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Characterization of T Cell Activation and Function in Cancer Immunotherapy Using High-Throughput Cytometry

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Abstract

T cells are pivotal in the immune system's response to cancer, with their ability to recognize and eliminate tumor cells. However, challenges such as poor tumor recognition and penetration into solid tumors hinder their efficacy. Novel immunotherapies, including bispecific antibodies, tumor infiltrating lymphocytes (TILs), and chimeric antigen receptor (CAR) T cells, have been developed to enhance T cell responses against cancer. To facilitate rapid monitoring of T cell function, a high-content, multiplexed assay using high-throughput flow cytometry has been developed. The iQue® Human T Cell Activation Kit allows simultaneous assessment of cell phenotype, activation markers, proliferation, viability, and cytokine secretion in a single well assay.

These studies present data on antibody-mediated T cell activation, TILs assay models, and CAR-T cell activation assays, demonstrating the kit's ability to evaluate T cell responses to various stimuli. Results indicate that the iQue® platform can effectively characterize T cell activation and function, providing insights into the concentration-dependent effects of therapies, the impact of environmental factors on TILs, and the antigen specificity of CAR-T cells. The platform's high-throughput capability and minimal sample volume requirements make it an ideal tool for developing new immunotherapies, with the potential to predict on-target off-tumor effects and aid in the rapid identification of therapeutic candidates.

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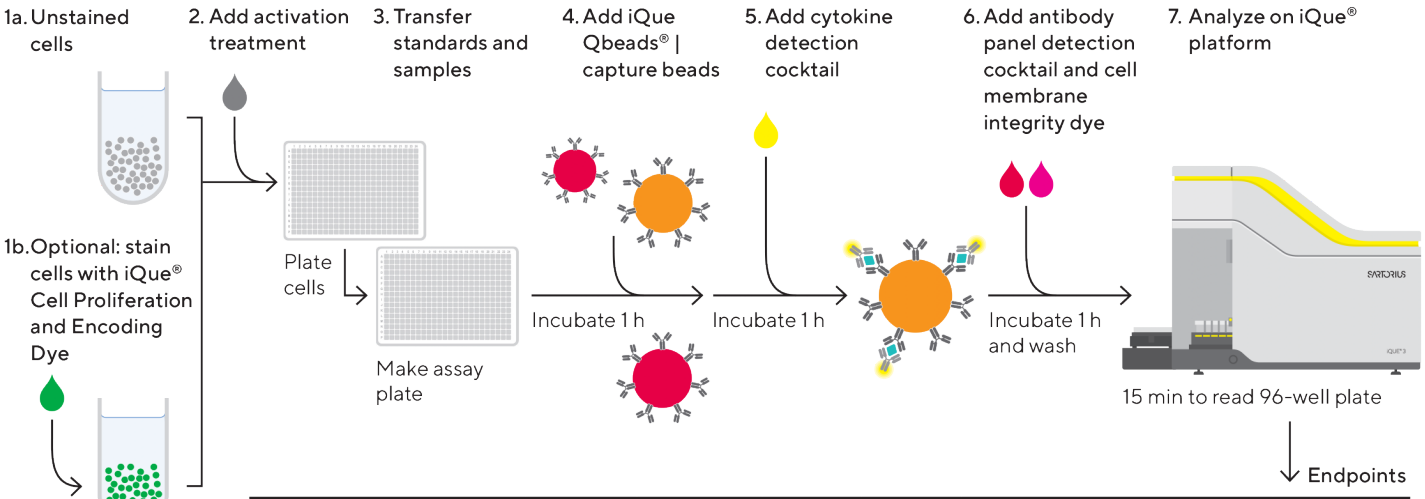
Introduction

T cells play a critical role in immunosurveillance and clearance of cells infected with viruses and bacteria, as well as cancerous cells. Upon recognition of tumor antigens and co-stimulatory signals, T cells can become activated, proliferate, and consume or secrete various cytokines that support T cell health and/or effector functions. Recognition and killing of cancerous cells is effectively performed by the actions of activated T cells, thus making them an attractive therapy against this disease.

Despite the innate tumor cell killing ability of T cells, research is ongoing to combat challenges such as poor tumor recognition, targeting, and solid tumor penetration. Discovery of novel immunotherapies that specifically target and enhance the T cell response against cancer, for example, bispecific antibodies, tumor infiltrating lymphocytes (TILs), and chimeric antigen receptor (CAR) modified T cells, have revolutionized cancer treatment paradigms and are rapidly expanding research areas.[1,2] Not limited to immuno-oncology, bispecific antibodies are valuable in making the connection between immune cells and their target cells, increasing the directed killing towards those targets.

This approach has yielded some success in the form of bispecific T cell engager (BiTE) antibodies in the fight against cancer. To overcome issues with tumor recognition, the development and optimization of therapeutics involving TILs or CAR-T cells that are engineered to recognize specific tumor proteins have gained much attention.[3]
Thus, there exists a need for development of robust and relevant strategies to monitor novel therapeutics for sufficient activation and antitumor efficacy while minimizing toxicity concerns. This includes assessment of cell health, viability, phenotype, and function.

