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Phenotypic and Functional Characterization of CAR-T Cells with Advanced Flow Cytometry and Live-Cell Analysis

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Introduction

The successful use of immunotherapies to help combat cancer has expanded rapidly in the last few years, with many therapies now approved for clinical use. The precision of the immune system allows a more targeted approach to killing cancer cells, while sparing healthy cells, when compared to traditional chemotherapeutic strategies. One key area of advance has been in the use of gene-modified cell therapies with the introduction of chimeric antigen receptor (CAR) T cells leading the field. The CAR construct is designed to interact with a specific surface epitope or antigen present on the tumor cell, which once in close proximity enables the T cell to kill the tumor cell. Where specific antigens can be identified on the tumor cells, CAR-T cells display targeted effects and, as they are sourced from the patient (known as autologous therapy), there is a lack of rejection. CD19 targeted CAR-T cell therapies, for example Kymriah® (Novarits) or Yescarta® (Kite/Gilead)[1], have shown clinical success against liquid tumors common in lymphoblastic leukemia and non-Hodgkin's lymphoma. Despite this progress, obstacles remain, for instance, the high cost and technical difficulties of phenotyping, profiling and purifying immune cells.[2,3] Also, while some patients have been highly responsive to treatment, others were refractive, and uncovering the mechanistic basis for these differing outcomes is an active area of research. In more recent years, research has progressed to explore the introduction of CAR constructs into alternative immune cells, for example CAR-NK or CAR-macrophages[4-6], and to investigate gene modified cells that target solid tumors.

This article will touch on the process of manufacturing and expanding cell therapy products with a focus on CAR-T cells. Key *in vitro* assays used to phenotype and assess function of these modified cells will be introduced with three case studies illustrating the utility of the iQue® Advanced High Throughput Flow Cytometry Platform and the Incucyte® Live-Cell Analysis System (Sartorius).

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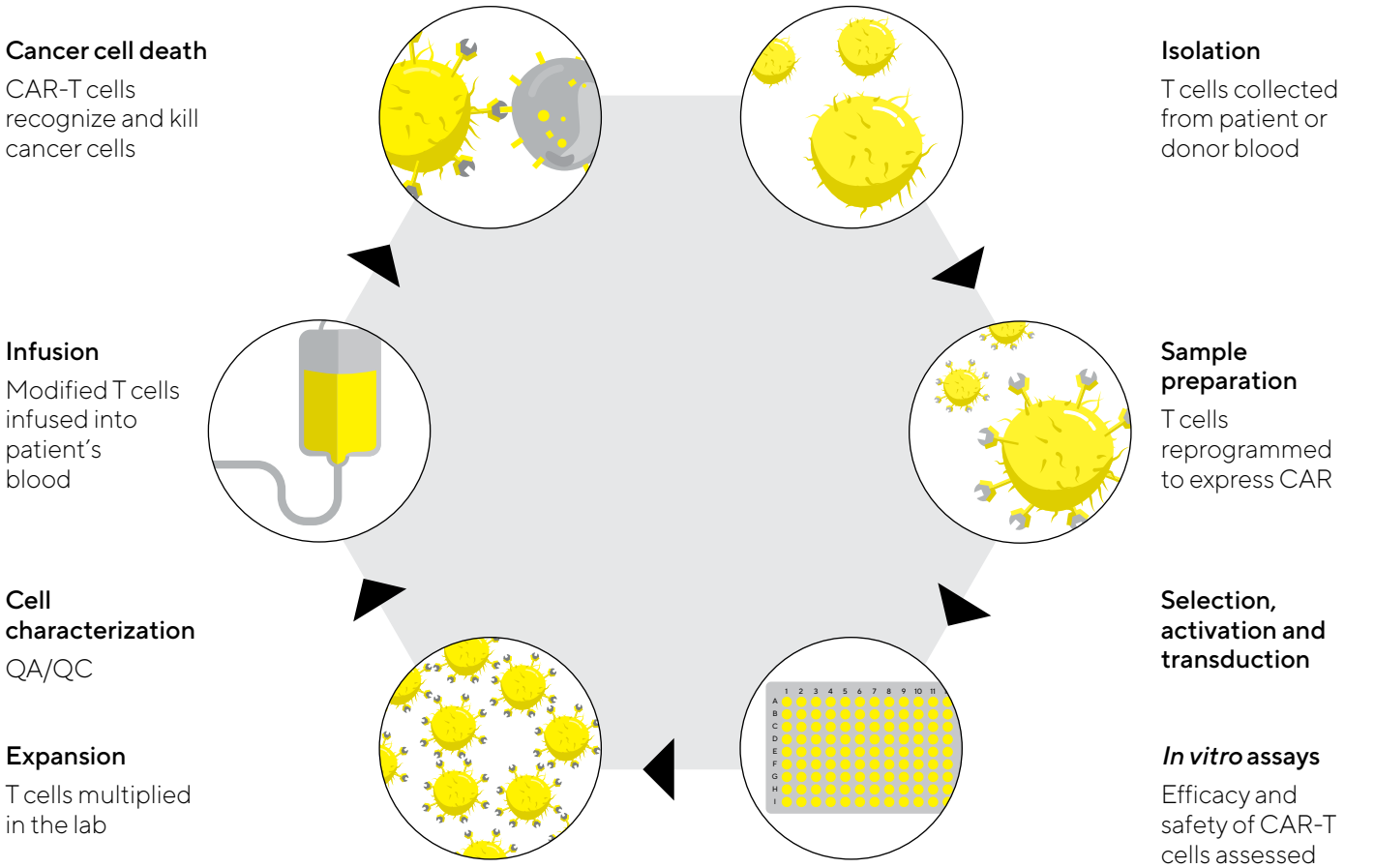
Manufacture and Expansion of CAR-T

In recent years, there has been a large focus on improving the efficiency and quality of CAR-T cells to support their continued clinical use. As shown in Figure 1, there are multiple stages involved in the development and expansion of autologous CAR-T material. At all stages, the resulting product needs to be assessed for quality and functionality, while reducing time from initial donation to re-introduction to the patient.

