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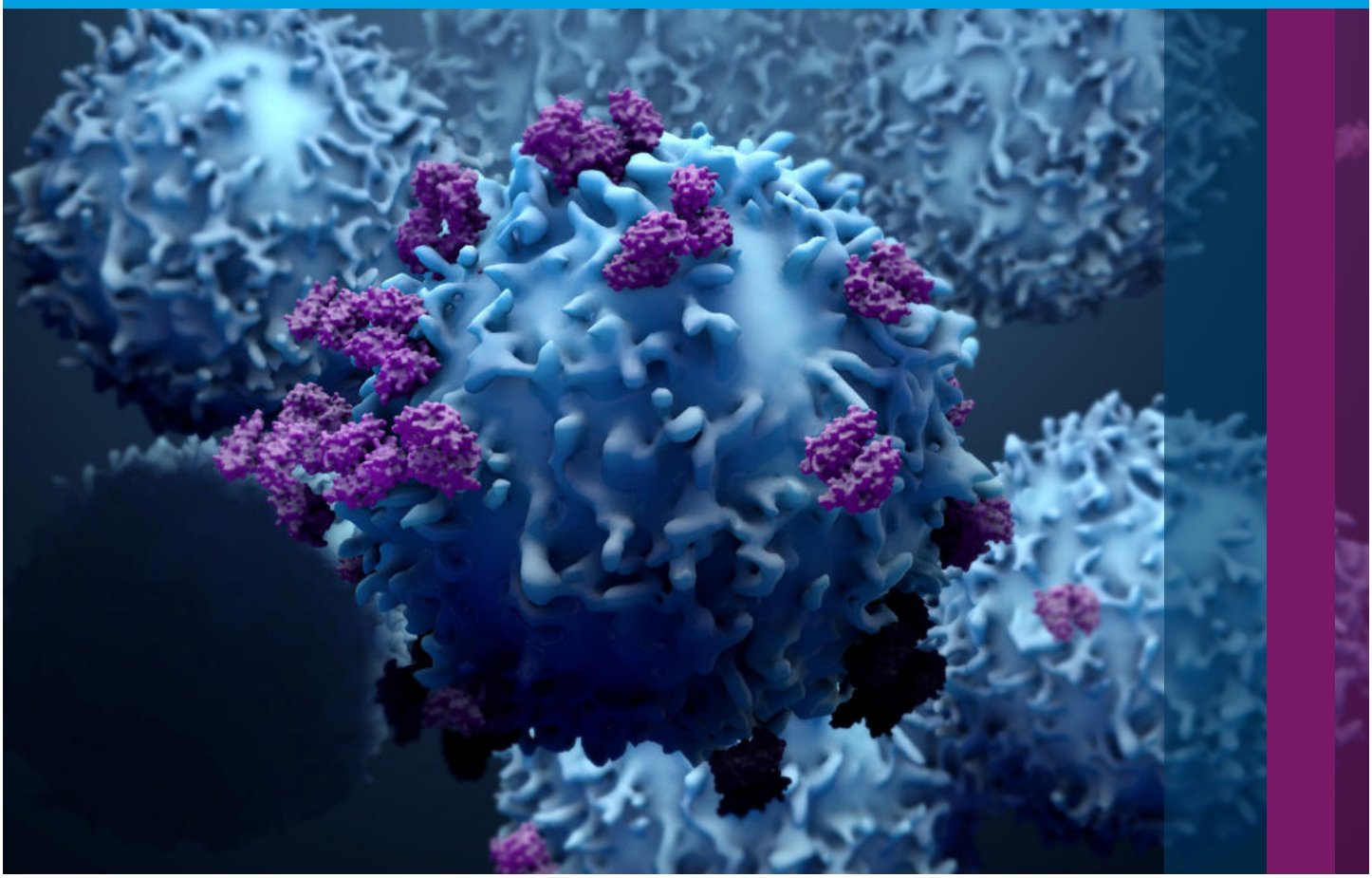
Evaluating Immune Cell Therapy Potency

From Discovery and Process Development to Manufacturing QC



Foreword

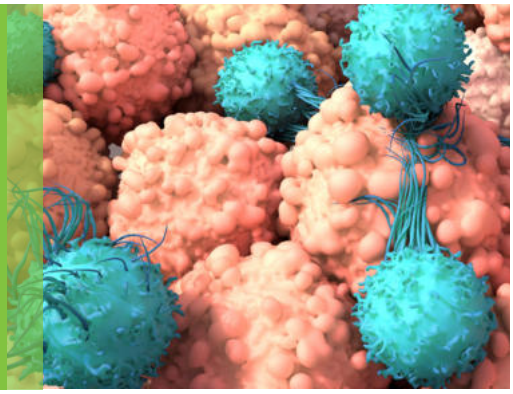
By harnessing the power of the immune system, adoptive cell therapies offer a promising approach for the treatment of numerous diseases including cancer, autoimmune and infectious diseases. In particular, autologous CAR T cell therapy provides an unparalleled adaptive response to hematological tumors – minimizing the risk of systemic side effects and bio-incompatibility.

Researchers and manufacturers must ensure reproducibility, efficacy, and potency before treatments reach the clinic. However, subject specific variability makes achieving product quality and consistency a challenge. Hence, efficient tools to monitor potency *ex-vivo* are essential to ensure safe and consistent cell therapy products.

This eBook will 1) provide an overview of the current state of CAR T therapies, 2) showcase xCELLigence Real-Time Cell Analysis (RTCA) technology as an accurate approach to evaluate the potency of novel adoptive cell therapies, and 3) show how this technology can be incorporated into research and development as well as manufacturing workflows of cell therapies.

Content Page

The Current State and Future of CAR T Cell Therapies



Cancer places a significant burden on public health worldwide. Accordingly, cancer drug discovery and development are at the core of innovative drug research in the biomedical area. As cancer incidence and mortality rates grow year by year, the demand for innovative and personalized anti-tumor drugs also increases.[1] In recent years, technologies such as adoptive cell therapies have entered a stage of rapid development.[2] Among them, Chimeric Antigen Receptor (CAR) T cell therapy has shown great potential against hematological tumors.[2]

The global cell therapy market size was valued at $7.8 billion in 2020 and is expected to expand at a compound annual growth rate (CAGR) of 14.5% from 2021 to 2028. The autologous therapies dominated the 2020 revenue share owing to the presence of a substantial number of approved products for clinical use.[3] China is at the forefront in the development of these innovative biopharmaceutical products with the most CAR T cell therapies under clinical studies worldwide, leading to more CAR T cell therapy partnerships/approvals in the rest of the world.[4]

CAR T cell therapy is currently a highly personalized therapy and faces several challenges to achieve mass production, i.e., a complicated manufacturing process, a long production cycle and an imperfect regulatory system.[5] This article will explore recent CAR T-cell therapy successes and how to overcome the current challenges.

CAR T cell therapy in a nutshell

Different from traditional anticancer drugs, CAR T cell therapy is a living drug

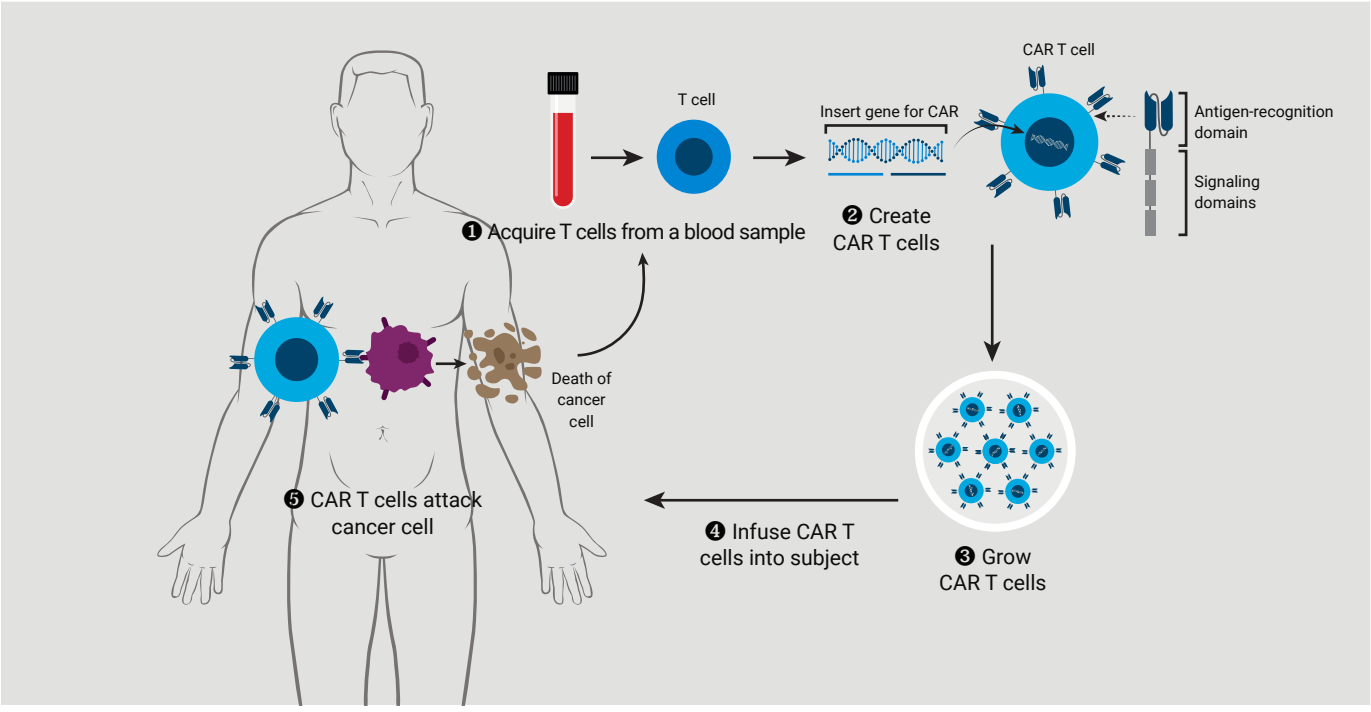


Figure 1. Steps involved in CAR T cell therapy. CARs have two domains; a tumor antigen recognition domain and an intracellular signalling domain that helps T cells to survive and grow once re-introduced into the subject.

and uses T cells to directly target the tumor cells.[1] This therapy starts with the isolation of immune cells from the individual's blood followed by selective isolation of T cell subsets. These T cells are subsequently genetically engineered to express a chimeric antigen receptor (CAR) on their surface. These CAR T cells are expanded *ex-vivo* and reinfused into the individual. Once inside the body, the chimeric antigen receptors enable CAR T cells to latch onto a specific antigen expressed on the individual's tumor cells and selectively kill them (Figure 1).[1] Since the United States Federal Drug Administration (FDA) approved the first CAR T cell therapy in 2017, six CAR T cell therapies have now been approved worldwide, including two CAR T cell therapies approved in China in 2021. A recent longitudinal study presents data of two individuals with chronic lymphocytic leukemia (CLL) that received CAR T cell therapy in a clinical trial in 2010. The study shows that the CAR T cells remain detectable at least a decade after infusion, with sustained remission in both individuals.[6]

CART cell development: a case study in overcoming challenges during manufacturing

One of the leading biotechnology companies in the research, development and manufacture of adoptive cell therapies in China is Guangzhou Bio-gene Technology Co., Ltd (Bio-gene). "The company is committed to developing a cost-effective new generation of CAR T cells to treat leukemia, lymphoma and other tumors in the hemopoietic system and as well as various solid tumors", explains Ding Wen, Director of Clinical Production Department.

Bio-gene has developed a CAR T cell therapy, called BG-1805, to treat children with relapsed and refractory acute myeloid leukemia (R/R AML). This therapy targets the receptor CLL1 which is highly expressed on AML blasts cells and leukemia stem cells, but not on normal hematopoietic stem cells.[7] In 2021, the company presented the results of a Phase I clinical trial demonstrating the safety and efficacy of BG1805. The preliminary results showed that the overall response rate reached up to 82%, and the overall remission rate reached up to 73% in 11 individuals with R/R AML. In terms of safety, the individuals only had grade 1-2 cytokine release syndrome (CRS), a common acute side effect of CAR T cell therapies, and there was no obvious immune effector cell-associated neurotoxicity syndrome (ICANS), which brings new hope to individuals with R/R AML.[8]

Ding Wen says that "when filing for the emerging cell and gene therapies, the major difficulty we face is to comply with regulatory requirements, such as Chemistry, Manufacturing

and Control (CMC) and Current Good Manufacturing Practices (cGMP). Manufacturers must control Critical Quality Attributes (CQA) to ensure that the quality of products meets the acceptable standard consistently in the production process. In addition, before using these products to treat individuals, their quality should be strictly controlled to ensure that they have excellent efficacy."

In this context, pharmaceutical companies need to develop reliable methods for product quality inspection to shorten the period of product release. To evaluate the efficacy of cell therapies, various *in-vitro* methods have been developed, such as luciferase, lactic dehydrogenase (LDH), cytokine detection and flow cytometry. However, all these methods require cell labelling, are labor-intensive and only provide information about the cytotoxic effect of products at a selected time point. To overcome these hurdles, Ding Wen explains that

"Bio-gene used Agilent's xCELLigence Real-Time Cell Analysis (RTCA) label-free platform for real-time release testing (RTRT). This technology can automatically detect cells in the whole process without labelling cells, which can greatly reduce the manual operation steps, and ensure the objectivity, accuracy and integrity of experimental data. This scheme can more accurately evaluate the biological characteristics, efficacy or potency of the product. More importantly, it can continuously detect the dynamic changes of cells in several hours or even dozens of days in real-time."

The xCELLigence RTCA technology supports real-time, label-free and long-time evaluation of the cell therapies efficiency. The technology offers an intuitive platform with enhanced repeatability and sensitivity as well as lower variability, thus promoting a faster development of cell therapies.

Upcoming cell therapy advancements

Although Bio-gene has made a great breakthrough, the medical technology is developing rapidly. CAR T cell therapy is an individualized treatment scheme, that currently is limited to the treatment of hematological tumors. However, CAR T therapy against solid tumors is expected to advance in the future. For example, combinations of CAR T cell therapy and oncolytic viruses are being explored for the treatment of solid tumors.[9]

Bio-gene is conducting investigator-initiated trials (IITs) on CAR T cell therapies for skin tumors and gastrointestinal tumors. "Only by focusing on the field of CAR T cell therapies, and insisting on technological innovation and process optimization, can high-quality products be produced. Only the CAR T cell therapies with excellent efficacy and reliable technology can truly benefit individuals", Ding Wen emphasizes.