To address the need for rapid monitoring of T cell function, we developed an optimized, high-content, multiplexed assay using high-throughput cytometry to measure T cell activation. The iQue® Human T Cell Activation Kit collapses the traditional workflow by evaluating cell phenotype, T cell activation markers, cell proliferation, cell viability, and secreted cytokines in a single well assay using a 96 or 384-well plate format.



Basic Cell ID		Cell Surface Activation Markers			Secreted Cytokines		Cell Viability	Cell Proliferation (optional)	Relative Cell Count
		CD69 (early)	CD 25 (late)	HLA-DR (even later)	TNFα	IFNγ			
CD3+	CD4+	+ | -	+ | -	+ | -	+	+ | -	+ | -	+	+
	CD8+	+ | -	+ | -	+ | -	+ | -	+ (Th1)	+ | -	+	+

Figure 1: Workflow Figure
"+" denotes highly expressed | secreted, "+|-"denotes partially expressed/ secreted, "-" denotes low or no expression/secretion (table insert).

Assay Principles

The iQue® Human T Cell Activation Kit has been designed to quantify T cell activation by measuring phenotype and function. Assay plates can be set up to look at signals from various T cell sources (e.g., PBMCs or CAR-T cells) using various methods of cell activation (e.g., Dynabeads™, effector and target cell co-culture). At the assay endpoint, an aliquot of the sample from each well is transferred into an assay plate. Cytokine analyses can be performed separately or multiplexed with the phenotyping components for added flexibility depending on research needs. To perform the assay, 10 µL of sample containing cells and supernatant together or separately were transferred to an assay plate and analyzed utilizing the iQue® Human T Cell Activation Kit protocol illustrated in Figure 1. Data was acquired on the iQue® High-Throughput Screening (HTS) Cytometry Platform using the violet, blue, and red (VBR) laser configuration and analyzed using the provided Forecyt® software template to quantify cytokines TNFα and IFNγ, T cell subsets (via CD3, CD4, CD8) and surface activation markers (CD69, CD25, HLA-DR), and cell viability via the iQue® Cell Membrane Integrity Dye provided in the kit. The kit also contains the optional iQue® Cell Proliferation and Encoding (B/Green) Dye that can be used to assess proliferation or to stain cells samples of interest (e.g., target cells). In these experiments, the encoder function of this dye was replaced with the use of stably transfected green-labeled target cells (Incucyte® Nuclight Green Lentivirus).

Methods

Antibody-Mediated T Cell Activation

Ramos cells modified to express a nuclear restricted green fluorescent protein (Incucyte® Nuclight Green Lentivirus) were seeded (15K/well) with PBMCs (5:1 effector-to-target; E:T) and either activated with increasing concentrations of an anti-hCD3xCD19 BiTE (10 ng/mL; 5-fold serial dilution), or an anti-hCD3xβGAL control antibody (10 ng/mL), or CD3/CD28 Dynabeads™ (75K beads/well; 3-fold serial dilution). Every 24 hours, 10 µL of supernatant was removed and cytokine analysis (IFNγ and TNFα) was performed using iQue Qbeads® found in the iQue® Human T Cell Activation Kit. At 72 hours, cells were dissociated and the numbers of live, green-labeled target cells were counted. T cell subsets and activation markers were also evaluated using the iQue® Human T Cell Activation Kit antibody panel.

TILs Assay Model

BT474, SKOV-3 or A549 cells were seeded in ultra-low attachment (ULA) plates and incubated for 72 hours to promote spheroid formation. Pre-activated (1:1 Dynabeads™ to cell ratio) PBMCs (5:1 E:T) were added for 24 hours. 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 TILs in the assay plate. Once the non-infiltrated cells had been separated, the spheroids and TILs were dissociated to create a single cell suspension. To examine the effect of fibroblasts in different donors, 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. Samples were assessed for T cell subset and activation marker expression using the iQue® Human T Cell Activation Kit.

CAR-T Cell Activation Assay

CD19, second-generation CAR-T cells (~50% transduction efficiency) or control matched-donor, mock-transduced T cells (Creative BioLabs) were placed into culture with CD19 antigen positive Ramos or CD19 antigen negative Jurkat cells at various E:T ratios. Samples were analyzed on Day 2, 4, and 7 using the iQue® Human T Cell Activation Kit and the iQue® Human T Cell Companion Kit (IL-2).

"On Target Off Tumor" CAR-T Profiling

HER2 high (AU565), HER2 low (MDA-MB-231) or HER2 negative (MDA-MB-468) cell lines were modified to express a nuclear restricted green fluorescent protein (Incucyte® Nuclight Green Lentivirus), seeded into ULA plates and allowed to form single spheroids over 3 days in the presence of Matrigel® (1.25%). Once formed, anti-HER2 CAR-T cells or mock transduced control T cells were added to the wells at various E:T ratios.