Figure 1
Development Cycle of CAR-T Material

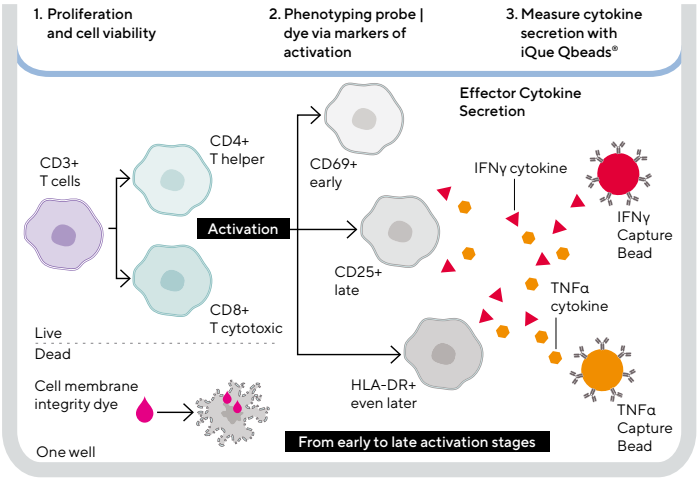


During early development, the CAR construct is optimized to ensure its specificity and engagement with the target of interest. Constructs typically include a recognizable marker, for example, protein L or GFP, which serves as an easy identifier of transduced cells. This marker is used following T cell reprogramming to assess transduction efficiency and can be used for CAR-T enrichment during downstream processing. Once CAR-T cells have been transduced, *in vitro* assays are used to profile the cells and to assess their functional reactivity to the target of interest. For example, flow cytometry can be used to assess the phenotype of cells in combination with functional readouts following a tumor killing assay.[7] These assays need to deliver reproducible and biologically relevant results. An example of the type of flow cytometry readouts that can be easily captured using a high throughput instrument like the iQue® platform are shown below (Figure 2). The panels shown also highlight the capacity for simultaneous quantification of secreted cytokine levels within the same sample.

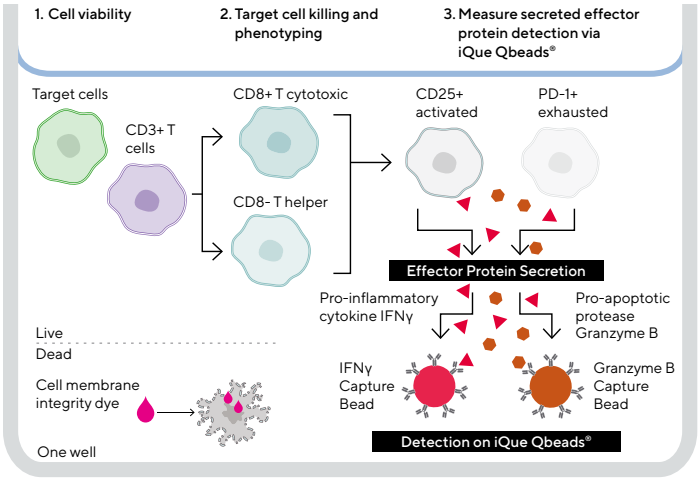
Figure 2

Overview of Phenotype and Function Kits to Characterize T Cells, Compatible With the iQue® Advanced Flow Cytometry Platform

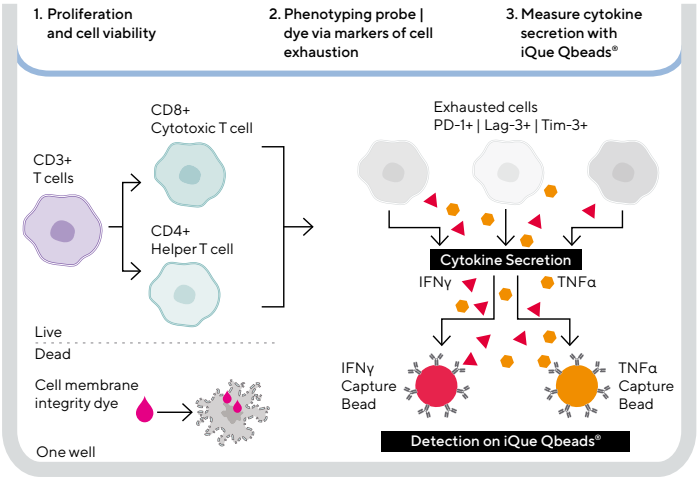
iQue® Human T Cell Activation Kit



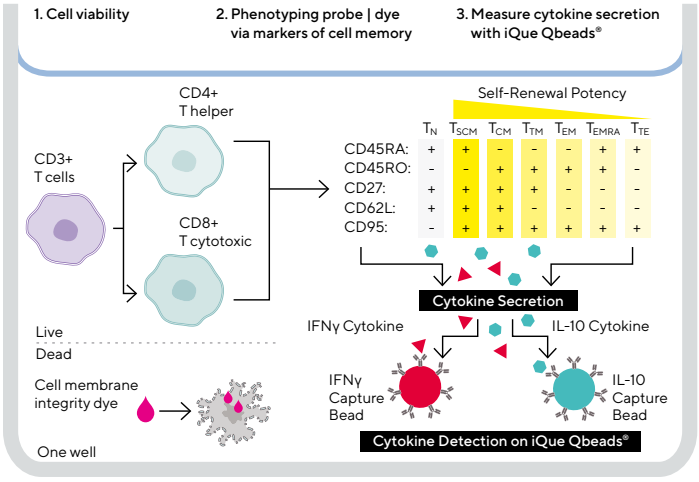
iQue® Human T Cell Killing Kit



iQue® Human T Cell Exhaustion Kit



iQue® Human T Cell Memory Kit



Once development and assessment of the CAR construct is complete, the cells are expanded to provide the quantities needed for re-introduction into the patient. During this phase, phenotype and function of the cells is quantified to ensure required cell profiles are maintained. Expansion is driven by the activation of the cells, often through non-specific mechanisms, for example, by the addition of anti-CD3 and anti-CD28. This can lead to rapid expansion in culture vessels, so nutrient depletion and cell densities need to be closely monitored. To accommodate larger scale CAR-T production (1–10 liters), cells may need to be cultured in stirred tank or wave bag bioreactors.[8,9] When producing clinical grade material, many additional control processes must be introduced to ensure integrity and quality of the samples. For example, cells must be thoroughly characterized, and heightened safety measures must be implemented and carefully documented. These processes are often time-sensitive, so it is important that rapid solutions are available. Real-time screening and analysis techniques enable continuous phenotypic and functional analysis over time to ensure a high-quality product is maintained.