Ding Wen thinks that the demand for CAR T cell therapies is different from that of traditional medicines and is more similar to that of medical technology which needs continuous cooperation with hospitals. The advantage of Bio-gene is that the company has reached stable clinical cooperation with more than 10 grade-A tertiary hospitals in China. At the same time, HEDY Group, Bio-gene's main investor, plans to build 20 oncology hospitals nationwide in the next five years, which will bring unique advantages to its marketing promotion in the future. Meanwhile, Bio-gene's R&D team continuously launches new products.

Conclusion

Adoptive cell therapies are unique and are a promising field for innovation in the future. Cooperation between different partners developing different types of cancer therapies, including cell and gene therapies, as well as traditional approaches such as radiotherapy and chemotherapy, will lead to treatment breakthroughs in the future. There will be greater development opportunities if all related parties cooperate with each other. At the same time, with the improvement of industry supervision and technical knowledge, the industrialization process of China's cellular immunotherapy industry will be further promoted.

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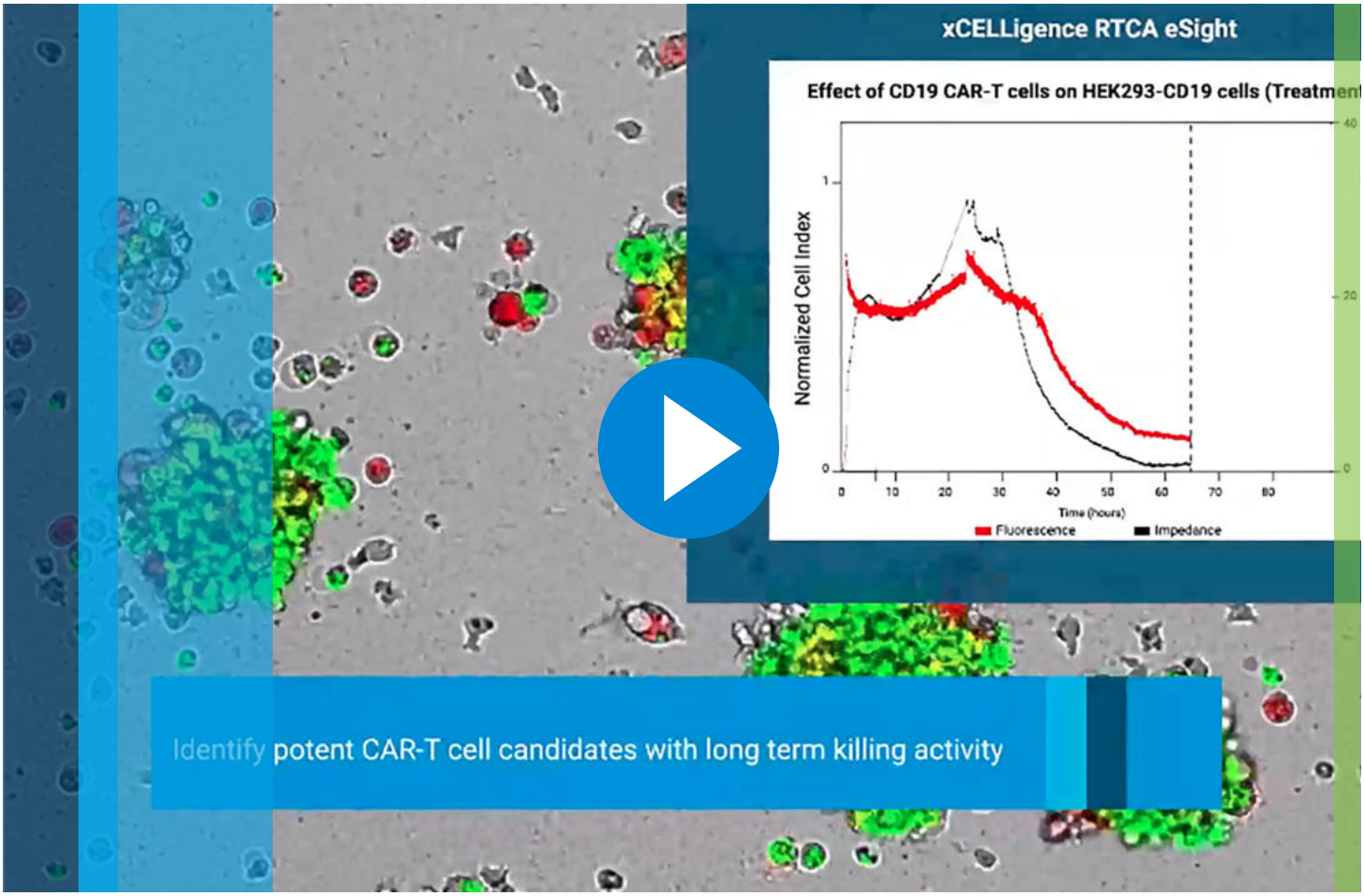
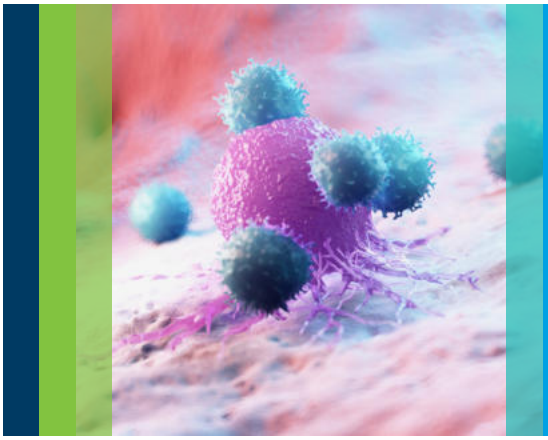


Gaining Deeper Understanding of CAR T Killing of Cancer Cells

The Agilent xCELLigence Real-Time Cell Analysis (RTCA) eSight provides automated real-time data from culture vessels inside your incubator

- Gain deeper insights into CAR T cell specificity, potency, persistence, and efficiency for killing of cancer cells
- Generate impedance and live-cell imaging readouts in parallel
- Identify potent CAR T cell candidates and make better informed research and process development decisions

Watch Now



Strategic Collaboration to Transform Manufacturing of Cell Therapies

Agilent and Lonza combine their technologies to increase the quality of therapeutic products

Cell therapy is a novel, personalized medicine which is becoming increasingly complex as the range of treatable diseases and conditions expands and different processes evolve. Cell therapy is not only used for treatment of cancer, but also genetic diseases, autoimmunity, infectious diseases and chronic diseases such as certain cardiac conditions. Due to the complexity of the products and subject-specific variability, product quality and consistency are significant challenges for cell therapy manufacturing. Since cell therapy products are largely defined by the process, minor changes during manufacturing have the potential of causing major changes in the product quality and efficacy. In this context, in-process controls and analytics are crucial to ensure a consistent product.

In a new collaborative effort, Agilent and Lonza combine their technologies to increase the quality of therapeutic products. This collaboration integrates the xCELLigence RTCA technology into the Cocoon® Platform's cell therapy automated manufacturing workflow to increase access to safe and efficacious cell-based therapies.

In this interview Yama Abassi, PhD, Head of Strategic Marketing and Business Development, Cell Analysis Division at Agilent Technologies, discusses the current challenges and solutions in cell therapy development and manufacturing.

Yama Abassi, PhD

Head of Strategic Marketing and Business Development,Cell Analysis Division, Agilent Technologies

Ash Board (AB): Why are product quality and consistency such significant challenges in cell therapy manufacturing?

Yama Abassi (YA): Cell therapy is a novel, personalized medicine which is becoming increasingly complex as the range of disease and conditions that could be treated with cells expands and different processes evolve. Cell therapy is not only used for treatment of cancer but also genetic diseases, autoimmunity, infectious disease and even considered for chronic diseases such as certain cardiac conditions.

There are various challenges to maintaining product quality and consistency in autologous cell therapy manufacturing. In autologous cell therapy, every batch is specific to the individual, meaning that we only have one opportunity to successfully manufacture safe, consistent, and high-quality cell therapy products. Cells taken from individuals will not only have genetic and phenotypic differences but will be influenced by the individual's unique medical history. It's vital to understand the functional status of the cells during manufacturing, which can be achieved by integrating cellular analytics into bioreactors to provide information about the cell status in real-time. The individuality and variations in starting material of cells coupled with potential changes in cell quality during manufacturing makes identification of critical quality attributes (CQA's) of paramount importance.

Having the right tools and equipment and validated CQA such as those that inform on immune cell persistence and potency during the manufacturing process, is vital for scientists to be able to produce safe and efficacious cell therapy products.

AB: Can you talk to us about the importance of in-process controls and analytics in the manufacture of cell therapy products?

YA: The motto in the field is that the 'process is the product', so it's very important to get that process right. To have effective personalized therapies, you must personalize the manufacturing process itself.

In-process monitoring of the actual cell therapies happens rarely in cell manufacturing at present but will grow in scale in the future. Successful in-process monitoring during cell manufacturing helps to control cell quality over time, gain a better understanding of how the cells will behave once they are manufactured and administered to the individual.

The bioreactor engineering process needs analytics throughout the entire workflow so scientists can monitor for CQAs in real-time, and ultimately produce a high-quality product which will act in the way intended.

AB: How does an understanding of CQAs enable the manufacturing process to be directed in real-time?

YA: It's important to have a solid understanding of the attributes of the cell. There are so many different facets to the cell itself. Those facets are measured by critical quality attributes. Whether a cell originates from an autologous donor or from a stem cell, the critical quality attributes determine whether the cell will provide therapeutic effect in a safe manner.

Real-time analytics of CQAs throughout the manufacturing processes determine whether the cell is ready for use and meets certain predetermined standard. Current processes use production quantity and time in the bioreactor to determine when the cell manufacturing process should finish. Having in-process analytics will help scientists better control the manufacturing process to produce optimal cell therapy products on a bespoke individual basis.

AB: Can you explain how integration of Agilent's analytics technologies and techniques into Lonza's Cocoon platform will benefit manufacturers of cell therapies?

YA: Cell therapy manufacturing is still in its relative infancy. Our partnership with Lonza, the largest CDMO in the world, will bring industry leading experience in biologic molecule manufacturing to cell therapy manufacturing through their Cocoon® bioreactor to our business. In turn, we will support Lonza with our broad range of analytical measurement tools for every step of the cell manufacturing process.

Previously scientists preferred to use molecular or non-functional measures in cell manufacturing due to their reliability and controllability. However, we're now learning that persistence and potency can only be measured by functional assays which provide a relevant measure of a cell's behaviour once administered to the individual. Agilent has the right analytical technologies for providing consistent and real-time

functional measurements in a variety of applications, such as assessing bioenergetics and metabolism for investigating persistence or real-time killing assay for measuring potency.

The question is, "can a functional measure be repeatable and reliable?" We believe that with more advanced tools like flow cytometers and state-of-the-art mass spectrometers, we can provide functional measures which weren't available previously.

Our work with Lonza will place Agilent sensors within the Cocoon® bioreactor workflow, and together we will research which key measurements are critical for different stages of the cell manufacturing process. Understanding exactly what to measure during these workflows is poorly understood, and our work with Lonza will fast track comprehension across the scientific community for advancing the production of safe and effective cell products.

AB: What impact do you see this having on the manufacture of autologous cell therapies?

YA: There is currently a failure rate of around 3 - 10% where cells do not meet the quality needed for use. In-process monitoring will increase the percentage of good quality cells available at the end of the manufacturing process.

Firstly, through increased analytics capabilities, we will be able to standardise and better understand key CQAs for producing more reliable and safe cellular therapies. Secondly, by reducing failure rates, cell manufacturing workflows will become more efficient, scalable, and affordable.

AB: The integration is set to deliver the first "fit-for-purpose" solution for scaled manufacturing. Can you expand why this has not been possible before, and what impact it will have on the industry?

YA: Cell therapy manufacturing is a new territory. Traditionally pharma and biopharma has been focused on small molecule production and biologics like antibodies and proteins. We have not been faced with the challenge to create fit-for-purpose cell therapy solutions until now.

Developing a "fit for purpose" solution means creating measurements designed to work within a bioreactor workflow which guide decision making as far as quality, potency, safety, and purity of the cells are concerned.

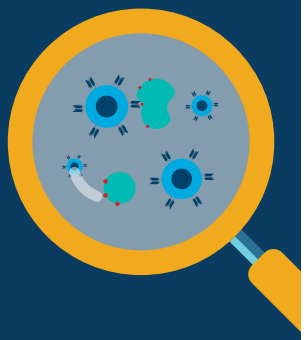
Both Agilent and Lonza are aware that there is nothing like this available to date.

Our first step will be to implement off-line or at-line analysis – to make it more automated and easier to feed samples into the bioreactor. Once we have a better understanding of the critical parameters needed for every manufacturing run or critical measurements, the second step will be to make them in- or on-line so they are able to measure within that bioreactor environment.

We believe that the fruits of our partnership will be provide a solution which can support the production of cell therapy products in a scalable and effective way, in turn transforming the future of medicine.

Yama Abassi was speaking to Dr. Ash Board, Editorial Director at *Technology Networks*.

