spheroids were dissociated and cells analyzed using the iQue® Human T Cell Memory Kit. (A) Schematic showing surface marker expression changes on T cells as they develop into different T memory phenotypes. From naïve (T_N) to Stem Cell Memory (T_{SCM}) to Central Memory (T_{CM}) to Transitional Memory (T_{TM}) to Effector Memory (T_{EM}) to Terminal Effector (T_{TE}) T cells through to cell death. (B)–(D) Graphs showing % of CD3+ cells at each stage of T cell memory development. IL-10 release is also shown.

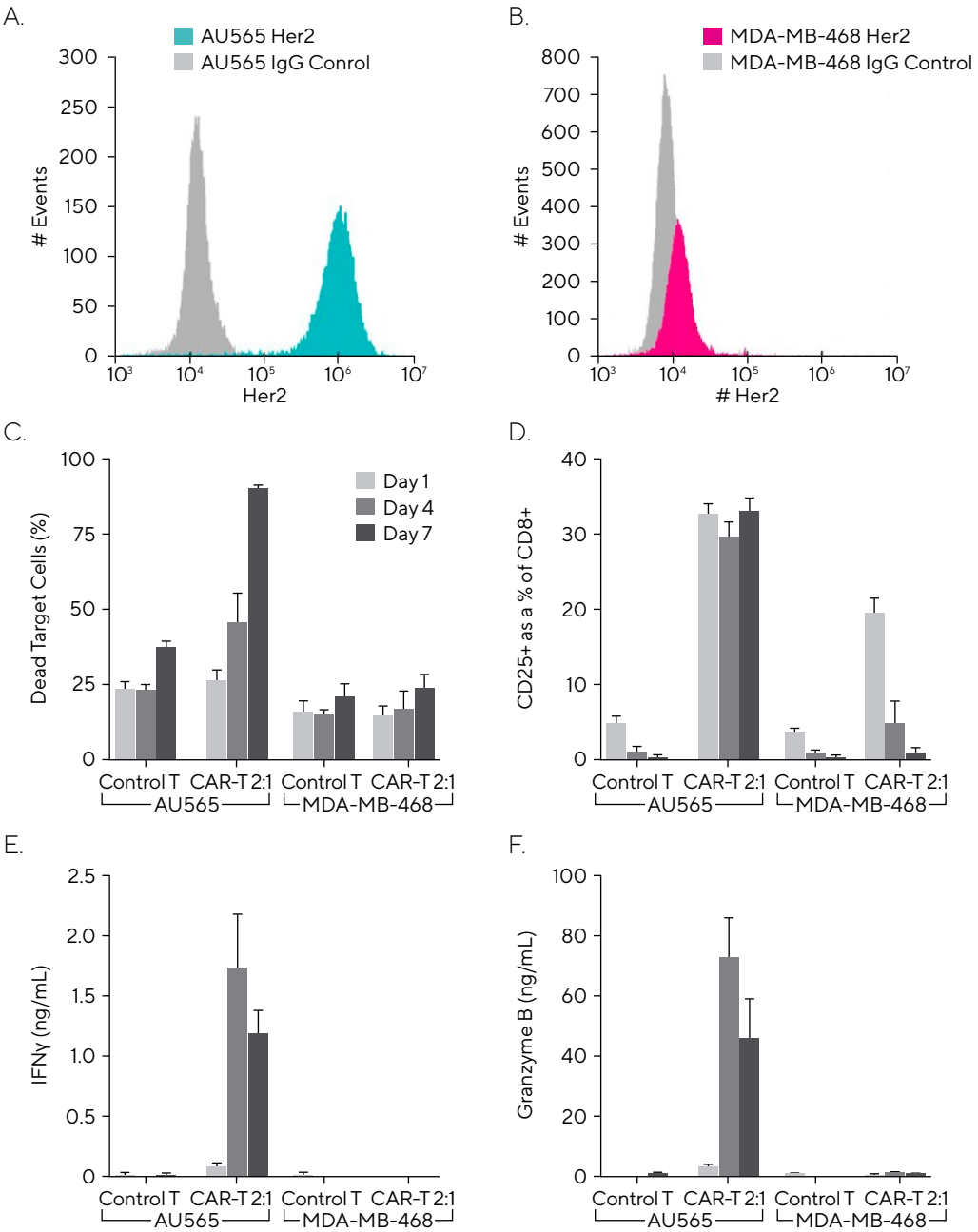
Anti-HER2 CAR-T Killing is Specific to HER2 Positive Tumor Spheroids

The next experiments aimed to use the iQue® 3D ICK assay workflow to look at the activity, killing capability, and specificity of CAR-T cells targeted towards HER2, a marker which is overexpressed in many breast cancers. So far, clinical success with CAR-T cells has been focused on treatment of liquid tumors, for example with CD19 targeted CAR-T approved for treatment of acute lymphoblastic leukemia, but much research is still ongoing to find suitable targets for solid tumors, which would hugely open up the potential uses of CAR-T therapy.[12]

To assess the specificity of the anti-HER2 CAR-T cells towards HER2 overexpressing tumor cells, we created target spheroids using both a HER2 high expressing cell line, AU565 cells, and a HER2 'negative' cell line, MDA-MB-468 cells. These cell types gave median fluorescence intensity (MFI) values (with IgG background control subtracted) of 1.0×10^6 and 4.2×10^3 for the BV421 labeled HER2 antibody, respectively, when measured using the iQue® (Figure 7A and B). The spheroids were incubated with anti-HER2 CAR-T cells (2:1 E:T) or non-transduced control T cells from the same donor in replicate plates which were analyzed on Days 1, 4, and 7.