The phenotype of the final CAR-T cell product is of great importance because it strongly links to their clinical potency. Much of the interest in this field has been focused on the influence of exhaustion and memory phenotypes on CAR-T function.[10] For a prolonged anti-tumor response, it is critical that populations of functioning CAR-T cells are maintained once re-introduced into the patient. This relies on preservation of the cells' self-renewal potency coupled with a lack of exhaustion, meaning their ability to kill tumor

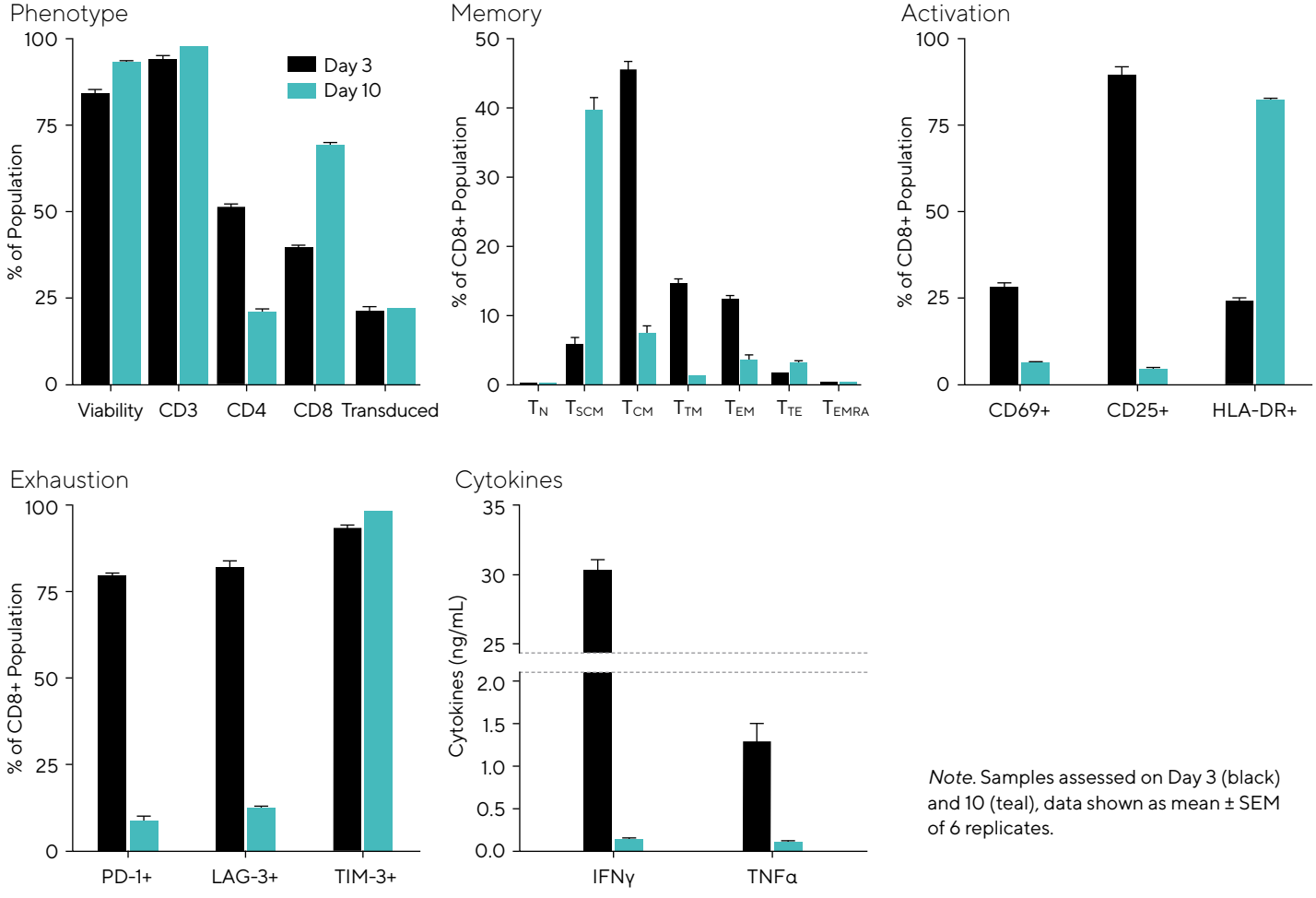
cells is sustained. Some features can be built into the CAR construct to improve longevity of signal[2,3], but expansion protocols can also influence the balance of phenotypes. There are key phenotypes that can be tracked *in vitro* to determine that these parameters have been maintained. Large populations of memory T cells such as stem central memory (T_{SCM}) or central memory cells (T_{CM}) are desirable as they have high self-renewal capabilities. Terminally differentiated cells, such as terminal effector cells (T_{TE}), are undesirable because they have lost their ability to self-renew. Markers such as PD-1, LAG-3 and TIM-3 are important indicators of exhaustion. Expression of these markers will often fluctuate during the expansion phase due to the stimulation added to drive activation and expansion of the T cells.[11] More recently, interest has also been directed towards determining the optimal ratio of CD4 and CD8 cells in a CAR-T product.[12,13]

The data below (Figure 3) shows an example phenotype profile of CAR-T cells during a 10-day expansion process with anti-CD3/anti-CD28 activation beads as a static culture in flasks (cells supplied by Dr. Qasim Rafiq's lab at University College London). Samples were analyzed on Day 3 and 10 post CAR transduction using the iQue® Human T Cell Kits. The data quantifies the general T cell population for CD3, 4 and 8 alongside viability and transduction efficiency across the sample days. For this example, the Day 10 memory phenotypes display a higher proportion of the desired T_{SCM} and T_{CM} cells with negligible populations of the more differentiated phenotypes of effector memory (T_{EM}), T_{TE} and effector memory cells re-expressing CD45RA (T_{EMRA}). The activation profile shows early activation markers, CD69 and CD25, are more highly expressed on Day 3 and reduce by Day 10, while HLA-DR, a later marker for activation, increases from Day 3 to 10. PD-1 and LAG-3 display a similar trend to the early activation markers, in that they are initially high but reduce by Day 10. Interestingly, TIM-3 expression remains high throughout. Both IFNγ and TNFα concentrations are high in the Day 3 sample but dramatically drop by Day 10.