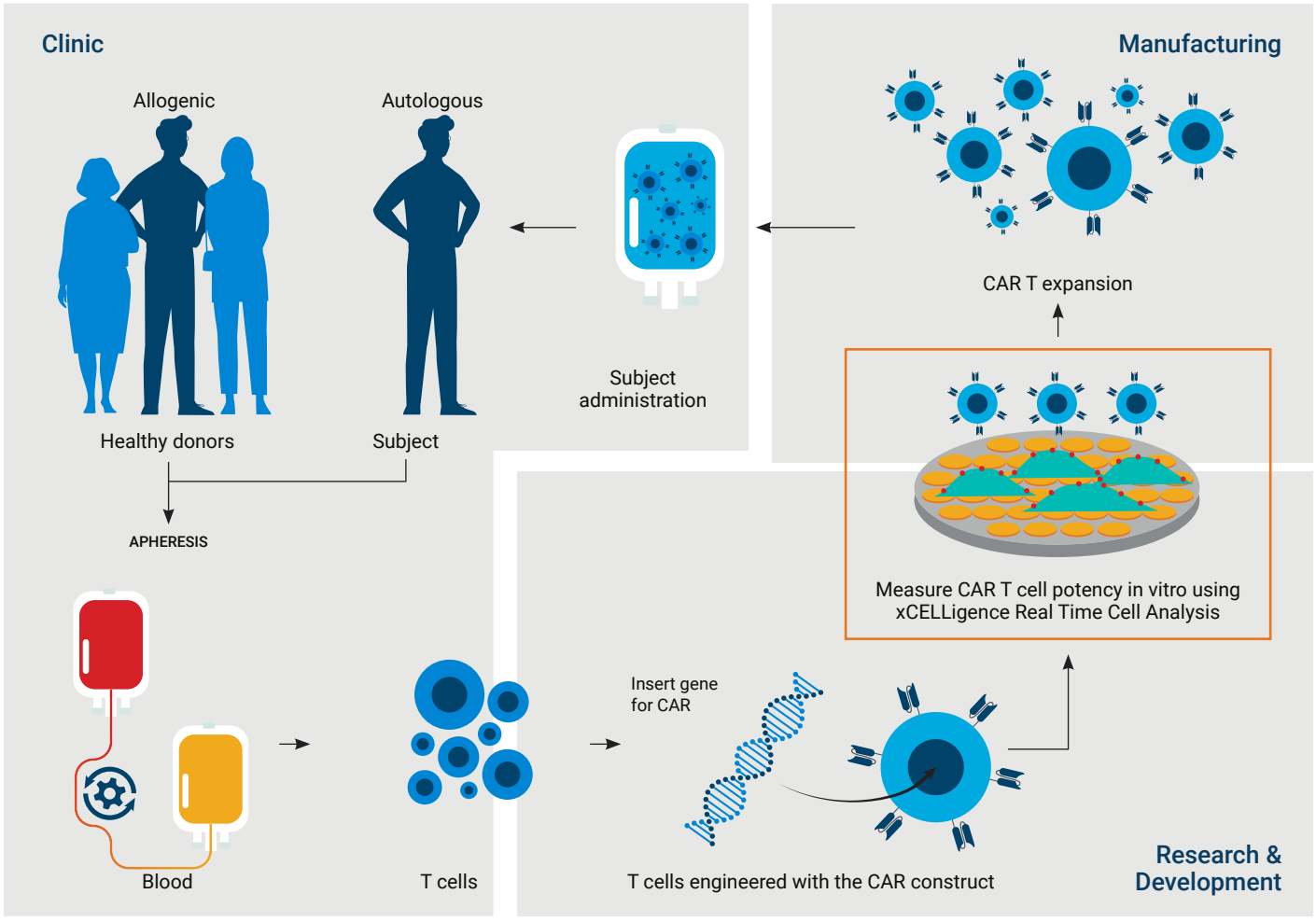
Investigating the Potency of Cell Therapies



Traditional immune cell potency assays are labor-intensive and require the collection of multiple timepoints using a combination of techniques. The Agilent xCELLigence Real-Time Cell Analysis (RTCA) assay offers an alternative to the traditional approaches. This label-free assay provides a complete view of immune cell killing while closely mimicking activity *in vivo*. This infographic explores how xCELLigence technology can be incorporated into the cell therapy discovery and process development workflow for manufacturing of engineered immune cells.

Measuring Potency *In Vitro* Accelerates Immune Cell Therapy Development

By measuring the immune-cell-mediated killing of cancer cells accurately, reproducibly, and in real-time, researchers are able to select the most effective cell therapy candidates to potentially move to the clinic.

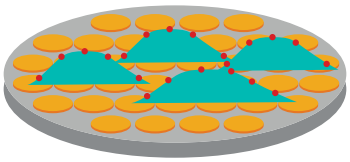


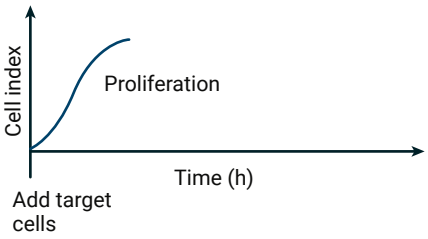
Simplifying Immune Cell Therapy Potency Assessment

The xCELLigence RTCA assay can be used to quantify immune cell killing of cancer cells continuously and automatically, from minutes to days, without the use of labels. Simply seed cells and add immune effector cells to the E-plates and begin monitoring immune cell killing kinetics at physiological conditions.

STEP 1

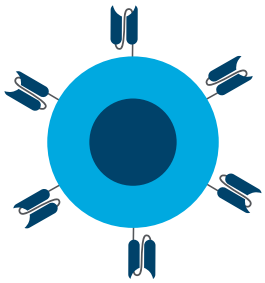
Adherent target cells (such as tumor cells) are seeded into the Agilent E-plate wells. As cells proliferate and attach to the biosensors, the Cell Index (CI) increases.

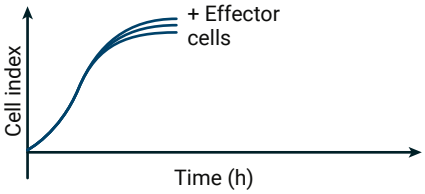




STEP 2

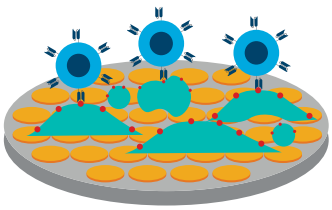
Non-adherent effector cells (such as CAR T cells) are added to the wells. The CI remains unchanged upon this addition.

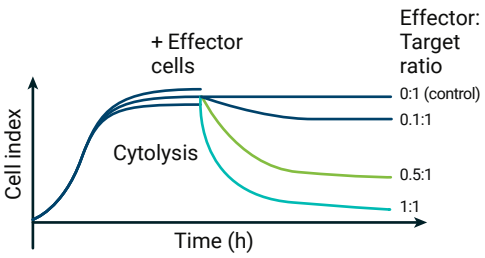




STEP 3

The CI will decrease if the effector cells kill the tumor cells. Thus, the cell therapy's potency is sensitively and precisely detected using the accompanying GMP compliant software.





Cell index decreases as target cells are killed

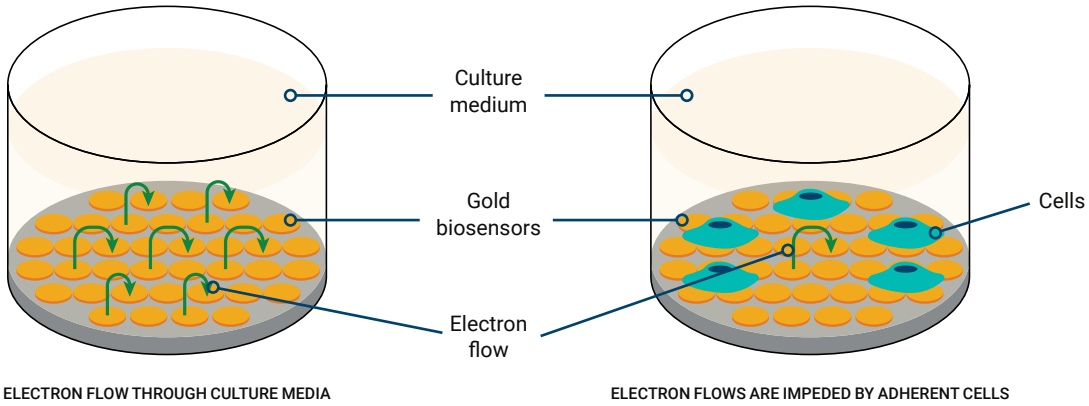
Key Features

- Non-invasive kinetic measurement of cell behavior at physiological conditions
- Convenient and simple workflow
- Highly sensitive and reproducible *in vitro* data to better predict *in vivo* response
- Assess critical process parameters for cell therapy development and manufacturing

Real-Time Cellular Impedance Explained

The xCELLigence RTCA assay enables non-invasive monitoring of parameters such as cell number, cell size and cell-substrate attachment quality, with enhanced sensitivity and in real-time.

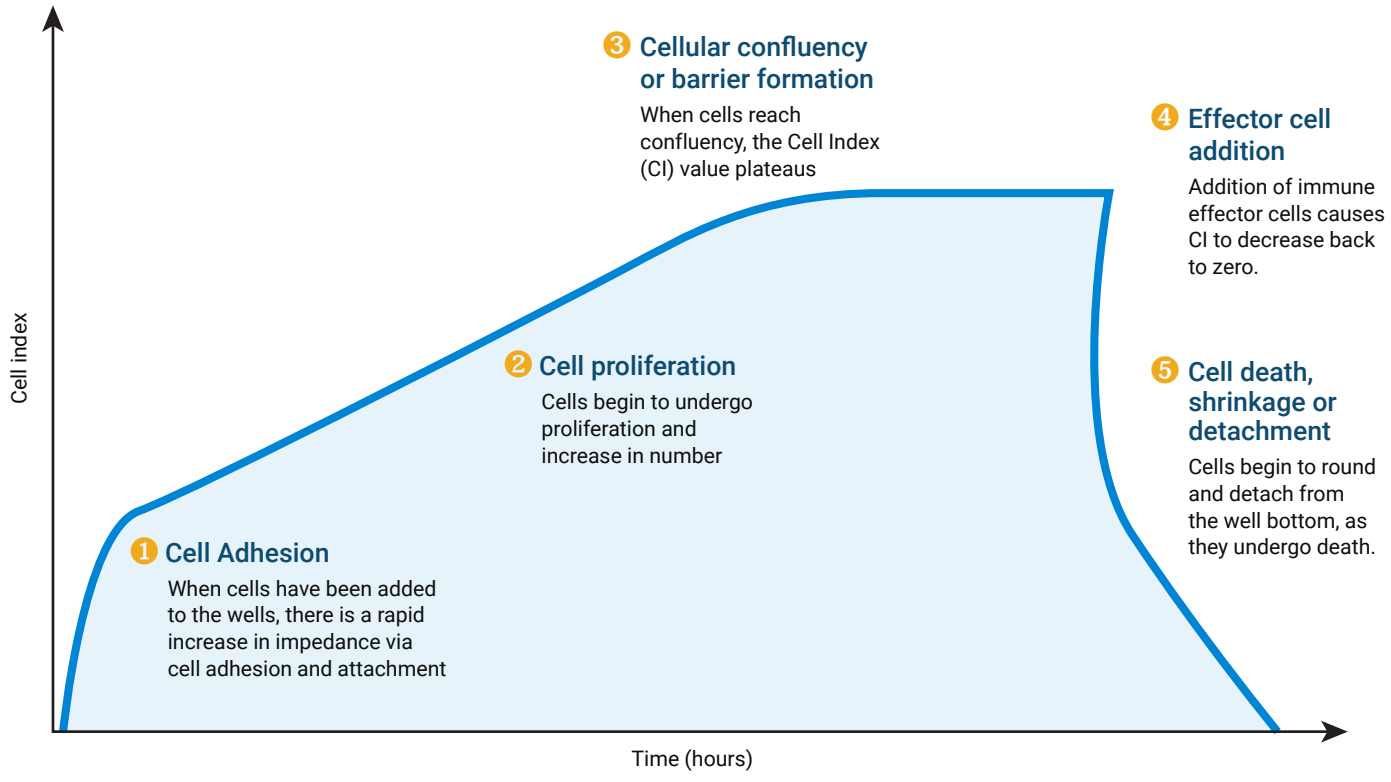
How is cellular impedance detected?



Electron flow is disrupted by cells adhered to the biosensors. Impedance increases when more cells are attached to the surface. The magnitude of this impedance is dependent on the number and size of cells as well as the cell-substrate attachment.

How is cell growth measured using cell index?

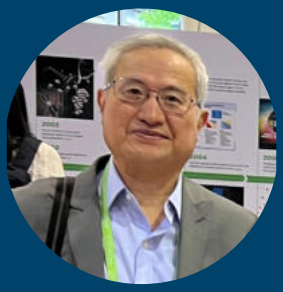
The impedance of electron flow caused by adherent cells is reported using a unitless parameter called Cell Index (CI):



Scientists Advancing Cell Therapy Development with xCELLigence RTCA

"The high throughput xCELLigence RTCA assay is really helping us determine which TCRs to advance to the clinic. Other assays produced high background, low sensitivity (10:1 E:T ratio), are time and labor intensive, and not high throughput. Our goal is to evaluate several TCRs against multiple cell lines, at different E:T ratios, which can be easily performed using xCELLigence RTCA."

Kristin Anderson, PhD, Senior Research Associate, Greenberg Lab, Fred Hutchinson Cancer



"Not sure where to start with how phenomenal the xCELLigence platform is. As a CRO, the MP (multi-plate) version is the workhorse of the over 80-90 CAR T and CAR-NK projects already completed. The real-time kinetic readout has accelerated the patent and IP positions of many of our clients. The ease-of-use is also wonderful. Plate cells, 24hr later add compound or CAR T, and walk away for the next 80 hours, leaving our scientists to continue all their other experiments. I can attest to the fact that xCELLigence MP data has been very predictive in animal models and has led to clinical study initiation in just 9 months from the first experiment!"

John Wu, PhD, CEO, ProMab Biotechnologies

Recent Publications Showcasing xCELLigence Technology

Golubovskaya V, Zhou H, Li F, *et al*. Novel CS1 CAR T cells and bispecific CS1-BCMA CAR T cells effectively target multiple myeloma. *Biomedicines*. 2021;9(10):1422. doi: 10.3390/biomedicines9101422.

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Lee MY, Robbins Y, Sievers C, *et al*. Chimeric antigen receptor engineered NK cellular immunotherapy overcomes the selection of T-cell escape variant cancer cells. *J. Imm. Ther. Canc*. 2021;9:e002128. doi: 10.1136/jitc-2020-002128.

Anderson KG, Voillet V, Bates BM, *et al*. Engineered adoptive T-cell therapy prolongs survival in a preclinical model of advanced-stage ovarian cancer. *Cancer Immunol. Res*. 2019;7(9):1412-1425. doi: 10.1158/2326-6066.CIR-19-0258.

Application Note

Immunotherapy Research

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Ex Vivo Phenotyping and Potency Monitoring of CD19 CAR T Cells

Using a combined flow cytometry and impedance-based real-time cell analysis workflow

Authors

Lauren Jachimowicz, Ming Lei, Peifang Ye, Yan Lu, Xiaoping Ji, Yu Yan, Garret Guenther, and Nan Li

Agilent Technologies, Inc.

Abstract

The development of cell-mediated immunotherapies has revolutionized cancer research as well as the study of the immune system. One of the most promising types of cell therapy involves the genetic engineering of novel chimeric antigen receptor (CAR) T cells to target cancer cells. To efficiently determine optimal CAR construction, researchers must develop a robust screening protocol to identify the ideal parent T cell populations and evaluate their cell killing potency. Here, we describe a workflow using a combination of the Agilent xCELLigence RTCA analyzer and Agilent NovoCyte Quanteon flow cytometer to thoroughly evaluate and characterize CAR T cells.

Introduction

Advancements in immunotherapy have altered the available treatments for cancer, using the specific ability of the immune system to recognize and kill cancer cells. A novel class of immunotherapy, CAR T cells, involves genetically engineering T cells to target a tumor antigen. Currently, adoptive T cell therapies are used for the treatment of B cell malignancies. However, significant challenges remain in the application to many cancers, including the treatment of solid tumors, side effects such as cytokine release syndrome, and long development timelines. The ideal universal receptor structure for highly potent CAR T cells is undergoing continuous improvements, with new generations of CAR structures being developed to maximize T cell longevity and cytotoxicity. More recent versions of CAR structures include costimulatory molecules and signaling molecules that help with better T cell function and persistence *in vivo*. The development of novel CAR T cells requires fast and in-depth evaluation of their potency to ensure efficacy and the identification of any nonspecific effects such as antigen-independent signaling.