On Day 2, 4, and 7, samples were analyzed on the iQue® HTS platform. Supernatants were collected for secreted protein analysis before cultures were gently dissociated to remove Matrigel® and break up the spheroids. Samples were assessed for phenotype and function using the iQue® Human T Cell Activation Kit.

Results and Discussion

Antibody-Mediated T Cell Activation

BiTE antibodies, such as Blinatumomab, are being used clinically for treatment of B-lymphocytic leukemia alongside other late-stage cancers.[4] The CD3xCD19 construct, simultaneously engages the CD3 on T cells and the tumor associated antigen, CD19, present on B cells. This interaction causes the clonal expansion and activation of T cells as well as direct contact between CD3+ T cells and CD19+ tumor cells, resulting in tumor specific cell lysis. To model the therapeutic effects on B cell leukemia *in vitro*, an anti-hCD3xCD19 BiTE antibody was evaluated alongside a control antibody targeting CD3 or Dynabeads™.

Cytokine production was evaluated from the supernatant samples taken daily. The CD3xCD19 BiTE induced production of TNFα and IFNγ in a concentration dependent manner, reaching maximal concentrations of 1.5±0.3 ng/mL and 1.3±0.3 ng/mL by day 3, respectively (Figure 2A and B).

Interestingly, CD3/CD28 Dynabead™ stimulation evoked vastly increased production of both cytokines, with TNFα reaching 3.8±0.2 ng/mL and IFNγ 14.4±2.5 ng/mL. Comparison to the levels of cytotoxicity as measured by target cell count (Figure 2C) highlight that despite the low level of cytokine release, the BiTE antibody induces relatively high levels of target cell death compared to CD3/CD28 stimulation.

On day 3 post-treatment, cells were lifted from the assay plate and labeled with the iQue® Human T Cell Activation Kit antibody panel. As expected, the CD3/CD28 Dynabeads™ induced concentration-dependent increases in the proportions of CD69, CD25 and HLA-DR positive populations, yielding comparable EC_{50} values for each marker of 4690, 16958 and 13865 beads/well, respectively (Figure 3A). The maximal population percentages were 29% for CD69, 69% for CD25 and 32% for HLA-DR.