This type of profiling data can help support optimization of expansion processes and the complete understanding of the phenotype ratios present in the final product.

Figure 3

CAR-T Phenotyping Using iQue® T Cell Characterization Kits During an Anti-CD3/Anti-CD28 Driven Expansion of Transduced T Cells



Note. Samples assessed on Day 3 (black) and 10 (teal), data shown as mean ± SEM of 6 replicates.

Quantification of the Functional Activity of CAR-T Cells

The following case studies will be used to exemplify the power of the combined use of the iQue® Advanced Flow Cytometry Platform and the Incucyte® Live-Cell Analysis System for the functional profiling of CAR-T cells. All data shown has been generated using commercially obtained CAR-T cells (Creative BioLabs) which were supplied as frozen cultures of transduced T cells alongside control mock transduced T cells from a matched donor. The CAR-Ts have been transduced with a second-generation CAR construct, specific for either CD19 or HER2, with reported transduction efficiencies of around 50% for these samples.

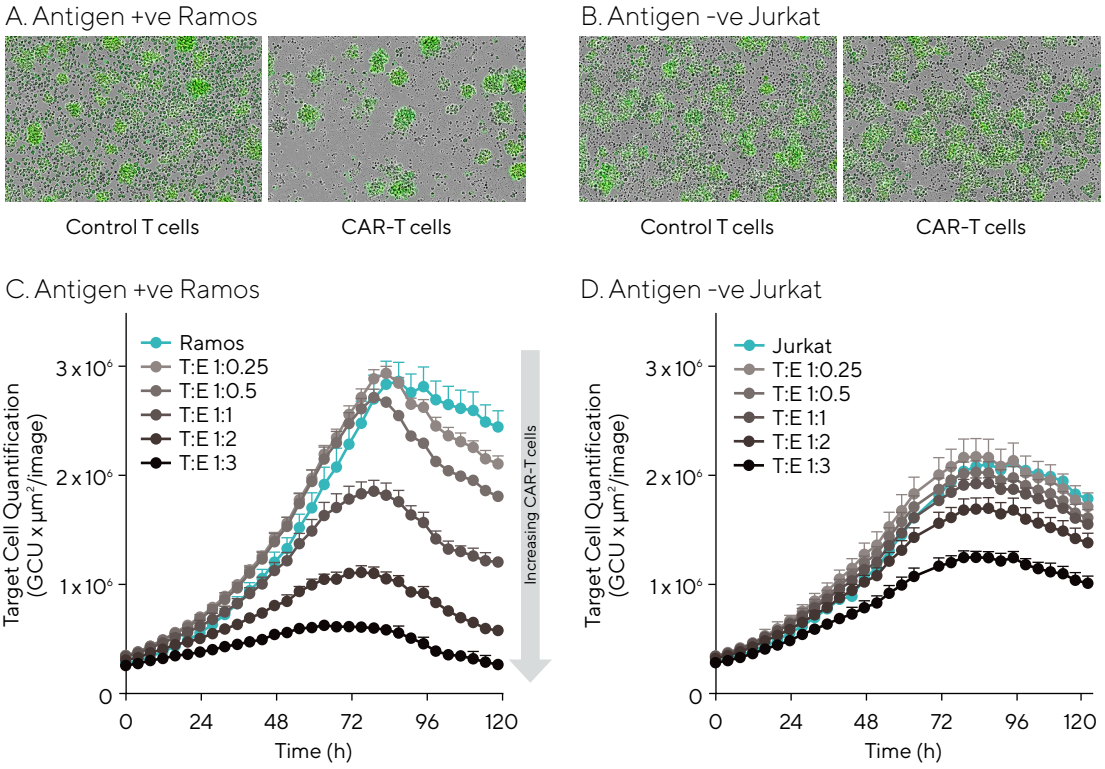
Case Study 1: Specific Killing Profile of CD19-Targeted CAR-T Cells

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 Incucyte® immune cell killing assay. CD19 antigen positive Ramos or CD19 antigen negative Jurkat cells were seeded in combination with T cells at various target to effector ratios (T:E). The target cells were transduced to express a nuclear restricted green fluorescent protein (Incucyte® Nuclight Green Lentivirus) to aid quantification. Images of the co-culture were collected over the next four days and quantified for area of green fluorescence in each well. Images showed a clear reduction in the antigen positive Ramos cells when they were co-cultured with anti-CD19 CAR-T cells (Figure 4A), which was not seen with antigen negative target cells or with mock transduced T cells (Figure 4A and B). Quantification of images demonstrates a clear CAR-T cell density related decrease in Ramos target cells over time (Figure 4C). Maximal effect was measured using a T:E ratio of 1:3, representing a 73.2 ± 0.7% reduction in target cell numbers at 72 hours. There was some death of antigen negative Jurkat cells at the higher CAR-T ratios (Figure 4C), representative of 36.5 ± 2.6% of Jurkat cells at 72 hours. This effect highlights the possibility of off-target events either by non-transduced T cells in the culture or due to the high number of effector cells in the well. No killing was induced by mock transduced control T cells, unless they had been non-specifically activated with CD3/CD28 Dynabeads® (ThermoFisher) (data not shown).