Unlike other cytolytic endpoint assays, the Agilent xCELLigence RTCA continuously monitors CAR T cell cytolytic activity in real time over multiple days. To determine the quality of the CAR T cells under investigation, orthogonal Agilent NovoCyte flow cytometry assays can be performed to evaluate T cell activation, differentiation, and exhaustion. Here, we combined impedance-based real time cell analysis (RTCA) and flow cytometry workflow for *ex vivo* cytolytic potency monitoring of CD19-specific CAR T cells (CART19). We also examined phenotypic and functional responses to antigen exposure over time. The potency evaluation and characterization of CAR T cells were performed in several ways:

- CAR expression and T cell phenotyping
- Cytolytic potency by an RTCA cytotoxicity assay
- Cytokine production in response to antigen with a flow cytometry multiplex cytokine detection assay
- Characterization of CAR T cell state following antigen-specific activation.

This powerful workflow can be used to easily measure the cytolytic capacity of CAR T cells in conjunction with an in-depth analysis of T cell cytokine production, cell differentiation, and activation state.

Experimental

Effector and target cell culture and characterization

HEK-293 cells were maintained at 37 °C/5% CO_2 in MEM/EBSS (HyClone, catalog number SH30024.01) supplemented with 10% FBS (Gibco, catalog number 16050-122). These cells were engineered to express CD19 using lentiviral transduction. Expression of CD19 on the surface of HEK-293 cells was verified by flow cytometry with an Agilent anti-CD19 PE antibody (part number 8920007).

CD19-specific CAR T cells were constructed using PBMCs from a healthy donor and cultured for nine days with anti-CD3/anti-CD28 beads with the addition of 200 IU/mL of IL-2 in the same growth media as target cells.

CAR expression on T cells

CAR expression was analyzed in T cell populations using Agilent anti-CD3 FITC (part number 8931016), anti-CD4 PE-Cy5 (Biolegend 300510), Agilent anti-CD8 PE-Cy7 (part number 8931024), Agilent anti-CD19 APC (part number 8930007), anti-CD16 APC-Cy7 (BD, 561726), and anti-CD56 BV605 (Biolegend 318334) antibodies, and a CARTEST-19 kit provided from the CAR T manufacturer. The CAR T kit consisted of a CD19 antigen fused to a Fc tag, followed by a secondary anti-FC PE antibody. 1×10^6 CAR T cells were stained with live/dead AViD stain followed by incubation of cells with Fc block buffer (D-PBS containing 10% heat-inactivated human serum, 0.5% BSA, and 0.5% heat-inactivated FBS). Following this, cells were stained with the primary CAR T antibody for one hour on ice, then excess antibody was removed with the addition of 4% bovine serum albumin followed by centrifugation. Cells were then resuspended in antibody cocktail with anti-CD3, anti-CD4, anti-CD8, and secondary CAR T cell antibody for 30 minutes at 4 °C. Following incubation, cells were washed with the addition of 1% BSA and resuspended for flow cytometry acquisition. All analyses were performed on an Agilent NovoCyte Quanteon.

Characterization of CAR T cells and cytokine production

CAR T cells were analyzed before addition into the T cell cytolytic assay and at 40 and 88 hours after addition. Cells were stained with live/dead AViD stain followed by incubation of cells with Fc block buffer (D-PBS containing 10% heat-inactivated human serum, 0.5% BSA, and 0.5% heat-inactivated FBS). Excess stain was removed with the addition of PBS and the solution was centrifuged for five minutes at 300 g. The antibodies used are specified in Table 1, which provides information on antibody clone and fluorophore used. Cells were stained with the antibody cocktail for 30 minutes on ice. Following antibody incubation, cells were washed with the addition of 1% BSA and resuspended for flow cytometric analysis. FMO controls

were made by staining a sample with all antibodies except the one in which background was to be assessed.

Cytokine production bead-based multiplex assay

50 µL of cell supernatant was collected 24 hours after addition of T cells into the T cell cytolytic assay. IL-2, 4, 6, 10, 17A, IFN-γ, TNF-α, soluble Fas, soluble FasL, Granzyme A, Granzyme B, Perforin, and Granulysin in the cell supernatant were detected following the kit manufacturer's instructions, Human CD8/NK Panel, Biolegend, catalog number 740267.

CAR T cell cytolytic assay

The CAR T cell cytolytic assay was monitored on the Agilent xCELLigence MP, impedance measurements were taken every 15 minutes. The same growth medium and conditions were used as described for the target cells. After measuring background impedance using 50 µL of media/well, 10,000 target cells, in 100 µL of media, were added to each well. Proliferation was monitored for 23 hours, then 50 µL of media was removed and replaced with 50 µL of CD19 CAR T cells. Total numbers of T cells added differed to achieve E:T ratios of 0.06, 0.12, 0.25, 0.5, 1, 2, or 4. When using the impedance data, % cytolysis = [1 – Normalized $CI_{treatment}$/Normalized CI_{target} only] × 100

Results and discussion

Determination of T cell CAR expression and T cell state

Assessment of CAR expression and CAR T-cell phenotype is essential during CAR T discovery and for quality control during manufacturing. A CAR construct that targets CD19, a receptor expressed on B cell lymphoma cells was generated and used to transduce peripheral blood mononuclear cells (PBMCs). After generation, cells were expanded *ex vivo* and examined for the expression of CAR (Figure 1). *Ex vivo* expansion of PBMCs with anti-CD3 and anti-CD28 antibodies activates the T cell receptor and exclusively expands T cell populations. After nine days of culture, over 99% of live cells were CD3+ demonstrating that T cells were specifically expanded, and only small numbers of other cells were present. Approximately 50% of the total cells expressed chimeric antigen receptor (CAR), specifically 50% of CD4+ T cells and 46% of CD8 T cells, demonstrating successful stable transduction of the CAR construct.

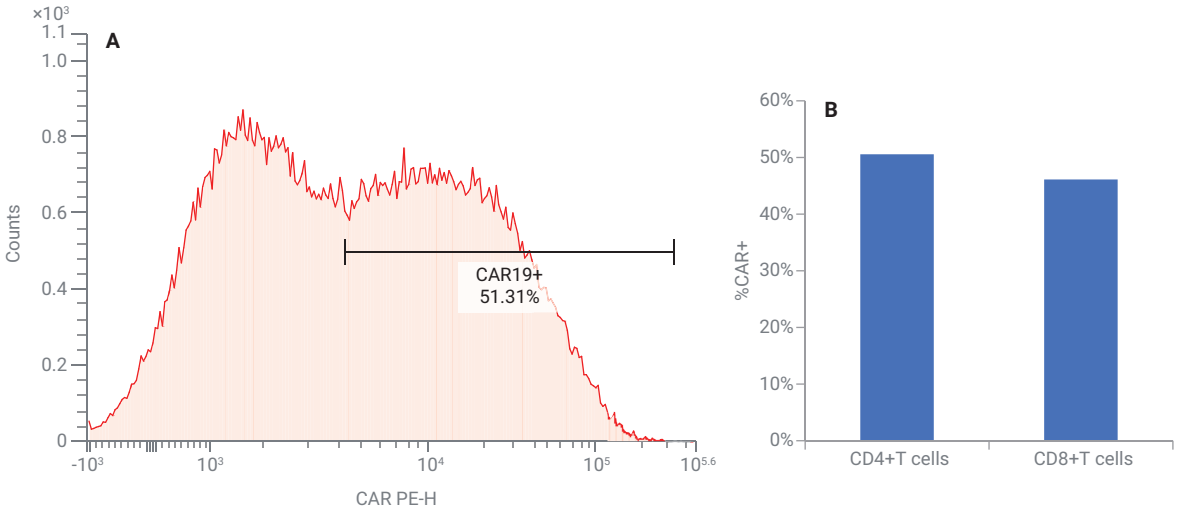

Figure 1. CAR expression and T cell phenotype of CART19 cells. CART19 cells were prepared by a collaborator for commercial use. Human PBMCs were transduced with a CD19 CAR construct or a mock vector and CAR19 expression was evaluated on T cell populations by flow cytometry. CAR T cells were stained with live/dead AViD stain, anti-CD3 FITC, anti-CD4 PE-Cy5, anti-CD8 PE-Cy7, anti-CD19 APC, anti-CD16 APC-Cy7, and anti-CD56 BV605 antibodies. The final CAR expression in total live cells was evaluated by staining with CD19 antigen with a FC tag and anti-FC PE antibody (A). Expression of CART19 in CD4+ and CD8+ subpopulations (B).

To further characterize the CART19 cells, a 12-color immunophenotyping flow cytometry panel was devised (Table 1) to examine T cell differentiation, activation, and exhaustion markers and provide insight into the status of CAR T cells. In this immunotherapy panel, after removal of dead cells and debris, T cells are identified by CD3 and divided into CD4 and CD8 T cell subsets (Figure 2A). The following differentiation states of T cells were identified using the expression patterns of CD45RA and CCR7; naïve-like (CCR7+CD45RA+), effector (CCR7-CD45RA+), effector memory (CCR7-CD45RA–), and central memory (CCR7+CD45RA–) T population. The naïve-like T cell population can be

Table 1. A 12-color flow cytometry panel was developed for analysis of differentiation, activation, and exhaustion of CART19 cells.

	Fluorochrome	Clone	Description
Dead cells	Aqua	AViD	Dead cells
CD3	BV570	UCHT1	T lineage
CD4	BV785	OKT4	
CD8	FITC	SK1	
CD45RA	BV650	HI100	Differentiation
CCR7	PE-Cy7	G043H7	
CD95	PE-Dazzle594	DX2	
CD25	BV421	M-A251	Activation
CD127	PE-Cy5	A019D5	
PD-1	APC	EH12.2H7	Exhaustion
TIM-3	PE	F38-2E2	
LAG-3	BV605	11C3C65	

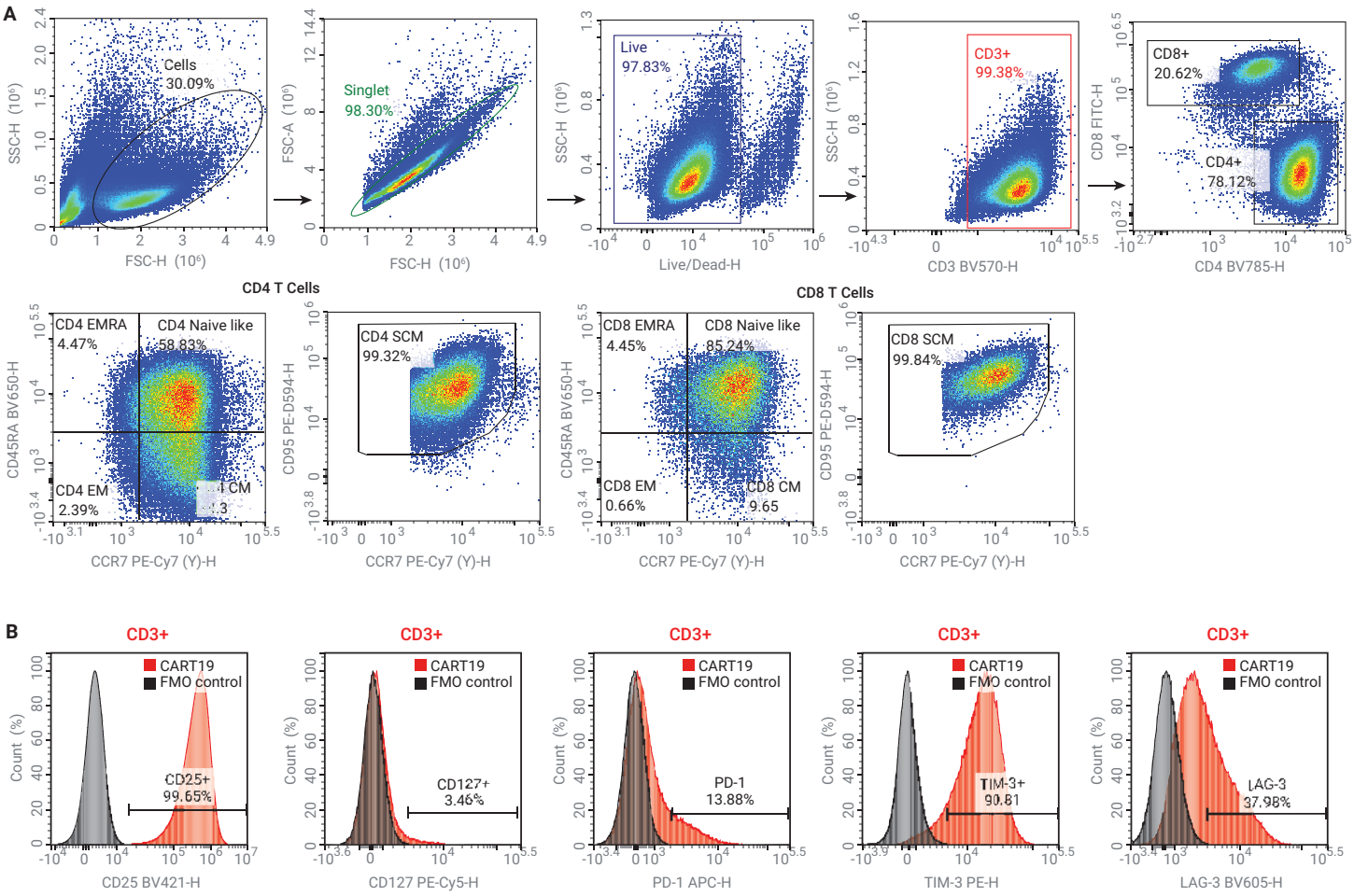

Figure 2. CART19 cell immunophenotyping before T cell cytolytic assay. CART19 cells stained with the 12-color immunophenotyping panel described in Table 1 (A). FMO controls are shown for CD25 BV421, CD127 PE-Cy5, PD-1 APC, TIM-3 PE, and LAG-3 BV605 (B).

further separated into stem cell-like memory T cells (CD95+) and true naïve T cells (CD95–). CAR T cells before addition to the cytolytic assay consisted of ~20% CD8 T cells and ~80% CD4 T cells. A substantial proportion of the cells were CCR7+CD45RA– central memory T cells (34% CD4+ cells, 9.38% CD8+ cells) and CCR7+CD45RA+CD95+ stem cell-like T cells (58% CD4+ cells and 85% CD8+ cells), demonstrating that most CAR T cells derived from PBMCs develop a memory-like phenotype in response to CD3/CD28 stimulation *in vitro*. Development of a memory like phenotype after *in vitro* expression has been demonstrated previously to be beneficial for CAR T cell persistence *in vivo*. Expression of T cell activation markers CD25 and CD127 as well as presence of co-inhibitory receptors PD-1, TIM-3, and LAG-3 provides an in-depth analysis of T cell status. After T cell activation and expansion, the CART19 cells express high levels of CD25 and express co-inhibitory receptors (exhaustion markers) PD-1, TIM-3, and LAG-3. While normally upregulated after T cell activation, sustained high expression of these co-inhibitory receptors can inhibit T cell cytolytic function. For efficient cancer immunotherapy, it is important to prevent CAR T cell exhaustion. Therefore, monitoring the expression of activation and exhaustion markers on novel CAR T candidates is essential for the development of new adoptive cell therapies. An in-depth investigation of CAR T cell candidates can be achieved, through the assessment of T cell phenotype and activation status, combined with functional assays, such as T cell cytolytic activity and cytokine production.