Figure 7

Targeted Activation and Killing of HER2 Positive Tumor Cells by Anti-HER2 CAR-T Cells



Note. (A) and (B) AU565 or MDA-MB-468 cells were incubated with BV421 labeled IgG control or HER2 antibodies and their fluorescence measured using the iQue® platform. (C)-(F) Incucyte® Nuclight Green labeled HER2 positive AU565 or HER2 negative MDA-MB-468 spheroids were incubated with either anti-HER2 CAR-T cells or nontransduced T cells from the same donor at a 2:1 ratio. On Days 1, 4, and 7, 10 µL supernatant samples were taken for cytokine analysis before spheroids were dissociated and cells were analyzed using the iQue® T Cell Activation and iQue® T Cell Killing kits.

There was a clear, temporal increase in death of the HER2 positive AU565 spheroids in the presence of the anti-HER2 CAR-Ts, up to 89 ± 0.4% by Day 7 (Figure 7C). This increase was not seen with the control T cells or with the HER2 negative MDA-MB-468 cells, which were only 23 ± 2% dead in the presence of the CAR-Ts on Day 7. This indicated that the anti-HER2 CAR-Ts had specifically killed the HER2 positive tumor cells, suggesting an interaction between the anti-HER2 CAR-T construct and the overexpressed HER2. This was supported by the activation marker expression (CD25) data which saw a specific upregulation on the CAR-Ts across the time course in the presence of the AU565s, but much lower-level expression in the presence of the MDA-MB-468s (Figure 7D). Moreover, cytokines IFNγ and granzyme B were only produced in the AU565 and anti-HER2 CAR-T co-culture (Figure 7E and F).

We also looked at anti-HER2 CAR-T killing of another HER2 'low' cell type, MDA-MD-231 cells (MFI 1.1×10^4) (data not shown). In contrast to the HER2 'negative' MDA-MB-468s, we saw very high levels of killing, activation marker expression, and cytokine release by the CAR-Ts in the presence of this cell type, suggesting that very low HER2 expression on targets can initiate the killing response. This may explain some of the catastrophic effects that occurred when anti-HER2 CAR-Ts were tested in clinical trials.[13] Many body cells express low levels of HER2, which meant that 'on-target, off-tumor' events of the CAR-Ts resulted in one patient experiencing multiple organ failure which ended the trial. Research is being carried out into how to develop clinically viable HER2 targeting CAR-Ts, for example by looking at reducing the affinity of the HER2 construct so it only affects HER2 overexpressing tumor cells.[14] The experiments in Figure 7 highlight how the specificity and activity of CAR-T cells can be tested *in vitro* using this 3D ICK advanced flow cytometry workflow.

Conclusions

This application note demonstrates the value of using the iQue® platform in conjunction with the validated suite of iQue® T cell characterization kits to assess immune cell phenotype and function in 3D advanced cell models. Importing the pre-set gating templates into the iQue Forecyt® software facilitates quick, multi-parameter analysis, enabling broad *in vitro* characterization of a drug's effect on immune cell infiltration and killing of tumors can be profiled in minimal time. The experiments in this note have highlighted the advantages of this workflow, including:

1. Spheroids are dissociated using a reproducible, easy-to-follow workflow which facilitates the evaluation of 3D models using advanced flow cytometry. This enhances the wealth of information obtained, compared to traditional techniques such as imaging, and allows investigation of solid tumor therapies whereas in the past we were limited to liquid tumors.
2. Infiltrated immune cell populations can be analyzed and compared to their non-infiltrated counterparts. Both the numbers and phenotypes of TILs can be important indicators of clinical outcomes and are impacted by many environmental and physiological factors. The ability to analyze these *in vitro* could aid the development of novel therapeutics.
3. Fluctuations in target cell viability, marker expression, and cytokine release can be monitored throughout T cell killing of spheroids. These are used to measure the rate of immune cell killing and the associated changes in activation, exhaustion, and memory status of the T cells. This gives broad insight into the immune effects and specificity of an immunotherapeutic of interest.

Together, these benefits create a powerful tool with the potential to enhance biological research and drug discovery applications.

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North America
Sartorius Corporation
565 Johnson Avenue
Bohemia, NY 11716
USA
Phone +1 734 769 1600
Email: orders.US07@sartorius.com

Europe
Sartorius UK Ltd.
Longmead Business Centre
Blenheim Road
Epsom
Surrey, KT19 9QQ
United Kingdom
Phone +44 1763 227400
Email: euorders.UK03@sartorius.com

Asia Pacific
Sartorius Japan K.K.
4th Floor, Daiwa Shinagawa North Bldg.
1-8-11, Kita-Shinagawa 1-chome
Shinagawa-Ku
Tokyo 140-0001
Japan
Phone +81 3 6478 5202
Email: orders.US07@sartorius.com

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For questions, email: AskAScientist@sartorius.com

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