Figure 4

CD19-Targeted CAR-T Cell Killing of Antigen Positive Target Cells



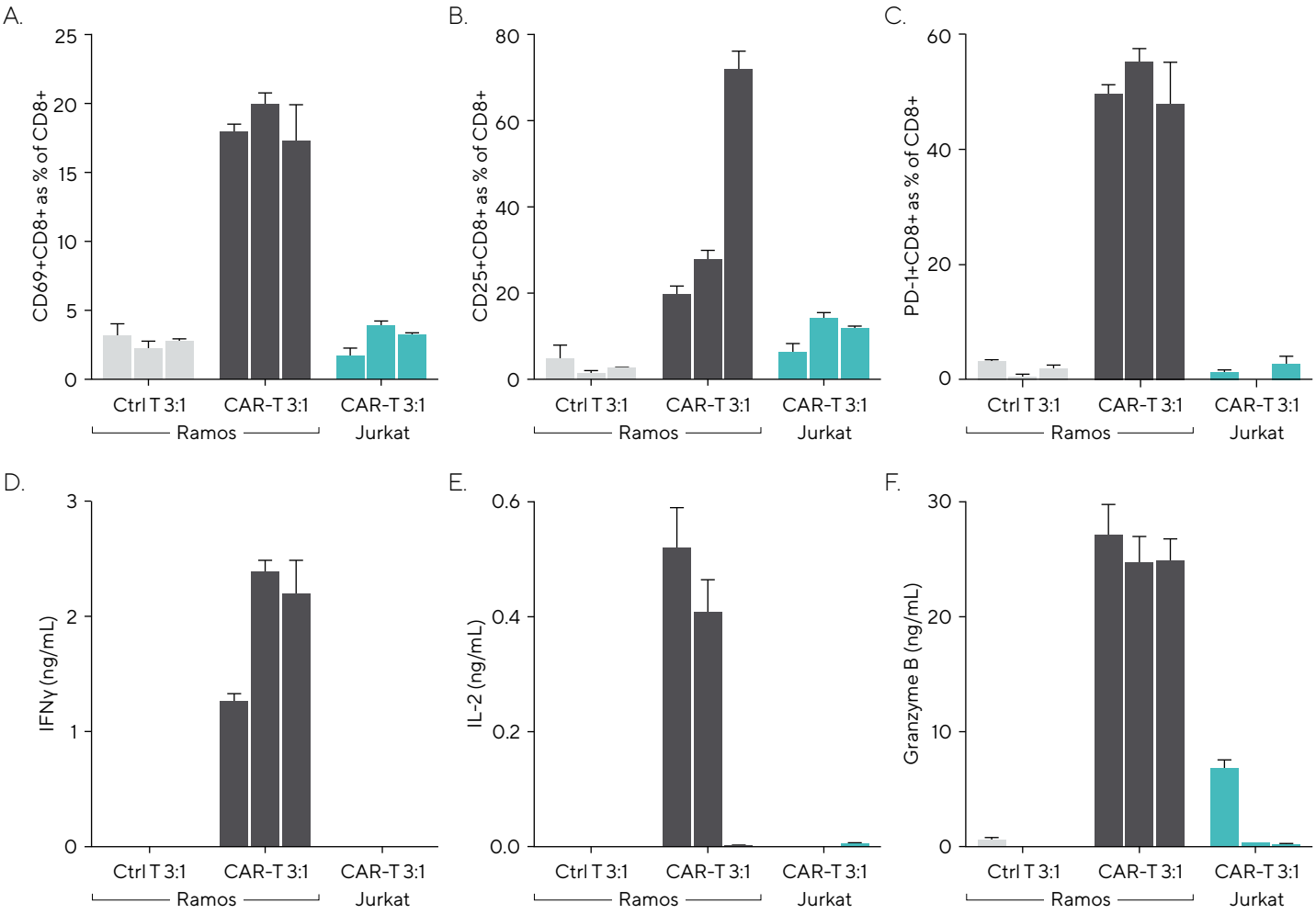
Note. A co-culture of Incucyte® Nuclight Green labeled Ramos or Jurkat cells with either anti-CD19 CAR-T or control T cells was set up at various T:E ratios in a 96-well plate. Cultures were imaged in the Incucyte® every 4 hours over 4 days and quantified for green fluorescent area. Images taken at 72 hours (A) show a clear reduction in green area of Ramos cells in combination with CAR-T cells (1:2 T:E). Time course graphs (B and C) demonstrate increased killing of antigen positive Ramos cells compared to antigen negative Jurkat cells. Data shown as mean ± SEM of 3 wells.

After Incucyte® images had been collected, on Day 2, 4, and 7, samples were subsequently analyzed on the iQue® platform to assess phenotype and function using the iQue® Human T Cell Activation and iQue® Human T Cell Killing Kits, as well as quantification of IL-2 via the iQue® Human T Cell Companion Kit. The kits enable quantification of T cell surface markers and secreted proteins indicative of T cell activation and tumor cell killing. Results show that, when combined with antigen positive Ramos cells, there was a rapid upregulation of T cell activation markers CD69, CD25, and PD-1 (Figure 5A-C, 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 all 3 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. Release of Granzyme B, an indicator of cell killing, increased in co-cultures containing CAR-T cells, but only in the presence of antigen positive Ramos cells. For all secreted proteins, there was a general increase in levels with increasing CAR-T density.

Overall, this complete quantification demonstrates a clear antigen specific activation of anti-CD19 CAR-T cells as measured by both surface markers and secreted proteins in combination with the functional readout of killing antigen expressing tumor cells.

Figure 5

Antigen Specific Activation of Anti-CD19 CAR-T



Note. Samples were quantified on Day 2, 4, and 7 for surface marker expression and secreted protein using either iQue® Human T Cell Activation Kit or iQue® Human T Cell Mediated Killing Kit with iQue® Human T Cell Companion Kit (for IL-2). Graphs (A-C) show expression levels in CD8+ T cells of CD69, CD25 or PD-1, and graphs (D-F) show levels of IFNγ, IL-2 or Granzyme B. 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.

Case Study 2: Exhaustion Profiling of CAR-T Cells Under Antigen Challenge

Repeated exposure to tumor cell antigens can lead to CAR-T cell exhaustion. Examining the phenotypic profile of exhausted CAR-T cells can help to improve our understanding of how this exhaustion affects the longevity of the clinical response. To investigate this *in vitro,* CAR-T cell exhaustion was induced by continuously challenging the anti-CD19 CAR-T cells with the antigen positive Ramos cell line. Every 2–3 days for 10 days, the CAR-T cells were counted and re-stimulated with fresh Ramos cells (1:1 T:E). On Day 11, the stimulated CAR-T cells were counted and seeded into a 96-well plate with Ramos cells (1:1 T:E). A fresh batch of non-exhausted CAR-T cells were plated both in co-culture with Ramos and as a monoculture for comparison. Daily cytokine samples (10 µL) were taken from all wells of the assay plate and, after 72 hours, cells and supernatants were quantified using the iQue® Human T Cell Exhaustion Kit.