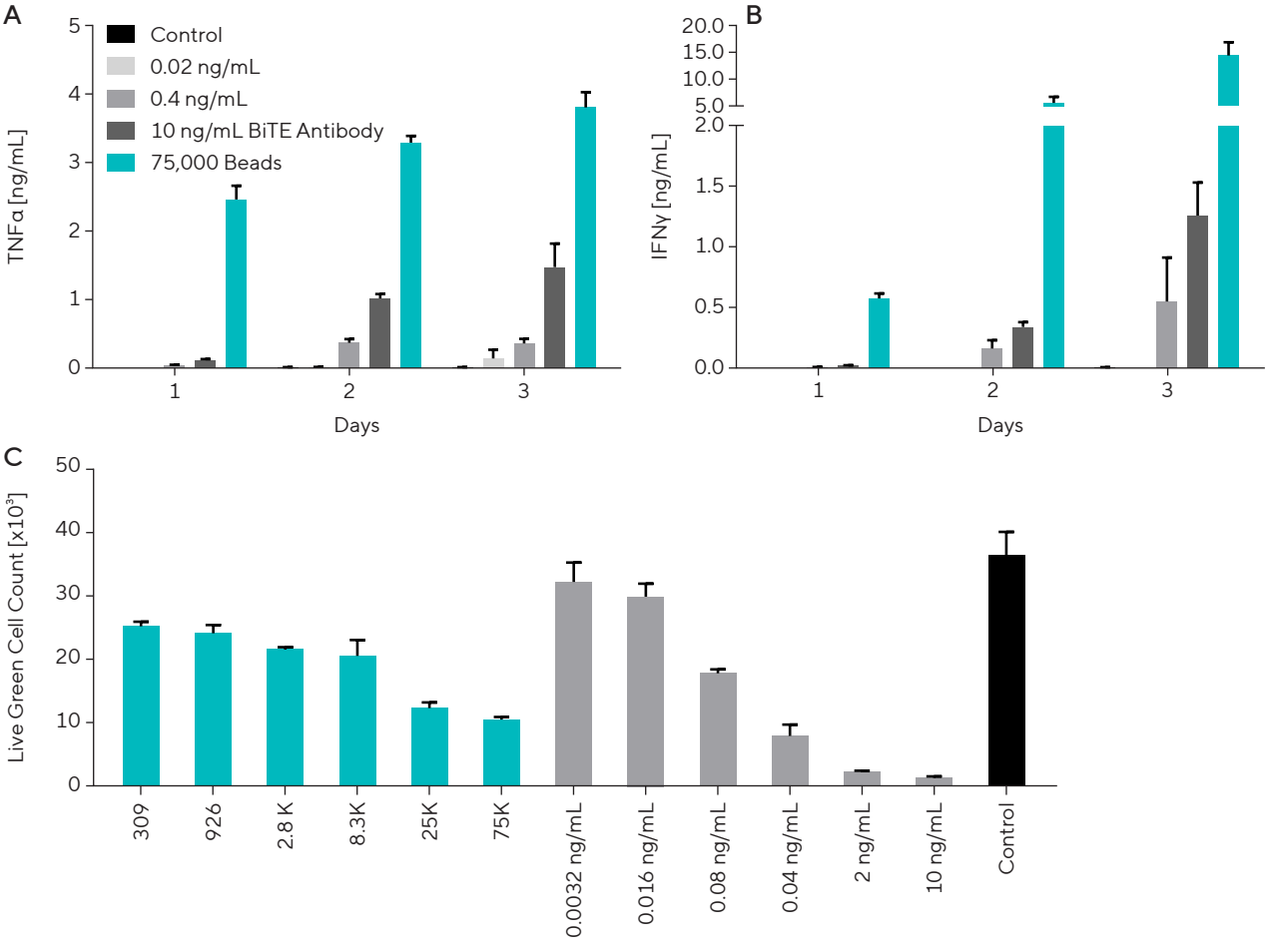


Figure 2: Temporal Cytokine Production and Immune Cell Killing in Response to CD3xCD19 BiTE Antibody
Daily supernatant samples (10 µL) taken for analysis of cytokine (TNFα and IFNγ) concentrations by the iQue® HTS platform (A and B). Temporal cytokine concentrations were compared to the level of immune cell killing as measured by the live green-labeled target cell count data in (C), with bars for CD3/CD28 Dynabeads™ in teal, BiTE in grey and CD3xβGAL antibody control in black.

In contrast, inclusion of the BiTE antibody displays a clear left shift in the CD69 expression pattern, with low concentrations (20 pg/mL) capable of inducing almost exclusive expression of this early activation marker (EC_{50} value of 5.5 pg/mL, Figure 3B). CD25 (mid activation) and HLA-DR (late activation) are induced but at much higher concentrations of BiTE, yielding EC_{50} values of 87 pg/mL and 72 pg/mL, respectively. Maximal subset percentages were 45% for CD69, 92% for CD25 and 46% for HLA-DR.

These data suggest different mechanisms of activation may yield altered proportions of CD8 positive subsets. The findings presented require further investigation to fully understand the implications of this observation.

Activation of Tumor Infiltrating Lymphocytes (TILs)

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] To investigate the phenotypic profile of these cells *in vitro*, a TILs assay model with wild type BT474 (breast cancer) spheroids and PBMCs, activated with a range of concentrations of CD3/CD28 Dynabeads™ was employed. After 24 hours in co-culture, single cell suspensions of non-infiltrated and infiltrated T cells from dissociated tumor spheroids were analyzed.

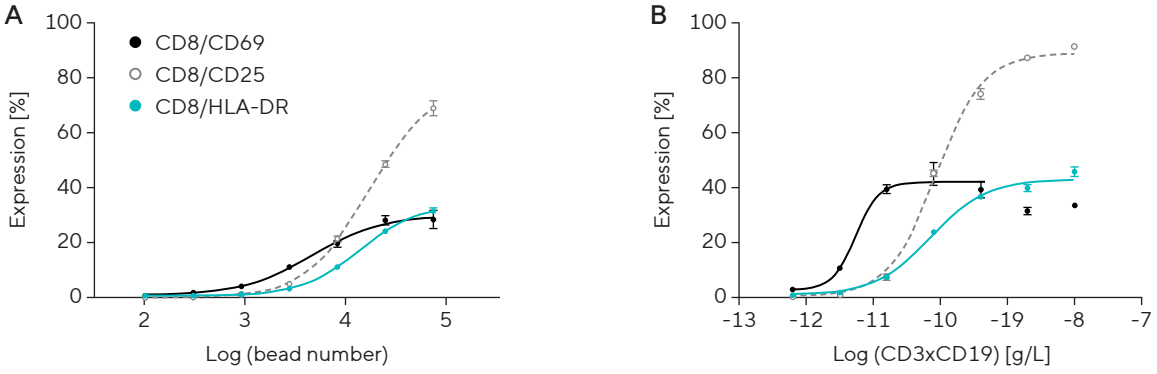


Figure 3: Immune Cell Activation in Response to CD3xCD9 BiTE Antibody
Concentration response curves to activation induced by (A) CD3/CD28 Dynabeads (100 to 75K beads/well) or (B) CD3xCD19 BiTE antibody (0.6 pg/mL to 10 ng/mL).