CD19 CAR T cell cytolytic activity measured by xCELLigence RTCA

The xCELLigence RTCA system uses a microplate with biosensors in the base of the wells to monitor cellular interaction with the plate surface. This cellular interaction is used as a measurement of cell concentration, adhesion, and morphology. The cell signal is recorded continuously throughout the assay at set intervals and represented as Cell Index, providing real-time analysis of the cells. Lymphocytes do not adhere to the bottom of the well and do not generate signal, therefore, in this assay, the Cell Index measurement is exclusively from the target cancer cells. As CAR T cells lyse target cancer cells, a decrease in the cell index is observed.

The cytolytic capacity of CART19 cells was measured using a T cell cytolytic assay. CART19 cells were cultured with CD19 expressing HEK-293 cells while real time measurements of T cell mediated cytolysis were taken with the xCELLigence instrument. One day before the addition of CART19 cells, HEK293 cells that ectopically expressed CD19 or controls were seeded. After the addition of CART19 cells, rapid cytolysis of HEK-293-CD19 cells occurs (Figures 3B to 3D) reaching more than 95% cytolysis within 24 hours post-CART19 addition (Figure 3C). There was almost no cytolysis from nonspecific CAR T cells, and it was only observed at very late time points of the coculture of effector and target cells (Figure 3D). The CART19 response was also dose-dependent, increased E:T ratios resulted in more rapid and a higher total percentage of cytolysis of target cells than lower E:T ratios. T cell cytolysis assays performed using xCELLigence RTCA allow rapid real-time assessment of CAR T function.

Upregulation of CD19 CAR T cell cytokine and cytolytic protein production

When CAR T cells are activated by the corresponding target cells, they release large amounts of cytokines important for T cell cytolytic activity and immune cell activation. CAR T adoptive transfer *in vivo* is marked by rapid elevation of several cytokines in serum including tumor necrosis factor-alpha (TNF-α), interferon γ (IFN-γ), interleukin 6 (IL-6), and interleukin-10 (IL-10). In addition to aiding in the immune cell response, an overabundance of cytokines can lead to cytokine release syndrome. It may be beneficial to monitor cytokine production in an *in vitro* model to better predict T cell efficacy and screen for an overexuberant cytokine response. Further characterization was performed by quantifying the amount of cytokine produced by CART19 while killing target cells. Cytokine levels were measured in the supernatant of CART19-HEK-293-CD19 cultures using a bead-based multiplex flow cytometric assay that measures multiple cytokines simultaneously. Higher E:T ratios of CART19 cells resulted in increased expression of cytokines IFNγ, TNFα, IL-2, and IL-10 as well as cytolytic proteins granzyme A, granzyme B, granulysin, perforin, and sFasL. Almost no cytokine production was observed from CART19 cultured with HEK-293 demonstrating that increased secretion of cytokines and cytolytic proteins was induced by CAR-dependent signaling.

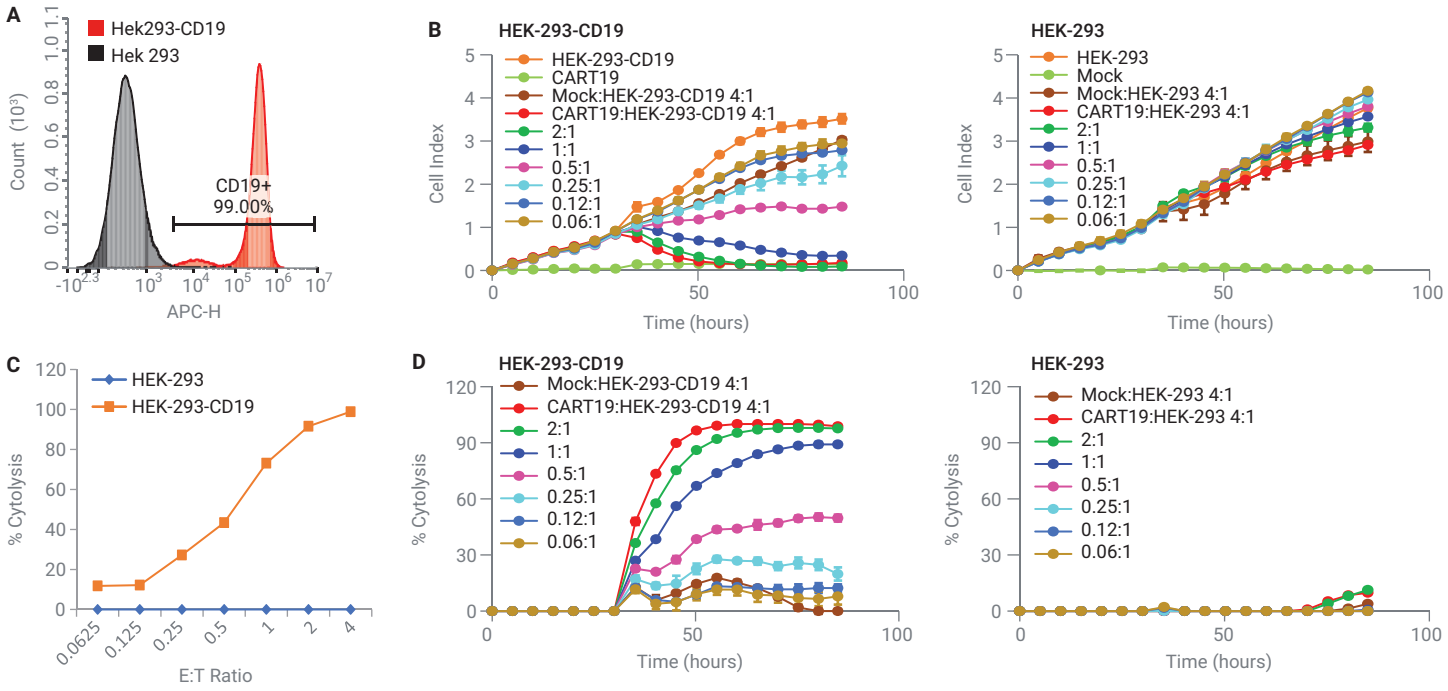

Figure 3. Evaluation of cytolytic activity of CART19 by the Agilent xCELLigence RTCA system. CD19 expression was evaluated on HEK-293 and HEK-293-CD19 cell lines by surface stain with anti-CD19 APC antibody (A). 10,000 HEK-293-CD19 and HEK293 cell were seeded in a 96-well E-Plate, and Cell Index (CI) measurements were taken every 15 minutes using an xCELLigence MP system. (B) After 24 hours, CART19 effector cells (left plot) or mock CAR T (right plot) were added at various E:T ratios ranging from 2:1 to 0.06:1. (D) The Cell Index plots are converted to % cytolysis by the xCELLigence Immunotherapy software. % Cytolysis was measured at 24 hours after CART19 addition with HEK-293 or HEK293-CD19 target cells (C).

Characterization of CD19 CAR T cell activation upon antigen stimulation

CAR T cells were also assessed at the end of the T cell cytolytic assay using the same 12-color immunophenotyping panel used to assess the CAR T cells before the cytolytic assay. This enabled the detection of any changes in the expression of T cell activation and exhaustion markers. Effector CART19 cells displayed a more activated T cell state at both 40 and 88 hours after T cell addition compared to control cocultures. CART19 cells that were cultured with CD19-HEK-293 cells showed increased FSC, higher expression of CD25, PD-1, TIM-3, and LAG-3 and downregulation of CD127. Combining CART 19 cytolysis measurements with flow cytometric assessment of T cell status and cytokine production may provide a comprehensive understanding of underlying mechanistic aspects of CAR T cell mediated killing.

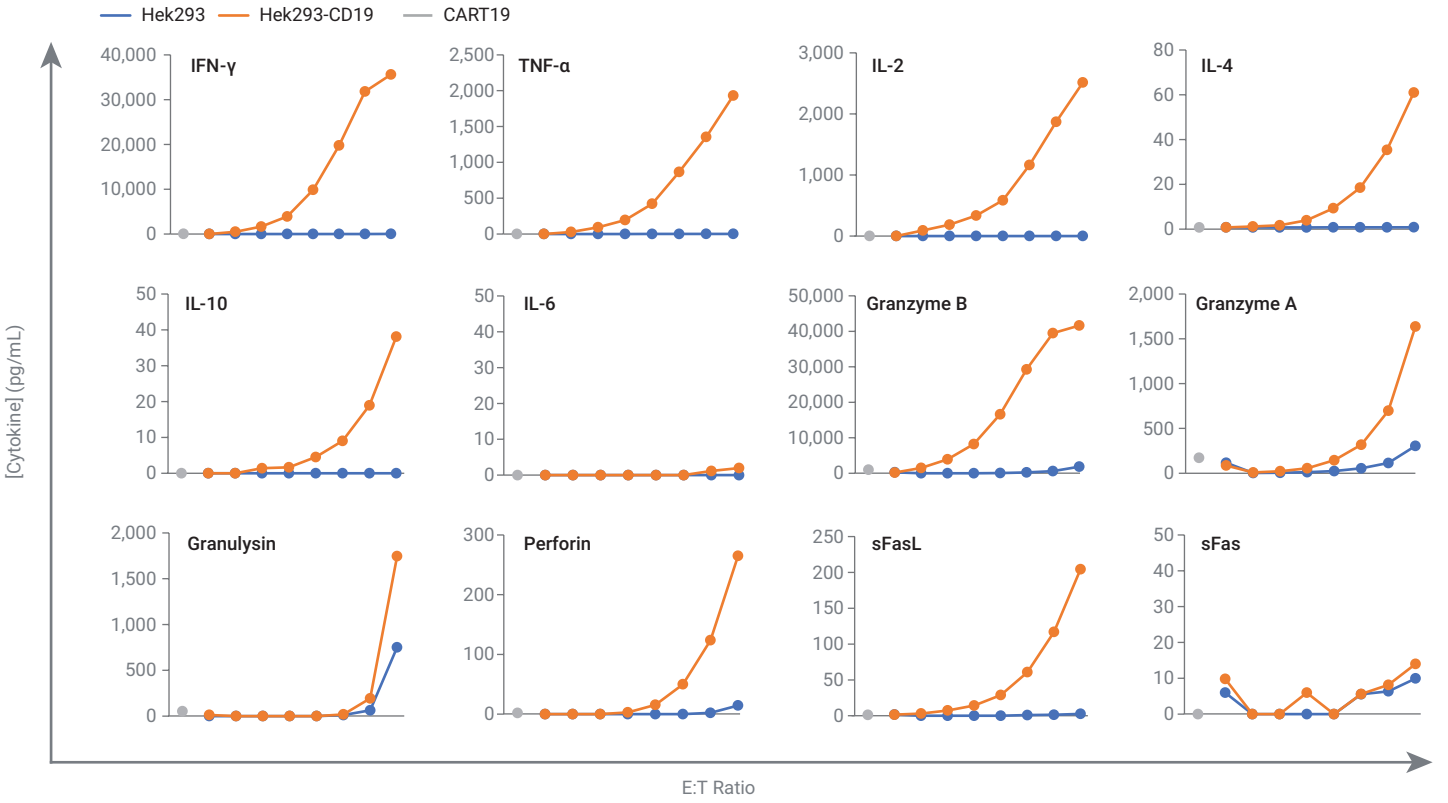

Figure 4. Cytokine production by CART19 after cocultured with HEK-293-CD19. CART19 cells were added at different E:T ratios 24 hours after HEK-293-CD19 or HEK-293 cells seeding. Supernatant was collected 18 hours later and the cytokine concentration was measured on the Agilent NovoCyte Quanteon flow cytometer using BioLegend LEGENDplex Human CD8/NK panel.

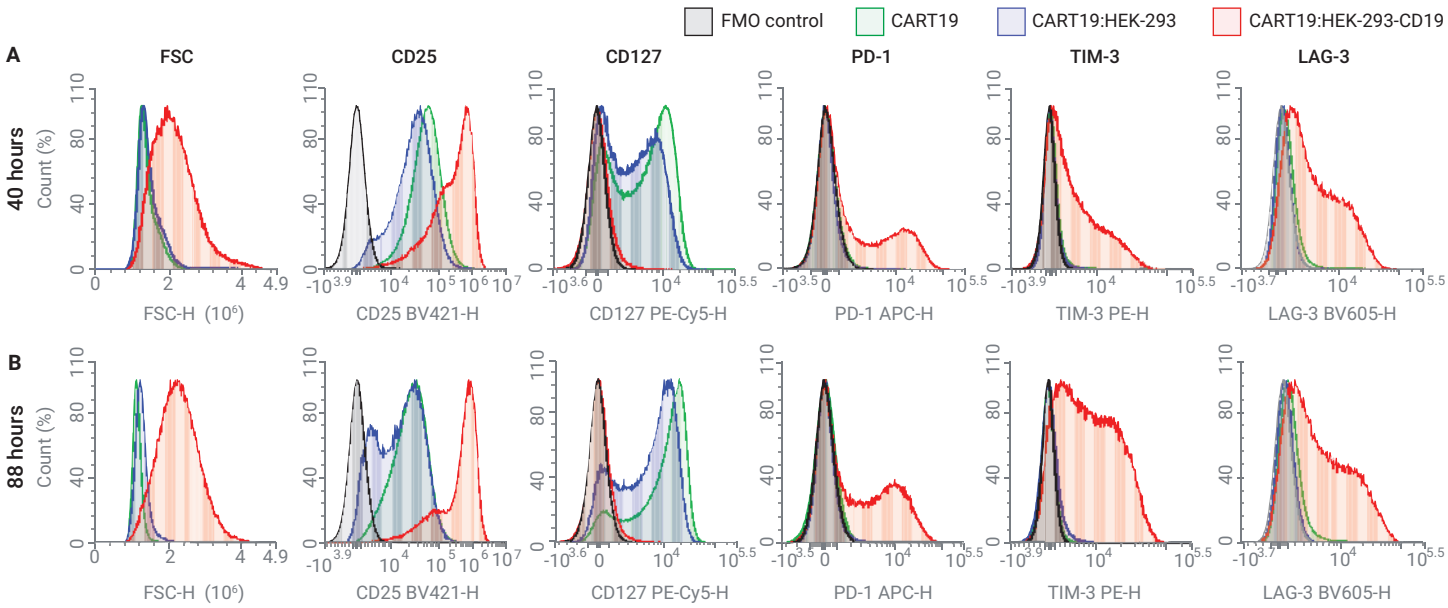

Figure 5. Characterization of CART19 cells after coculture with HEK-293-CD19. Cells were collected from CART and HEK-293 or HEK-293-CD19 cocultures after 40 hours (A) and 88 hours (B) at a 1:1 Effector:Target ratio. Cells were stained with the 12-color immunophenotyping panel described in Table 1. Expression of activation and exhaustion markers were compared.