Cytokine secretion, both of IFNγ and TNFα, was low in wells containing the exhausted CAR-T and Ramos cell co-culture (Figure 6A and B). Comparatively, fresh CAR-T cells secreted significantly higher levels of IFNγ and TNFα, with peak concentrations at 48 hours of 3.5 ± 0.2 ng/mL and 0.6 ± 0.1 ng/mL, respectively. Fresh CAR-T cells in monoculture produced low levels of each cytokine. This distinct loss of cytokine secretion in wells with the repeat antigen challenged T cells is a clear sign of their exhaustion.

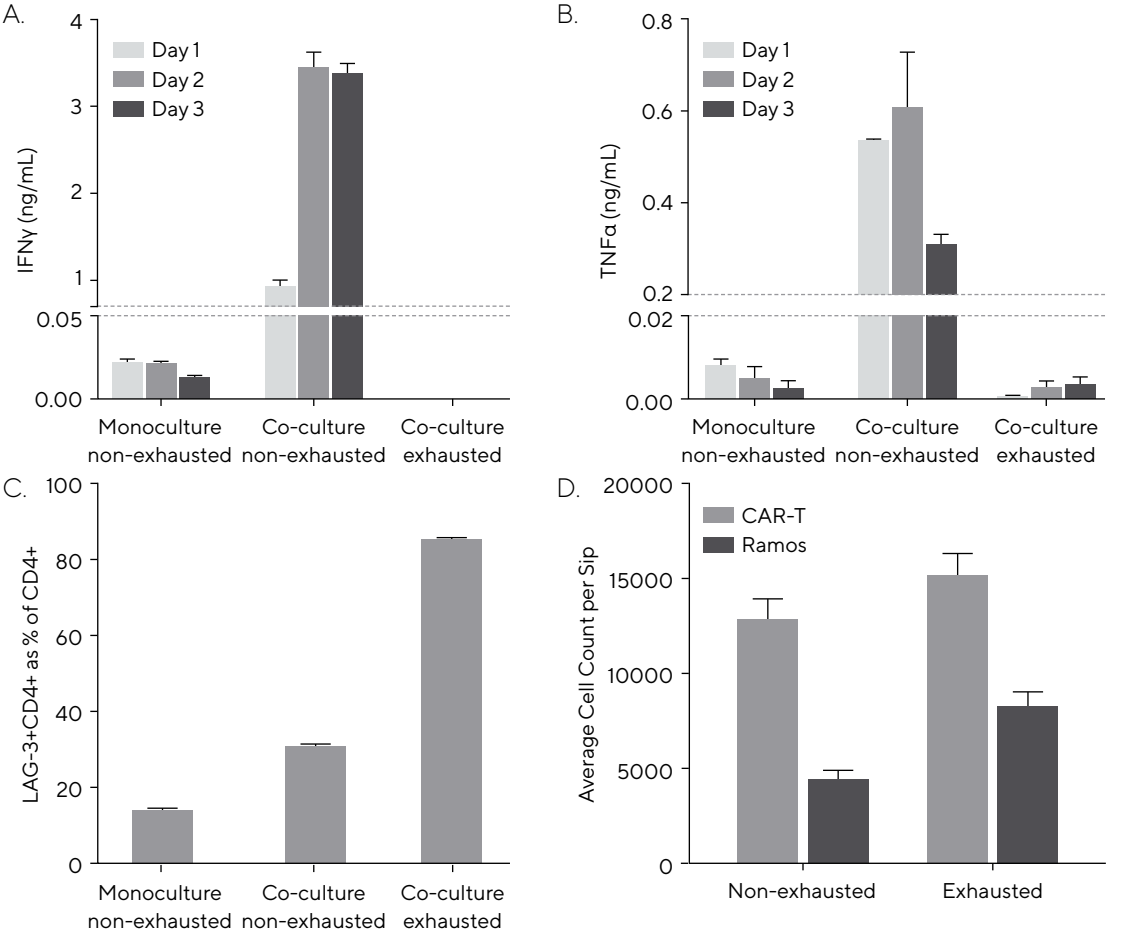
After 72 hours, expression of the LAG-3 exhaustion marker was highly elevated in the challenged CAR-T cells, with 86 ± 0.3% of the CD4+ population positive for this phenotype, whereas the freshly stimulated CD4+ CAR-T cells had just 31 ± 0.6% expression (Figure 6C). The CAR-T cell monoculture had a small population positive for LAG-3 (17 ± 0.8%).

A reduction in the exhausted CAR-T cells' ability to kill the target cells was also observed in the co-culture incubations (Figure 6D). Non-exhausted CAR-T cells were able to reduce Ramos cell numbers more effectively than exhausted CAR-T cells, with iQue® acquired values having an average of 4606 ± 463 cells per sip from wells containing non-exhausted CAR-T cells compared to 8483 ± 688 cells per sip with the exhausted CAR-T cells. The reduced ability of T cells to kill target cells is another hallmark of exhaustion.

Overall, this complete quantification demonstrates a clear antigen-specific driven exhaustion profile in these anti-CD19 CAR-T cells. The data demonstrates the utility of the exhaustion profiling kit in this type of cellular profiling.

Figure 6

Anti-CD19 CAR-T Cells Challenged With the CD19+ Ramos Cell Line Exhibited a Clear Exhaustion Phenotype



Note. Exhausted and non-exhausted anti-CD19 CAR-T cells were seeded separately at 50K/well. Incucyte® Nuclight Green labeled Ramos cells were then added at 50K/well (T:E ratio of 1:1). Non-exhausted CAR-T cells grown as a monoculture were used as controls. Cytokine samples were taken every 24 hours. After 72 hours, all cells and supernatant samples were analyzed using the iQue® Human T Cell Exhaustion Kit. Each data point represents mean ± SEM, n = 4 wells.