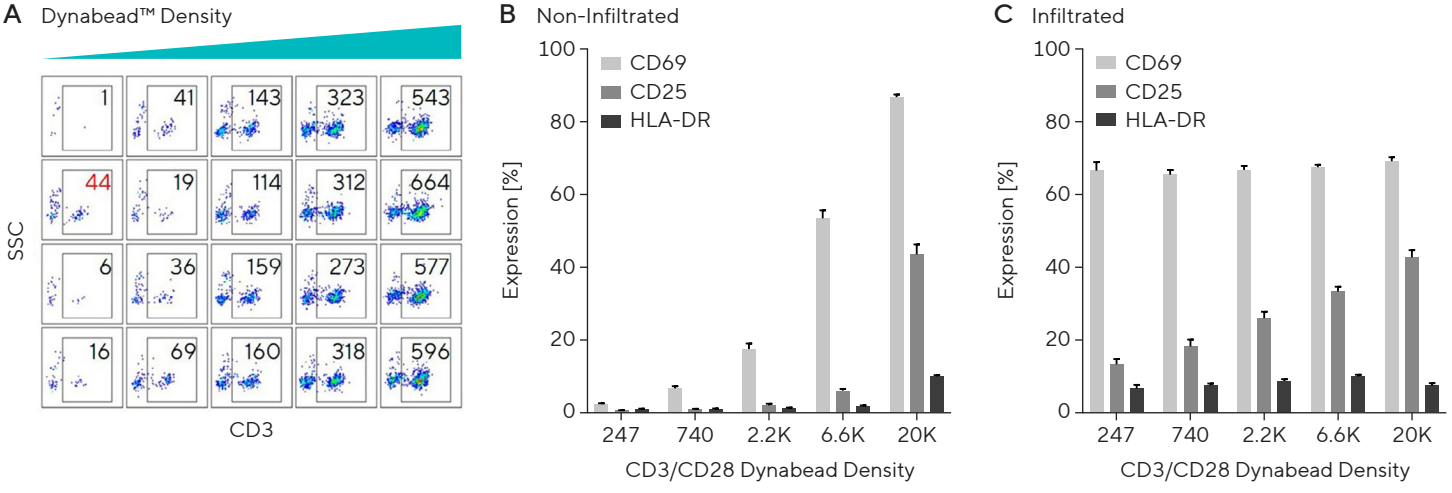


Figure 4: Comparison of the Activation Status of Infiltrated or Non-Infiltrated T Cells from Dissociated BT474 Spheroids
(A) Spheroids and TILs were dissociated to a single cell suspension and analyzed using the iQue® Human T Cell Activation Kit. Plate view shows individual well plots of side scatter (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. (B) and (C) Activation marker expression comparison between the non-infiltrated and infiltrated T cells.

A Dynabead™ concentration-dependent increase in infiltration of T cells into the spheroid was evidenced by the relative number of CD3+ TILs per well, as shown in the Forecyt® plate view diagram (Figure 4A). Figures 4B and 4C 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 topmost 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, elevated 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. These data show that activation marker expression on TILs is greater than on non-infiltrated T cells.

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.[8-10] 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 iQue® Human T Cell Activation 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 5A). 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:CD4 cells, respectively (Figure 5B). 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] 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 5C 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, possibly related to spheroid disruption during washing. Thus, these data demonstrate that tumor type and the addition of fibroblasts impacts the number and profile of TILs.

CAR-T Activation

CAR-T cells are designed to selectively target and kill tumor cells through interaction with a specific surface antigen while limiting off-target side effects. To demonstrate this specificity *in vitro*, anti-CD19 CAR transduced T cells or donor matched mock transduced T cells were used in an immune cell killing assay and functionally profiled at different time points using the iQue® HTS platform.

On Day 2, 4, and 7, samples were analyzed on the iQue® platform to assess phenotype and function using the iQue® Human T Cell Activation Kit, and quantification of IL-2 via the iQue® Human T Cell Companion Kit. Results show that, when combined with antigen positive Ramos cells, there was a rapid upregulation of T cell activation markers CD69 and CD25 (Figure 6A and B, respectively) on the CD8+ cells.

This upregulation demonstrated some time dependence, with the highest levels observed on Day 7, but there was little difference between CAR-T cell densities. Expression of activation markers was low in co-cultures with antigen negative Jurkat cells or in the presence of mock transduced T cells (< 7%). In the presence of Ramos cells, concentrations of secreted cytokines IFNγ and IL-2 (indicators of activation) increased at early time points, but then dropped by Day 7, indicating a transient response. Overall, this quantification demonstrates a clear antigen specific activation of anti-CD19 CAR-T cells as measured by both surface markers and secreted proteins.