Conclusion

The therapeutic potential of CAR T cell therapies has driven the development of these treatments, requiring rapid analysis of novel CAR T cell candidates. The combined Agilent xCELLigence RTCA and NovoCyte Quanteon flow cytometer cell analysis workflow provides researchers comprehensive and convenient results of cytolytic potency, cytokine secretion, CAR T characterization, and impurity analysis.

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RA.6848263889

Printed in the USA, October 27, 2020
5994-2377EN

Technical Overview

Cell Analysis

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Trusted Answers

Real-Time Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) Assays

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Introduction

Similar to T cells, natural killer (NK) cells secrete perforin and granzyme to induce target cell death. However, unlike T cells, NK cells do not express an antigen-specific receptor to guide them in target cell selection. During antibody-dependent cell-mediated cytotoxicity (ADCC) the potent killing capacity of NK cells is endowed with specificity via an exogenous antibody. While the variable region of an antibody is bound to a target cell antigen, its constant region can simultaneously be bound by the Fc receptor (CD16) of an NK cell. This tethering of the two cells in close proximity activates the NK cell to effect target cell killing.

Because of the generic antibody binding capacity of CD16, NK cells can be directed to attack an essentially unlimited array of targets, and ADCC is accordingly being pursued as a means to combat diverse types of cancer[1] and infectious diseases.[2,3] In contrast to other emerging immunotherapy strategies such as CAR T cells, a major advantage of ADCC is that the NK cells do not need to be removed from the patient and genetically engineered. To bring therapeutic ADCC to fruition, major efforts are being made to identify appropriate antigen targets, improve the affinity and specificity of antibodies for antigen targets, and improve the affinity of antibodies' constant region for the CD16 receptor (through amino acid substitutions and glycoengineering).[4] As an *in vitro* tool to assess the impact that these modifications have on target cell killing, release assays have traditionally been the gold standard.

As a significant improvement over release assays, this technical overview demonstrates a real-time ADCC assay run on the xCELLigence RTCA eSight. Within eSight's 96 well microplates, gold biosensors continuously track the health and behavior of target cells (cell number, cell size, cell-substrate attachment strength, and cell-cell adhesion) as they are exposed to effector cells and antibodies. Concurrently, eSight collects live cell images in brightfield and fluorescent (red, green, and blue) channels – providing an orthogonal perspective on the killing process. Whereas traditional release assays require significant hands-on processing

steps and only produce endpoint data, the eSight assay requires only the addition of cells + antibody and captures the full continuum of the killing process.

Using a combination of Trastuzumab and PBMCs as a model system, this study demonstrates differences in the impedance- and image-based data for HER2-negative versus HER2-positive target cells. The Trastuzumab concentration is subsequently titrated to produce dose-response curves that enable quantitative evaluation of efficacy using EC_{50} values.

Results and discussion

Tracking ADCC using imaging and impedance

As a first step towards establishing an ADCC assay on eSight, 10,000 target cells were seeded into E-Plate wells and allowed to attach and proliferate for 20 hours before being treated with Trastuzumab (2.5 µg/mL), PBMCs (E:T = 2:1), or a combination of Trastuzumab (2.5 µg/mL) + PBMCs (E:T = 2:1). Representative photos, taken 48 hours post treatment, are shown in Figure 1. Based on visual inspection, none of the treatment conditions were able to induce killing of the HER2-negative MDA-MB-231 cells within this time window. SKBR3 cells, which are HER2-positive, appear to be unaffected by Trastuzumab alone, display cytoplasmic shrinkage in the presence of PBMCs alone, and exhibit both cytoplasmic shrinkage and reduced cell numbers when exposed to a combination of Trastuzumab and PBMCs (Figure 1).

While the photos in Figure 1 are indicative of HER2-specific ADCC activity, this assessment is inherently qualitative. To quantitatively compare ADCC activity across the different cell lines and treatments, eSight's software was used to count the number of red target cell nuclei over time. Figure 2A demonstrates that eSight's segmentation mask (yellow outline) accurately identifies red target cell (MDA-MB-231) nuclei while appropriately excluding the unlabeled PBMCs. Plotting the number of these red nuclei over time clearly illustrates that these HER2-negative cells proliferate in a manner that is minimally perturbed by all treatments examined (Figure 2B). Using this same image-based approach to analyze the HER2-positive SKBR3 cells shows a very different situation (Figures 2C and 2D). While Trastuzumab alone has negligible impact on SKBR3 growth, treating these cells with PBMCs alone causes a ~30% reduction in the number of target cells by the 70-hour time point (Figure 2D). Importantly, using Trastuzumab in combination with PBMCs increases the killing efficacy markedly above that of PBMCs alone (Figure 2D). The above results are consistent with literature reports that

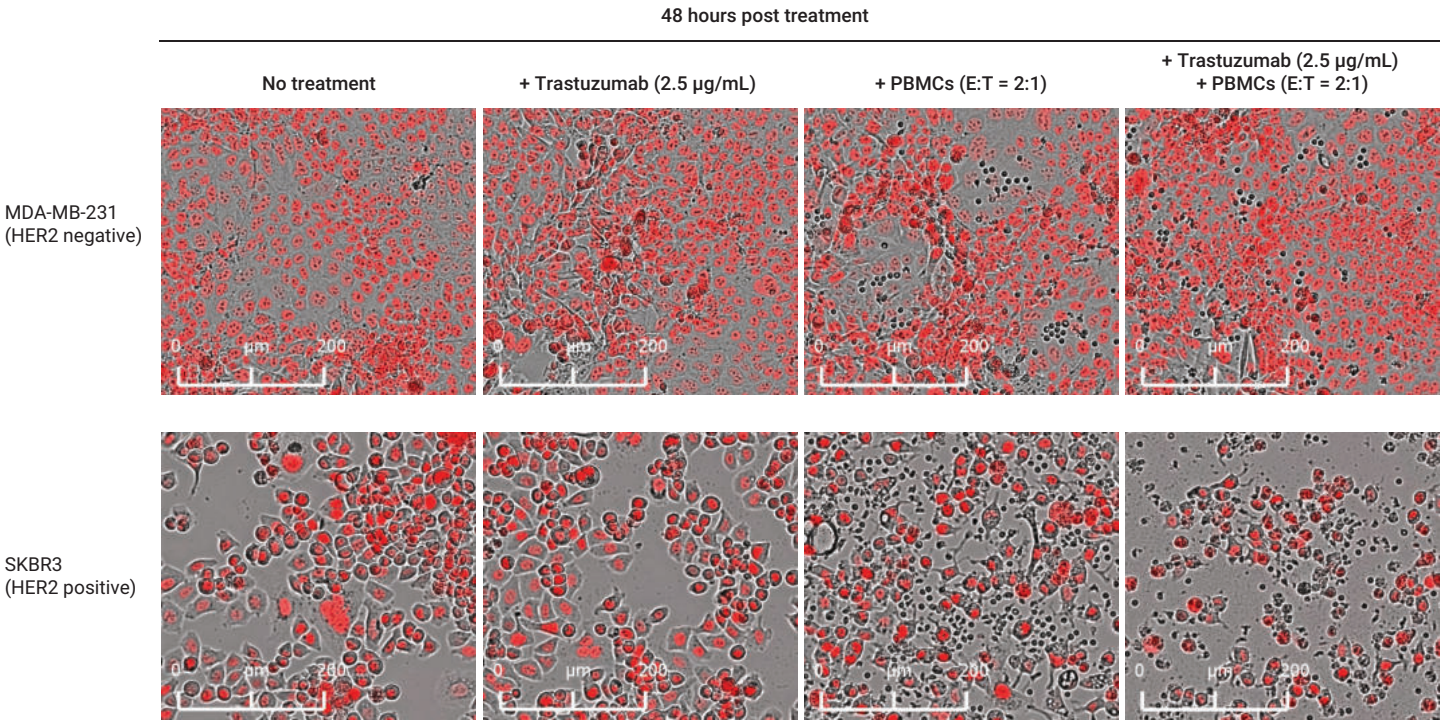

Figure 1. Image-based assessment of ADCC activity 48 hours post treatment. While target cells express nuclear-localized RFP, PBMCs are unlabeled. Scale bars = 200 µm.

Trastuzumab's ability to stimulate ADCC is dependent upon HER2 being expressed on the target cell's surface.[5]

As a means of corroborating the above image-based analyses, impedance data were collected simultaneously in the same wells of the E-Plate. Figure 2E confirms that none of the treatment conditions examined here has an impact on MDA-MB-231 growth over the 70 hour time course. When the HER2-positive SKBR3 cells are examined by impedance (Figure 2F), they also display killing trends that are consistent with those observed by imaging (Figure 2D). There is, however, one notable exception. Immediately after adding PBMCs there is a rapid and transient spike in impedance (Figure 2F, blue trace). The magnitude of this spike is even larger when PBMCs are added in combination with Trastuzumab (pink trace). To understand what is driving this behavior, images from time points spanning this impedance peak were analyzed. These photos (not shown here) demonstrated a perfect correlation between the surface area of the SKBR3 cells' cytoplasm and the magnitude of the impedance signal. This rapid expansion-contraction cycle is readily detected by impedance, but is impossible to detect by imaging when using the nuclear labeling scheme that was used in this study; even though the cytoplasm displays dramatic changes in surface area, the size of the red nucleus is unchanged (data not shown). If tracking these morphology changes in real time were deemed to be important, this could be accomplished simply by labeling the cytoplasm instead of the nucleus.

The fact that PBMCs induce a rapid and transient impedance spike in SKBR3 cells but not in MDA-MB-231 cells indicates that this is not a universal phenomenon. Indeed, when other target cells were tested under similar ADCC assay conditions, this impedance spike was not observed (data not shown). The magnitude and shape of impedance curves in ADCC assays are unique to each target cell line, antibody concentration, and E:T ratio, as shown in the next section.

Quantifying ADCC efficacy

With the goal of demonstrating that the eSight ADCC assay is applicable across broad target cell types, the Her2-positive ovarian cancer cell line SKOV3 was next used to study the impact of Trastuzumab concentration on killing efficacy when the E:T ratio was fixed at 10:1. Photos taken 48 hours post treatment qualitatively demonstrate the gains in killing efficacy that result from higher antibody concentrations (Figure 3A). Plots of the number of red target cells over time (Figure 3B) can readily be converted to plots of % cytolysis (Figure 3C). Plotting the area under these % cytolysis curves as a function of antibody concentration yields the dose response curve in Figure 3D. The same types of analysis plots were also generated using impedance data in Figures 3E to 3G. Note that the EC_{50} values determined by these two orthogonal approaches are in close agreement with one another (1.13 versus 2.06 ng/mL for imaging and impedance, respectively), which is typical in eSight assays.

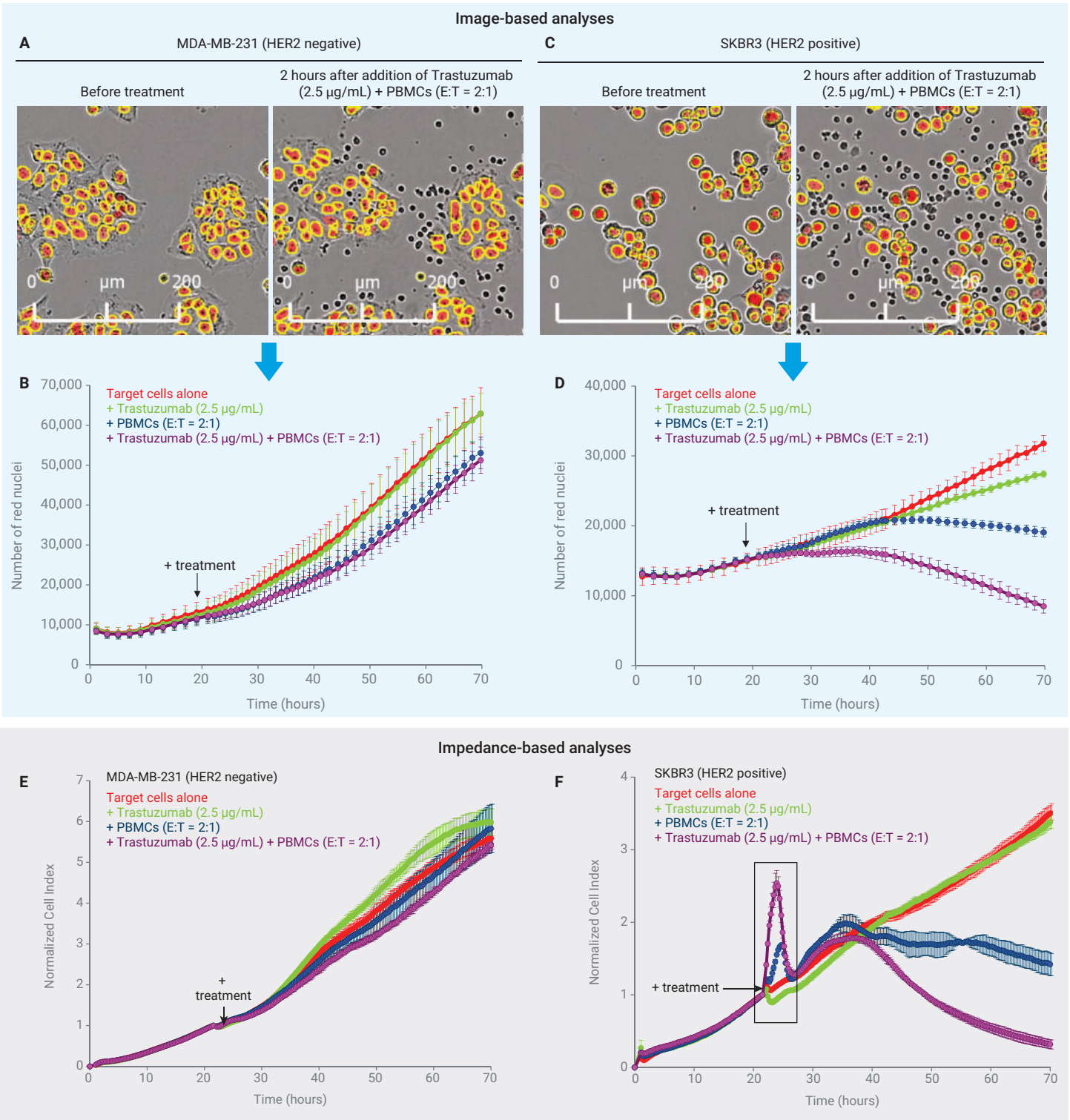

Figure 2. Quantification of ADCC activity using imaging and impedance. (A) For HER2-negative MDA-MB-231 cells, eSight's segmentation mask (yellow outline) accurately recognizes red target cell nuclei and appropriately excludes unlabeled PBMCs. (B) Plot of red MDA-MB-231 nuclei as a function of time. The different treatments were added at the 20 hour time point. Error bars reflect the standard deviation for triplicate wells. (C and D) Similar to panels A and B but for HER2-positive SKBR3 cells. (E) Real-time impedance traces for MDA-MB-231 cells. (F) Real-time impedance traces for SKBR3 cells. The black box encircles a rapid and transient increase in impedance that takes places immediately after the addition of PBMCs or PBMCs + Trastuzumab.