Case Study 3: Solid Tumor Killing and "On Target Off Tumor" Profiling with HER2 CAR-T Immune Cell Killing

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.[14, 15] 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.[16]

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. Three cell lines were profiled for their HER2 expression (Figure 7A) showing a spectrum of expression levels relative to IgG isotype control. AU565 display the highest expression of HER2 (median fluorescence intensity (MFI) with IgG background subtracted 1×10^6). MDA-MB-231, often used as triple negative, control line, in our experiments show a very low level of expression relative to IgG (MFI 1.1×10^4) while MDA-MB-468 show minimal expression (MFI 4.2×10^3).

The three cell lines, modified to express a nuclear restricted green fluorescent protein (Incucyte® Nuclight Green Lentivirus), were seeded into ultra-low attachment (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 T:E ratios. Spheroids were imaged in the Incucyte® for 7 days and green fluorescence intensity was quantified as a measure of spheroid health.

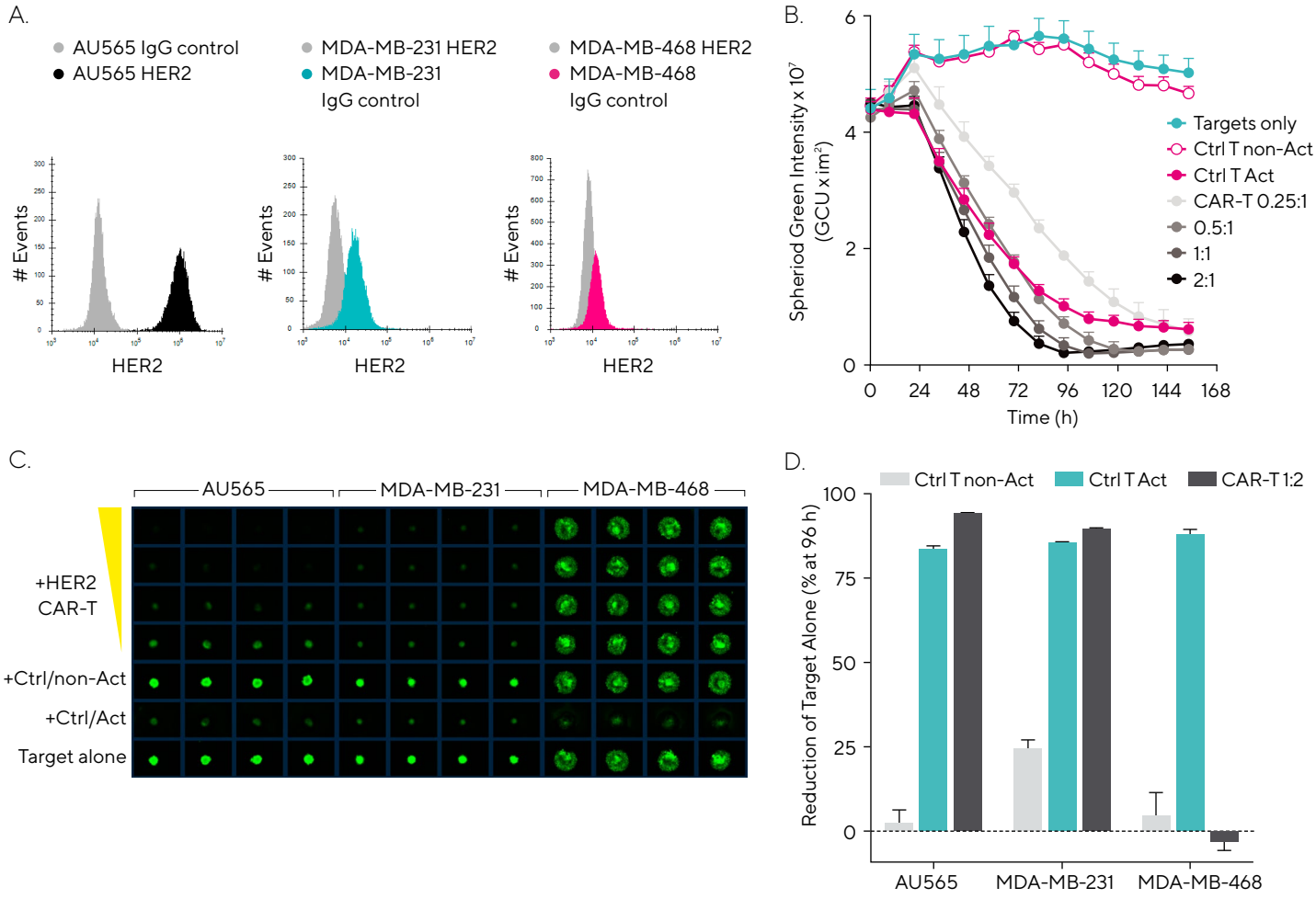
Results with the high expressing AU565 cells demonstrate a clear CAR-T cell driven reduction in green fluorescence (Figure 7B), indicating spheroid death (96% reduction at 96 h compared to target cells alone). No death was measured with non-activated, mock transduced T cells unless in the presence of CD3/CD28 Dynabeads®. When using low level expressing MDA-MB-231 cells, there was also a strong killing effect with the anti-HER2 CAR-T cells, indicating an "on target off tumor" effect (Figure 7C and D). The effect on MDA-MB-231 was seen across two separate CAR-T cell preparations and is in line with previously reported anti-HER2 CAR-T data.[16] The extent of killing measured was similar in strength to that seen with the AU565 cells (91% reduction at 96 h). When using MDA-MB-468 target cells, which have minimal expression of HER2, no CAR-T driven death was measured (Figure 7C and D).

As in the previous example, on Day 2, 4, and 7, samples were analyzed on the iQue® 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 and iQue® Human T Cell Killing kits. The results show an increase in CD69 and CD25 activation markers on the CD8+ population for both AU565 and MDA-MB-231 co-cultures with anti-HER2 CAR-T cells (Figure 8A and B). This effect was absent in the presence of mock transduced T cell. The 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 and Granzyme B levels using iQue Qbeads® detection as part of the kits. Once again both AU565 and MDA-MB-231 CAR-T co-cultures demonstrated high levels for both proteins while nothing was detected in the MDA-MB-468 co-culture wells.