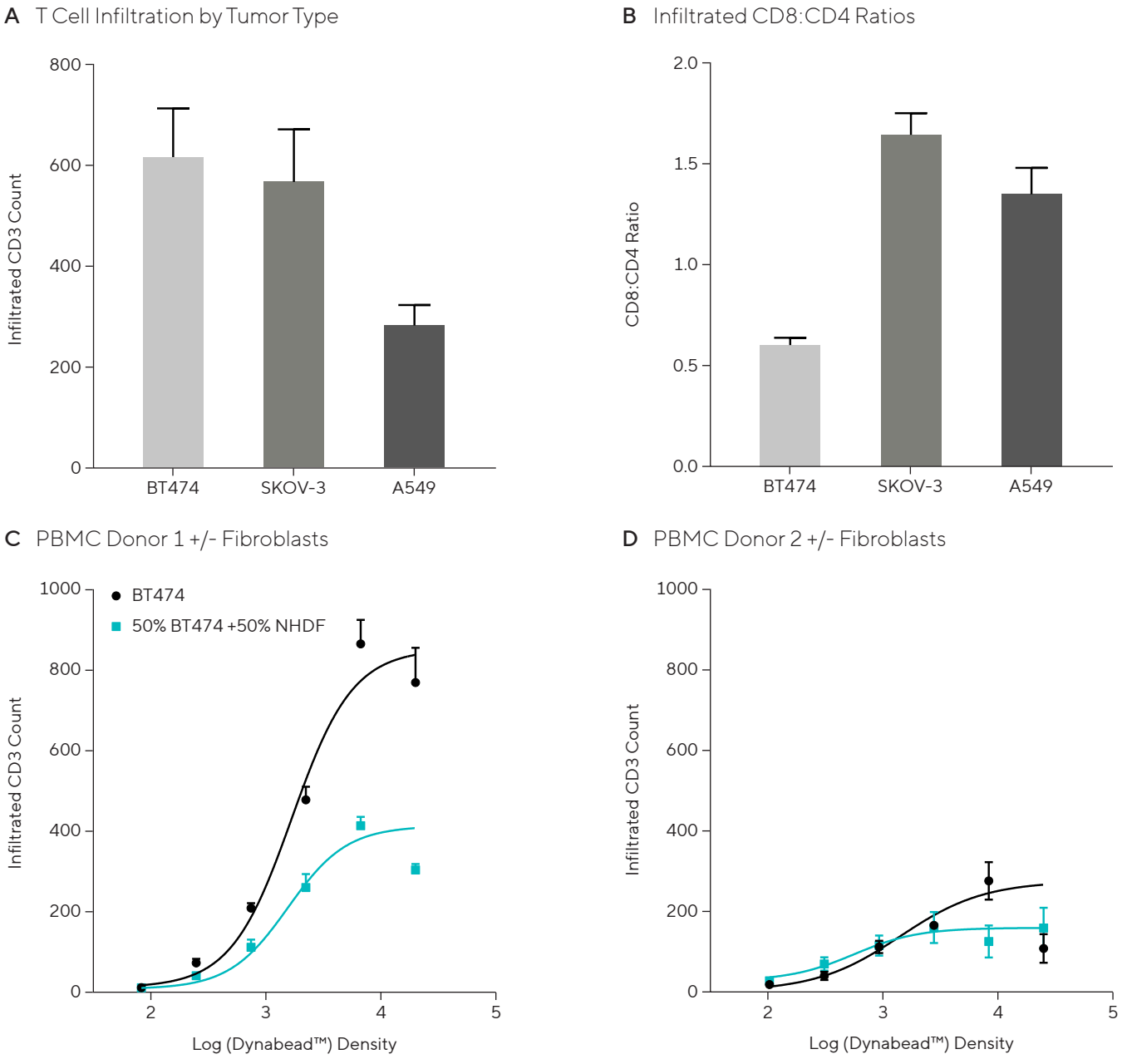


Figure 5: BT474 Spheroid Infiltration by CD3+ TILs is Affected by Tumor Type, the Presence of Fibroblasts (NHDF), and Immune Cell Donors
TILs subsets were analyzed using the iQue® Human T Cell Activation Kit. (A) and (B) Graphs show the average number of TILs (CD3+) per iQue® sip per spheroid type and 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.

Post clinical success of anti-CD19 CAR-T therapies for liquid tumors, there has been increased interest in applying similar therapies to solid tumors, for example, in the fight against breast cancer. An obvious target of interest in this area is the HER2 (ERBB2) receptor which has been identified to be over-expressed in many breast cancers. Unfortunately, in early trials, there were serious adverse events in the clinic linked to "on target off tumor" effects and further testing was stopped.[11,12] It was indicated that the CAR cells had attacked other "off tumor" cells throughout the body that expressed low levels of HER2 epitope and were, therefore, defined as "on target." There is additional evidence in the literature that the affinity of the CAR-T interaction with the HER2 antigen can also contribute to this effect.[13] To model potential "on target off tumor" effects *in vitro*, a spheroid co-culture with anti-HER2 CAR-T cells was used to mimic the immune killing of a solid tumor.