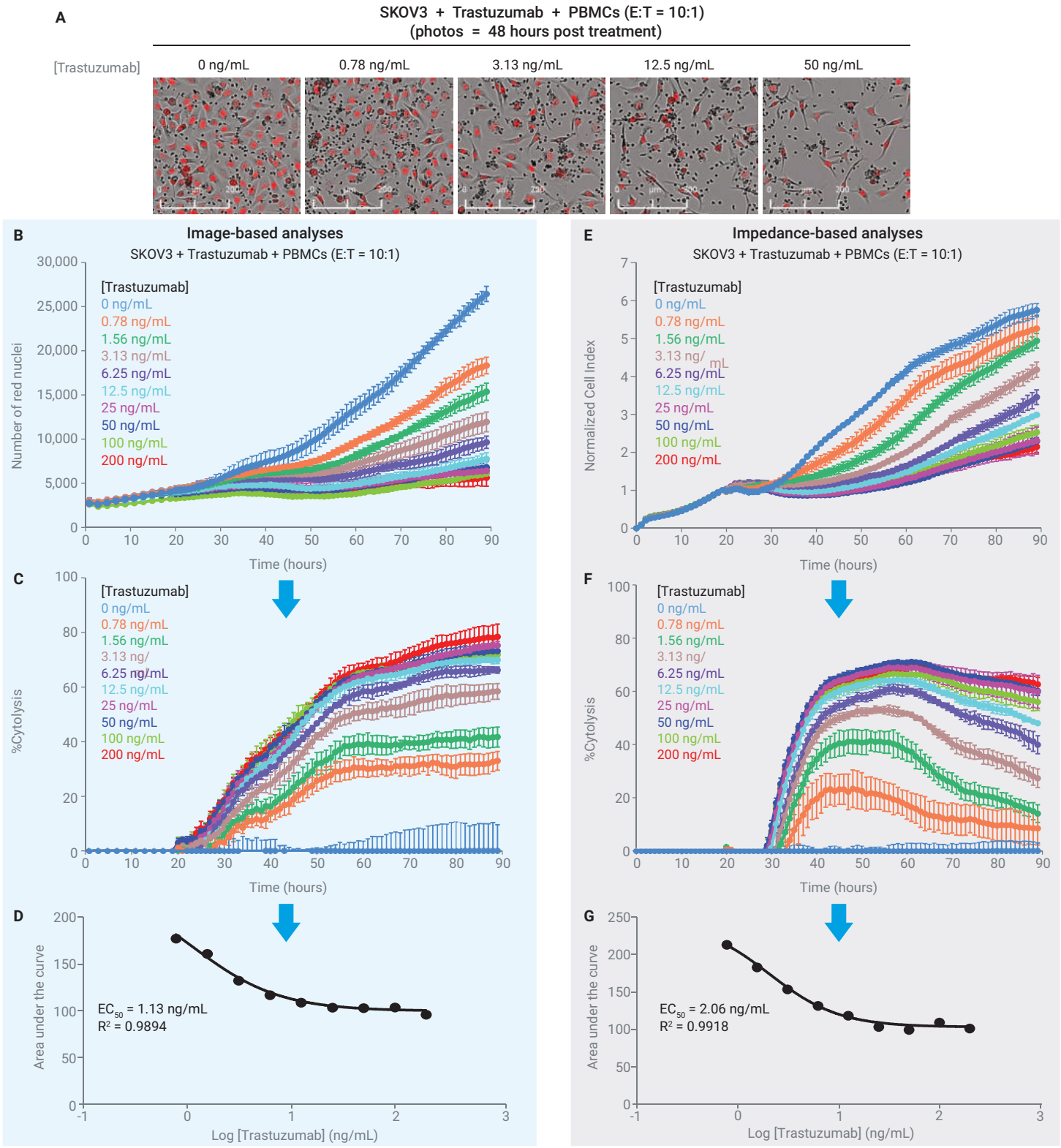

Figure 3. Titration of Trastuzumab concentration at constant PBMC density. (A) While leaving the PBMC density constant (E:T = 10:1), increasing the Trastuzumab concentration causes a clear increase in the extent of target cell (SKOV3) killing. (B) Tracking ADCC activity by plotting the number of red SKOV3 nuclei over time. (C) The data from panel B were converted to real-time % cytolysis plots. (D) Trastuzumab dose-response curve based on the area under the % cytolysis plots in panel C. (E to G) Similar to panels B, C, and D, except using impedance to track ADCC activity.

Conclusion

Perhaps the most salient feature of the eSight ADCC assay presented here is the minimal hands-on time that it requires. After seeding target cells and subsequently adding effector cells + antibody, the plates require no additional handling/processing steps. Being able to produce primary (impedance) and confirmatory (imaging) data simultaneously using the same population of cells (i.e., in the same well) is another major advantage of this assay. The same approach used here to study ADCC against adherent target cells can also be used for liquid cancers. Agilent has developed kits that use antibodies to tether liquid cancer cells to the bottom of E-Plates (including both the glass surface and the gold biosensors), making the destruction of these cells analyzable by both imaging and impedance.

In summary, eSight's impedance readout serves as an inherently sensitive, simple, and label-free means of screening and characterizing antibodies in ADCC assays. eSight's simultaneous imaging readouts provide confirmation of the impedance results without increasing the amount of hands-on time required to run the assay.

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Printed in the USA, May 26, 2022
5994-4955EN

Technical Overview

Cell Analysis

Agilent

Trusted Answers

Real-Time Potency Assay for CAR T Cell Killing of Adherent Cancer Cells

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Introduction

Harnessing the exquisite potency and molecular specificity of the immune system, and directing these towards cancer cell destruction, immunotherapy in its various forms has permanently changed the landscape of clinical oncology. Among the frontrunners that have helped to usher in this new paradigm are adoptive cell therapies such as chimeric antigen receptor (CAR) T cells. CARs are engineered proteins that consist of an extracellular antigen-binding domain that is linked, via a hinge region and transmembrane domain, to intracellular activation domains that drive T cell activation, proliferation, and target cell killing.[1] A few decades of CAR engineering efforts have made it clear that merely linking these protein domains together, like "beads on a string", is insufficient for generating an effective CAR; subtle differences in how the domains are linked to each other can greatly impact functionality.[1,2] While today's third-generation CARs are improved relative to their predecessors, a universally efficacious CAR architecture has not been identified and efforts to design CARs with novel or refined functionality continue to require empirical optimization on a case-by-case basis.[2]

During CAR optimization, biochemical/biophysical assays can be used to study critical quality attributes such as the antigen affinity of the extracellular domain[3] or the cytokine profile of CAR T cells stimulated by soluble ligands.[4] Although these reductionistic approaches are useful, their results do not necessarily correlate with cancer-killing efficacy within a more physiologically relevant context.[3] To rigorously compare different CAR constructs and identify unwanted behavior such as tonic (antigen-independent) signaling, it is widely acknowledged that CAR function should be evaluated within the complex milieu of the immune synapse, using bona fide cancer cell killing as the readout. For this purpose, release assays (^{51}Cr, LDH) have historically been the gold standard, but suffer from the fact that they require significant "hands-on" time and only provide end-point data. Requiring just target cancer cell seeding and a subsequent CAR T cell addition step, this study used the Agilent xCELLigence RTCA eSight to continuously monitor CAR T cell-mediated killing of cancer cells over the course of multiple days. Providing a direct and objective assessment of target cell number, cell size, and cell-substrate attachment strength, impedance biosensors embedded within the base of eSight microplates quantitatively track the continuum of target cell killing, spanning from early (reduced cell-substrate adhesion strength) to late (lysis) events.

Concurrently, eSight captures live-cell images in brightfield and three fluorescence channels (red, green, and blue), providing an orthogonal readout of the killing process. By combining the strengths of real-time impedance monitoring (simplicity, analytical sensitivity, and objectivity) with that of live-cell imaging (specificity of the readout), eSight increases the information richness of the CAR T cell killing assay without increasing the workload.

Monitoring CAR T cell killing activity by live-cell imaging

When left untreated for 48 hours, the RFP-expressing HEK-293-CD19 cells proliferate to the point of confluence (Figure 1A). However, after 48 hours of exposure to CAR T cells there is a very clear reduction in the number of target cells present. As expected, this killing response is dose-dependent, with the highest E:T ratios causing the most pronounced killing. As the E:T ratio is increased, the unlabeled/grey CAR T cells become more prominent in the field of view, and clustering of these T cells (which is a characteristic of activation) becomes more robust. Note that at late time points these T cell clusters contain a large number of red target cells which, because they display rounding/detachment and cytoplasmic shrinkage, appear to be progressing through apoptosis. Finally, when the assay is repeated using a fixed E:T of

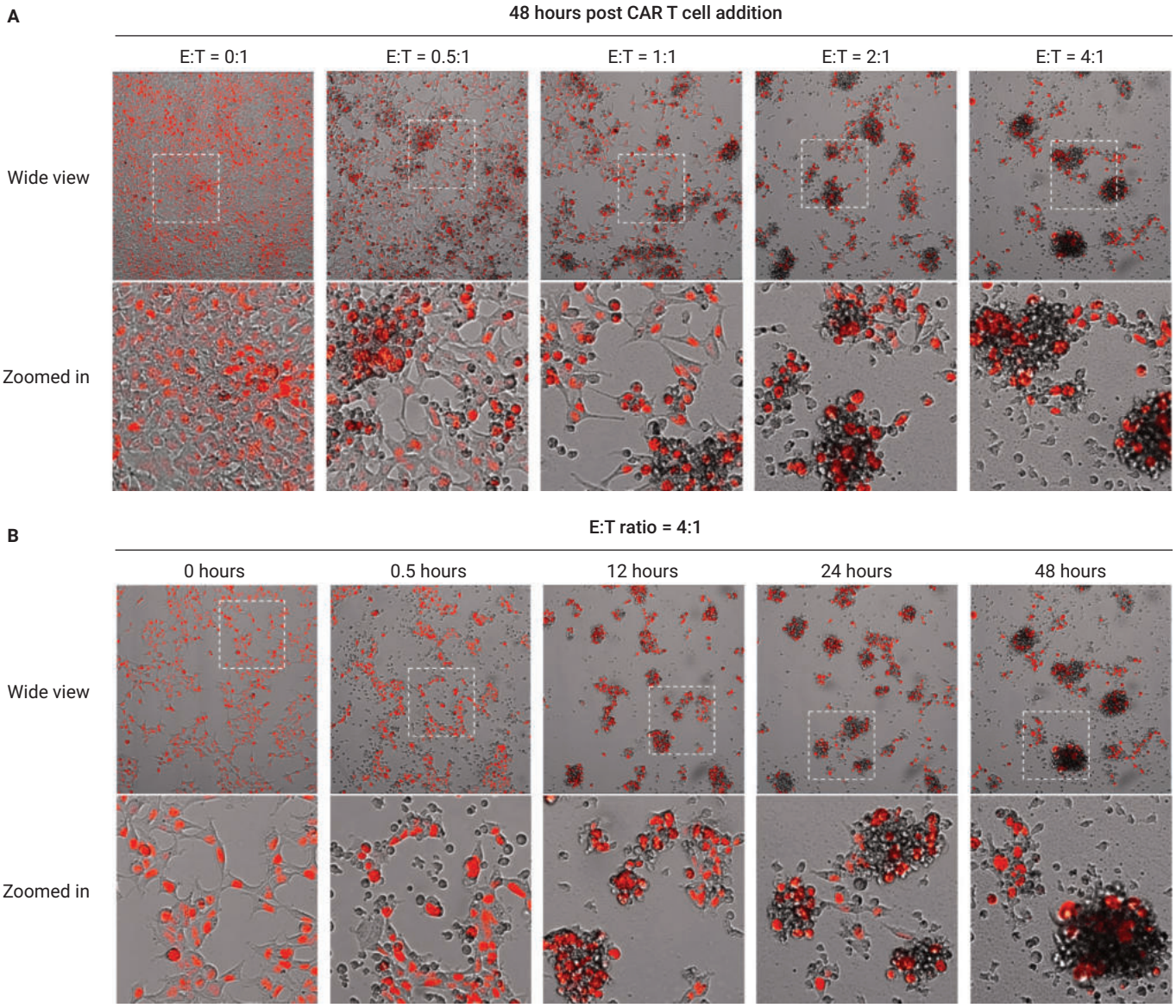

Figure 1. Killing of RFP-expressing HEK-293-CD19 cells by CD19 CAR T cells. (A) Comparison of different E:T ratios 48 hours post CAR T cell addition. The white squares in the upper panels denote the regions that are blown up in the lower panels. The unlabeled CAR T cells are grey. (B) Comparison of different time points for a constant E:T ratio of 4:1.

4:1, the time dependence of the killing response is clearly evident (Figure 1B).

Quantifying CAR T cell killing efficacy

The number of red target cells is plotted as a function of time in Figure 2A. Note that this plot excludes the first 18 hours of target cell proliferation; the zero-hour time point corresponds to the moment that CAR T cells were added to the well. At this time point, each well contains ~15,000 target cells, which is consistent with the published HEK-293 doubling time of 24 to 34 hours and the fact that 10,000 target cells were initially seeded. In the absence of CAR T cells, the target cells continue proliferating until the 50-hour time point (Figure 2A, black data trace). When CAR T cells are added at the very low E:T of 0.06:1, a killing response is not observed until the 30-hour time point (Figure 2A, orange data trace). Progressively increasing the E:T ratio causes the killing response to manifest at earlier time points and utlimately results in a greater number of target cells being destroyed.