Both the live-cell analysis and flow data indicate anti-HER2 CAR-T driven killing or 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. Similar data was observed in a 2D monolayer version of the assay (data not shown).

Figure 7

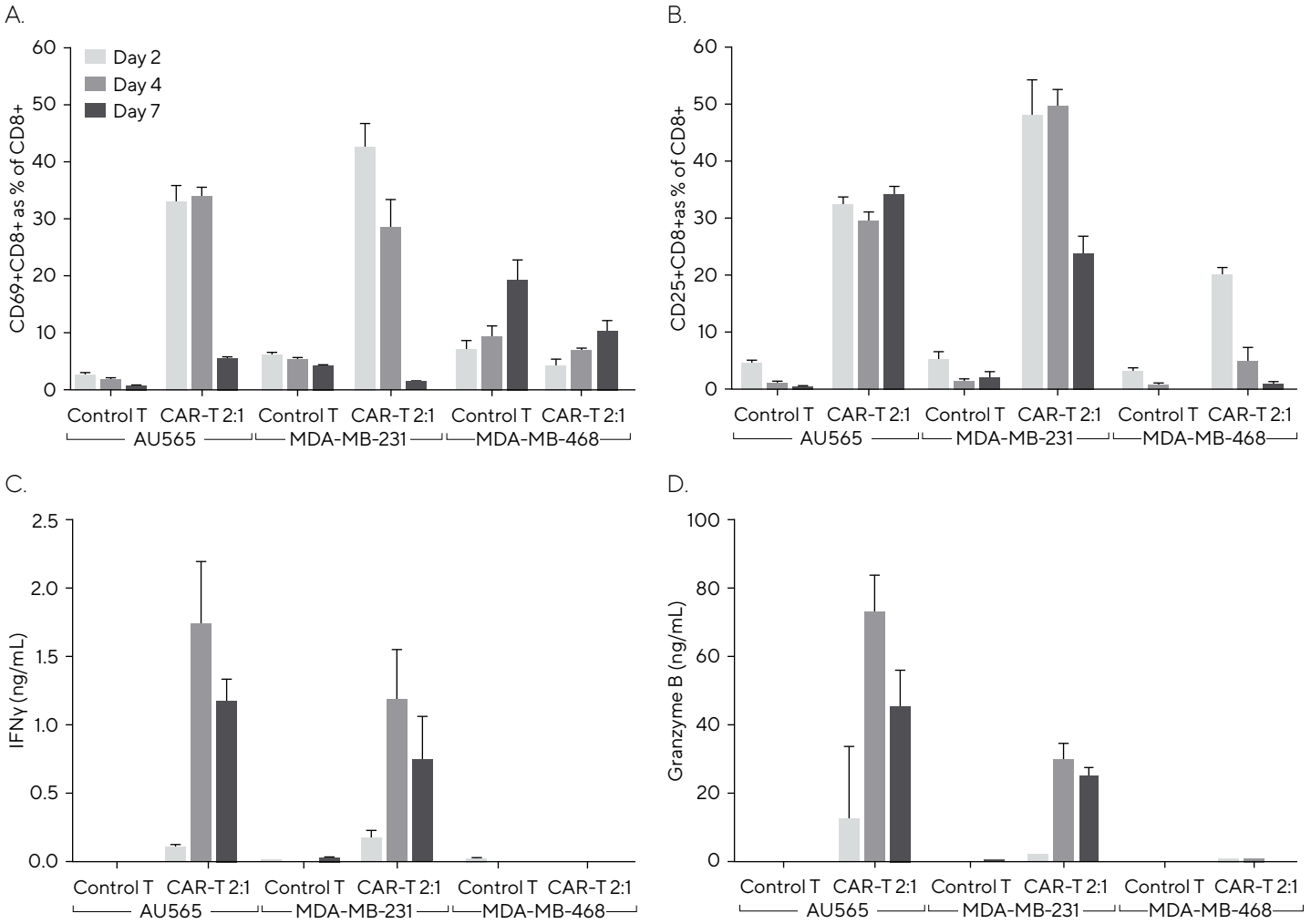
"On Target Off Tumor" Anti-HER2 CAR-T Driven Killing in a Solid Tumor Co-Culture Model



Note. Histograms (A) show AU565 > MDA-MB-231 > MDA-MB-468 for HER2 expression relative to IgG isotype control using iQue®. Time course graph (B) shows the reduction in AU565 spheroid green intensity over 7 days when in co-culture with CAR-T but not mock transduced T cells. The deep well view (C) and bar graph (D) indicate an "on target off tumor" effect when using MDA-MB-231 cells and no effect on MDA-MB-468 cells. All data shown as mean ± SEM of 4 wells.

Figure 8

"On Target Off Tumor" Activation of T Cells in a Solid Tumor Co-Culture Model



Note. Samples were quantified on Day 2, 4, and 7 for surface marker expression and secreted protein using either iQue® Human T Cell Activation Kit or the iQue® Human T Cell Killing Kit. Graphs (A and B) show expression levels in CD8+ T cells of CD69 or CD25, and graphs (C and D) show levels of IFNγ or Granzyme B 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.

Summary and Conclusions

The use of advanced cell therapies is rapidly developing, with increased focus on improving the efficiency of cell production for use in the clinic. Development is focusing on improving construct longevity, selectivity, manufacturing, and delivery to the patient. The potential switch to the use of allogeneic, off-the-shelf products offers a number of potential benefits for the clinic, such as reduced cost of manufacturing, improved long-term storage of cells, and increased consistency of larger batches. As mentioned, development is ongoing in the area of CAR-NK cells which have the benefit of HLA-independent therapies and are attractive for potential allogeneic therapies.

The data examples shared in this whitepaper demonstrate how the use of both live-cell analysis and advanced flow cytometry can add value when developing and characterizing T cell therapies. These techniques have value at multiple stages in the development and expansion of cell products and can be applied to multiple cell types.

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