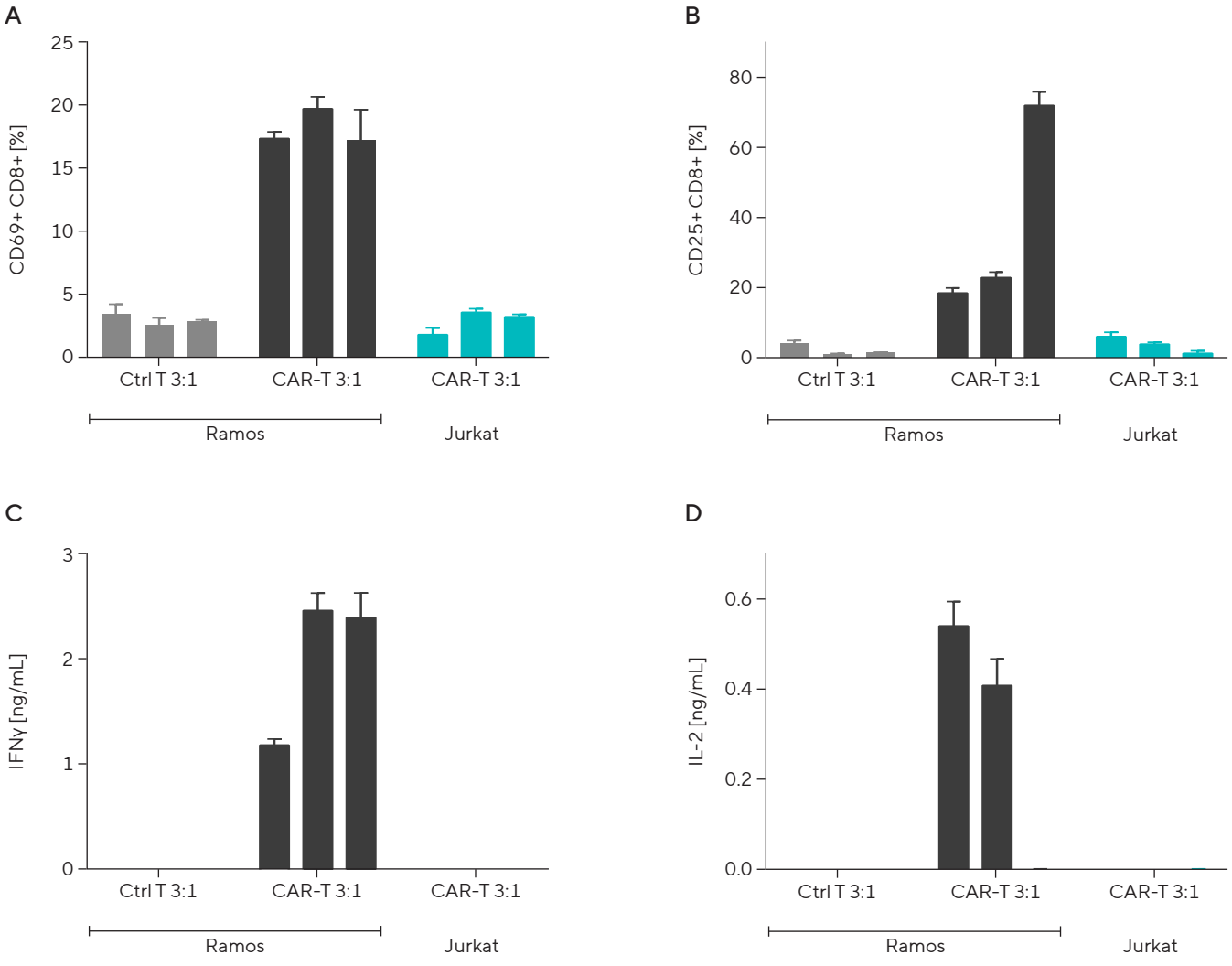


Figure 6: Antigen Specific Activation of Anti-CD19 CAR-T
Samples were quantified on Day 2, 4, and 7 for surface marker expression and secreted protein using the iQue® Human T Cell Activation Kit with iQue® Human T Cell Companion Kit (for IL-2). Graphs (A, B) show expression levels in CD8+ T cells of CD69 or CD25, and graphs (C, D) show levels of IFNγ or IL-2. Grey bars represent Ramos with mock transduced T cells, black bars are CD19 CAR-T with Ramos cells, and teal bars are CAR-Ts in combination with Jurkat cells. The 3 bars represent Day 2, 4, and 7, all data shown as mean ± SEM of 3 wells.

The results show an increase in CD69 and CD25 activation markers on the CD8+ population for both HER2 high expressing AU565 and HER2 low expressing MDA-MB-231 cell co-cultures with anti-HER2 CAR-T cells (Figure 7A and B). This effect was absent in the presence of mock transduced T cell. The HER2 negative MDA-MB-468 cells showed no change compared to control T cells for CD69 and low levels for CD25 which decreased by Day 4. Supernatants were assessed for IFNγ using iQue Qbeads® detection as part of the kits. Once again both AU565 and MDA-MB-231 CAR-T co-cultures demonstrated high levels while nothing was detected in the MDA-MB-468 co-culture wells.

These data indicate anti-HER2 CAR-T driven activation of T cells in co-cultures with high expressing AU565 and low expressing MDA-MB-231 cells, indicating the potential for "on target off tumor" effects with these cells. The lack of any activity in the presence of MDA-MB-468 cells demonstrates the expected specificity of the anti-HER2 CAR-T cells.

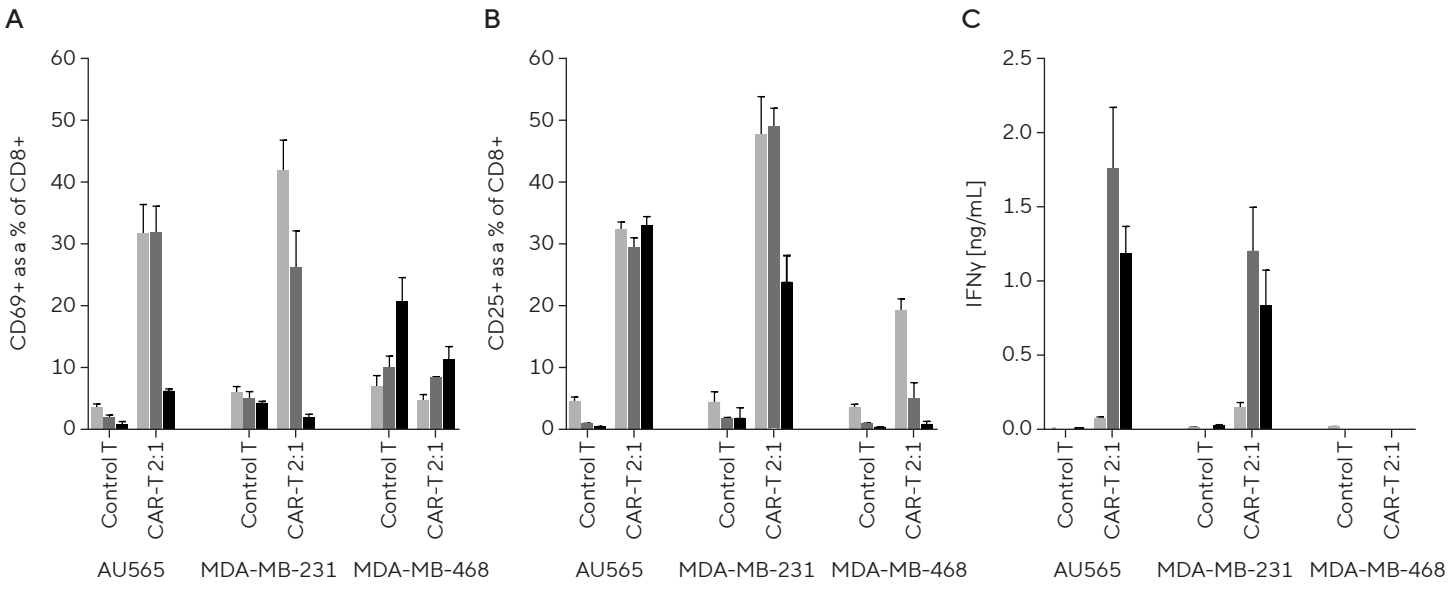


Figure 7: "On Target Off Tumor" Activation of T Cells in a Solid Tumor Co-Culture Model
Samples were quantified on Day 2, 4, and 7 for surface marker expression and secreted protein using the iQue® Human T Cell Activation Kit. Graphs (A and B) show expression levels in CD8+ T cells of CD69 or CD25, and graph (C) shows levels of IFNγ for each target cell co-culture with either non-activated mock transduced T cell or anti-HER2 CAR-T cells. The 3 bars represent Day 2, 4, and 7, all data shown as mean ± SEM of 4 wells.

Conclusion

This application note demonstrates the value of using the iQue® High-Throughput Screening cytometry platform in conjunction with the validated iQue® Human T Cell Activation Kit to assess immune cell phenotype and function during the development of antibody-mediated and cell-based cancer therapies using minimal sample volume.

The high-throughput iQue® HTS platform combined with the built-in, visual-based iQue Forecyt® software allows for assessment of multiparametric data of cell health, viability, phenotype, and effector function coupled with cytokine analysis from the same well, using simple workflows and minimal sample volumes. These functions enable broad *in vitro* characterization of therapeutic effects on immune cell activation and function including the following features.

- Concentration response curves give insight into concentration-dependent effects of BiTE therapy on T cell activation and secreted cytokines. In conjunction with target cell counts, this gives insight into effector functions against tumor targets. Development of novel therapeutic antibodies relies on rapid identification and characterization of candidate molecules as early in the development process as possible.
- 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.
- Antigen-specific activation by characterization of T cell surface markers and quantification of secreted proteins enable insights into tumor antigen specificity of CAR-T cells. Importantly, potential on-target off-tumor toxic effects can be exposed *in vitro* which may bear important consequences in a clinical setting.

Together, this method offers a rapid, robust, and convenient solution for characterization of cellular responses, and is ideal for assisting in the development of new immunological therapies

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