Tracking the killing response using impedance (Figure 2C) produces cytolysis curves that are largely similar to the image-based curves in terms of their time- and dose-dependency. One salient difference between the two readouts is the fact that at E:T = 4:1, the impedance response drops to zero, whereas the number of target cells never drops below ~5,000. This persistence of target cells even at late time points is consistent with the photos shown in Figure 1. That the impedance signal concurrently falls to zero suggests that these lingering target cells are no longer strongly adhered to the well bottom.

Consistent with this, nearly all of the red cells that are visible after 48 hours of exposure to CAR T cells at E:T = 4:1 are rounded and appear to be loosely resting on the well bottom (Figure 1).

Using the simple equations shown in the materials and methods section, both the impedance data and the image-based data were converted into % cytolysis plots (Figures 2B and D).

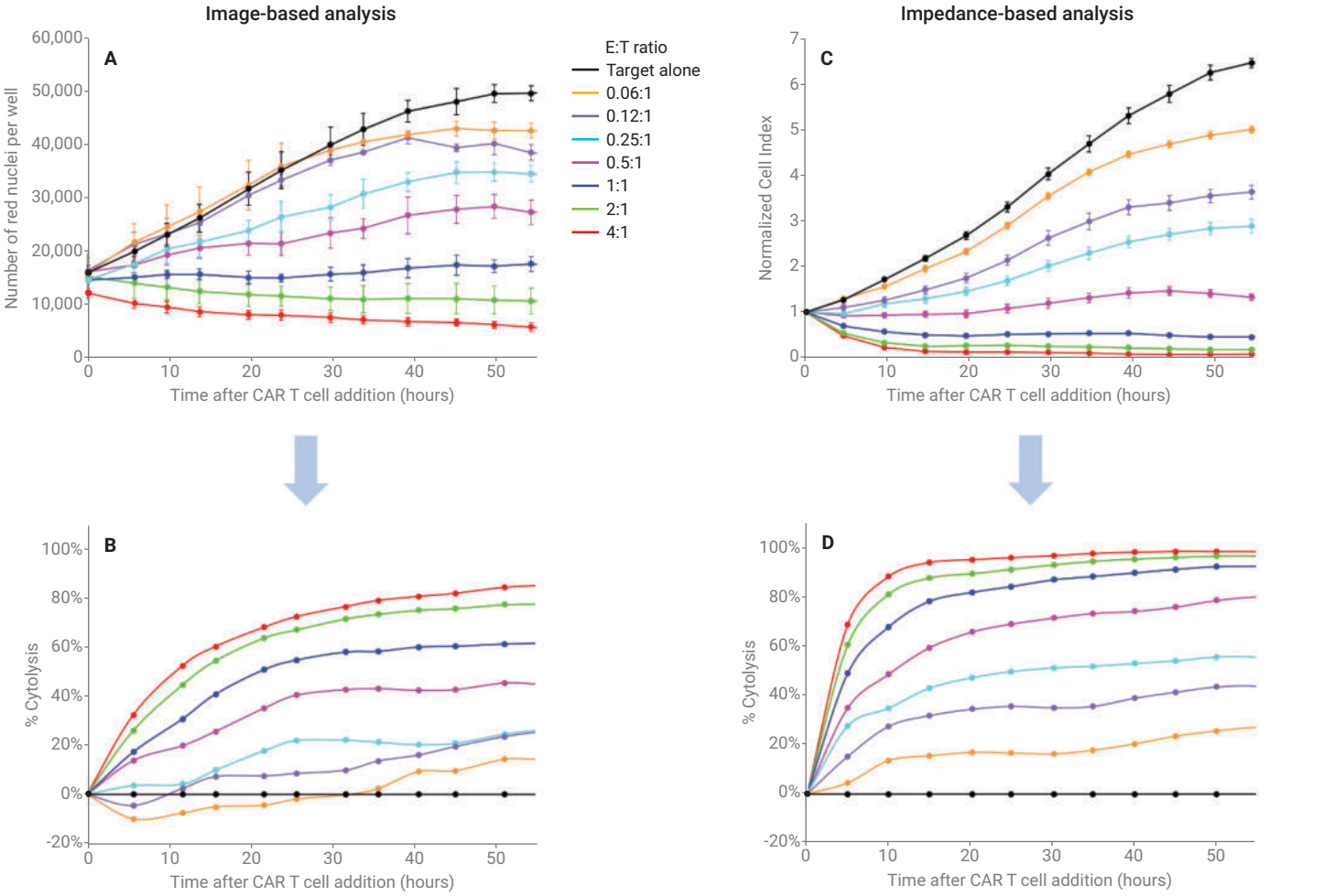

Figure 2. Time courses for HEK-293-CD19 killing by CD19 CAR T cells as measured by imaging (A,B) and impedance (C,D). While upper panels display the primary data, lower panels display % cytolysis, calculated as described in the materials and methods section. Assays were run in duplicate; error bars represent standard deviation.

When plotted this way, the kinetics of the killing responses are strikingly different between the two data sets. Even though both the impedance and imaging data were collected from the exact same population of cells (i.e., cells in the same well), for a given time point, the impedance signal universally gives a higher % cytolysis than the image-based readout.

Discussion

Beyond the benefit of having two independent measurement techniques, it is important to note the objectivity of the impedance readout, which is reported directly, without any processing or input from the user. Conversely, for eSight and all other imaging-based instruments, the raw image files get converted to outputs (such as number of red target cell nuclei) by user-informed algorithms where the expected size range, eccentricity, and brightness of cells must be defined. Although potential problems associated with this approach, such as interuser variability, can be minimized through proper training and consistent usage of the same segmentation parameters, having impedance as an objective comparator helps build confidence in the assay's results.

Although not used in this study, eSight can image cells in three different fluorescent channels. In addition to labeling target and effector cells with different colors, the third channel can be used to track cell death in general or apoptosis specifically through the use of annexin V- or caspase 3-specific reagents.

Conclusion

The xCELLigence RTCA eSight couples the simplicity, analytical sensitivity, and objectivity of real-time impedance monitoring with the highly specific readout of live-cell imaging to characterize CAR T cell killing efficacy with unparalleled ease and information richness.

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RA44334.6271643518

Printed in the USA, June 4, 2021
5994-3555EN

Application Note

Cell Analysis

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Trusted Answers

Tumor Cell Killing by T Cells

Quantifying the impact of a CD19-BiTE using real-time cell analysis, flow cytometry, and multiplex immunoassay

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Abstract

The ability to quantitatively correlate T cell biomarker expression or secretion with target cell killing is critical in tumor immunology studies. This application note demonstrates a workflow using the Agilent xCELLigence real-time cell analysis (RTCA) instrument with an Agilent NovoCyte flow cytometer to study both cytotoxic T lymphocyte (CTL)-mediated destruction of tumor cells and the corresponding secretion of cytokines and cytolytic proteins by CTLs. Bispecific T cell engagers (BiTEs) enhance the ability of CTLs to specifically recognize and eliminate tumors. This enhancement effect was evaluated using three distinct assays. Target cell death was monitored using an Agilent RTCA biosensor assay and a flow cytometry-based cytolysis assay, while secretion of cytokines and cytolytic proteins was quantified in a bead-based multiplex flow cytometry assay.

Introduction

Cancer immunotherapy is increasingly being evaluated as an approach to cancer treatment by harnessing the immune system to attack cancer cells. Both the adaptive and innate arms of the immune system play a pivotal role in a host's defense against tumors. CD8+ CTLs, a major component of the adaptive immune response, directly eliminate tumor cells by releasing cytolytic proteins such as granzymes, perforin, and granulysin, in addition to producing multiple cytokines.

The ability to correlate T cell biomarker expression/secretion with target cell killing is critical in both basic and applied studies of tumor immunology. Moving this research from the bench to the lab is critically important, but reliable tools are needed to translate *in vitro* protocols and results for use *in vivo*. This study uses a combination of biosensor-based technology and flow cytometry to evaluate both the target and effector cells in a T cell-mediated B cell killing assay. The Agilent xCELLigence RTCA biosensor technology provides a continuous readout of target cell viability, while flow cytometry allows simultaneous measurement of specific responses, such as target cell viability and effector cell function.

Representing a promising new class of therapeutics, BiTEs harness the power of the adaptive immune response by enhancing the ability of CTLs to specifically recognize and eliminate tumors. CD19-BiTEs are designed to bind CD3 on CTLs, as well as CD19 on cells of B cell lineage, simultaneously activating T cells and bringing them in close proximity to the B cells. This enhances the CTL effector function against various B cell-derived tumors. The capability of CD19-BiTEs to enhance the cytotoxic effects of T lymphocytes on a B-cancer cell line, Daudi cells, has been evaluated using three distinct assays (Figure 1). Target cell death was monitored using an RTCA biosensor assay and a flow cytometry-based cytolysis assay; while secretion of cytokines and cytolytic proteins were quantified in a bead-based multiplex flow cytometry assay to monitor the T cell response.

CD19-BiTE enhances T cell-mediated target cell killing in an xCELLigence RTCA biosensor assay

To demonstrate T cell-mediated target cell killing on the Agilent xCELLigence, human T cell killing of a B cell lymphoma cell line (Daudi cells) was assessed. Daudi cancer B cells were immobilized on an xCELLigence electronic microplate (Agilent E-Plate) that had been precoated with αCD40 antibody. Interaction between the cells and the gold micro-electrodes leads to increased electrical impedance, the magnitude of which correlates with cell number, size, and cell-substrate attachment quality. At 18 hours after the Daudi cells had been seeded, T cells enriched from primary PBMCs were added at an effector T cell to target Daudi cell ratio of 10:1. To measure if a BiTE can enhance T cell killing, CD19-BiTE or an αCD19 antibody control was also added. The impedance signal of the Daudi cell monolayer was recorded every 15 minutes, and is plotted using the unitless parameter Cell Index.

Uninterrupted growth and attachment of Daudi cells can be seen in wells with only Daudi cells (Figure 2, blue line), while no sustained impedance signal is generated from T cells alone (Figure 2, black line). Daudi cell growth is undisturbed by the addition of T cells and αCD19 antibody as a control (Figure 2, green line). However, a rapid decrease in Cell Index is observed with the addition of the CD19-BiTE, indicating that that Daudi cells are dying (Figure 2, orange line). These data demonstrate the capability of CD19-BiTE to enhance T cell-mediated B killing efficacy.

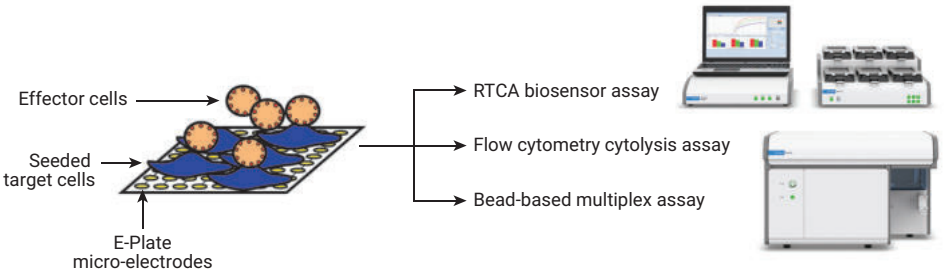


Figure 1. CD19-BiTEs enhance the cytotoxic effects of T lymphocytes on a B-cancer cell line, Daudi cells, using three distinct assays.

CD19-BiTE enhances T cell-mediated target cell killing in a flow cytometry-based cytolysis assay

The role of CD19-BiTE as a powerful facilitator of T cell-mediated killing of Daudi B cells was further confirmed using flow cytometry. Daudi cells were labeled with CFSE and cultured identically to the RTCA experiment described previously. At 48 hours after T cell addition, dead cells were detected with 7-AAD and acquired on the NovoCyte flow cytometer (Figures 3A and 3B). The presence of T cells and the αCD19 antibody did not significantly increase the frequency of Daudi cells (CFSE+) that were dead (7-AAD+) compared to the sample containing only Daudi cells. However, the addition of CD19-BiTE dramatically decreased the proportion of viable Daudi target cells; approximately 80% of Daudi cells were dead. These data correlate closely to the results obtained from RTCA, verifying that T cell-mediated B cell killing can be properly evaluated by flow cytometry or cellular impedance.

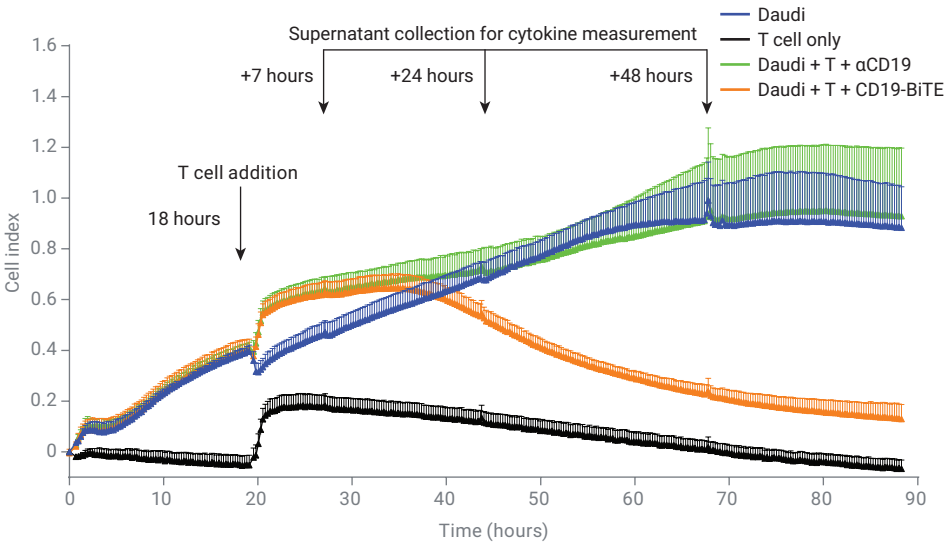


Figure 2. CD19-BiTE enhances T cell-mediated B cell killing measured by an RTCA biosensor assay. Daudi target cells were seeded at 50,000 cells/well in an Agilent 96-well E-Plate coated with αCD40 antibody. At 18 hours after Daudi cell seeding, enriched human effector T cells from primary PBMCs were added at a T:Daudi cells ratio of 10:1. Supernatant was collected at 7, 24, and 48 hours after the addition of T cells for protein measurement in the following experiment. Interaction of cells with gold micro-electrodes (biosensors) impedes the flow of electric current between electrodes. This impedance value is plotted as the unitless parameter Cell Index, and correlates with cell number, size, and cell-substrate attachment quality. The impedance signal was recorded every 15 minutes.

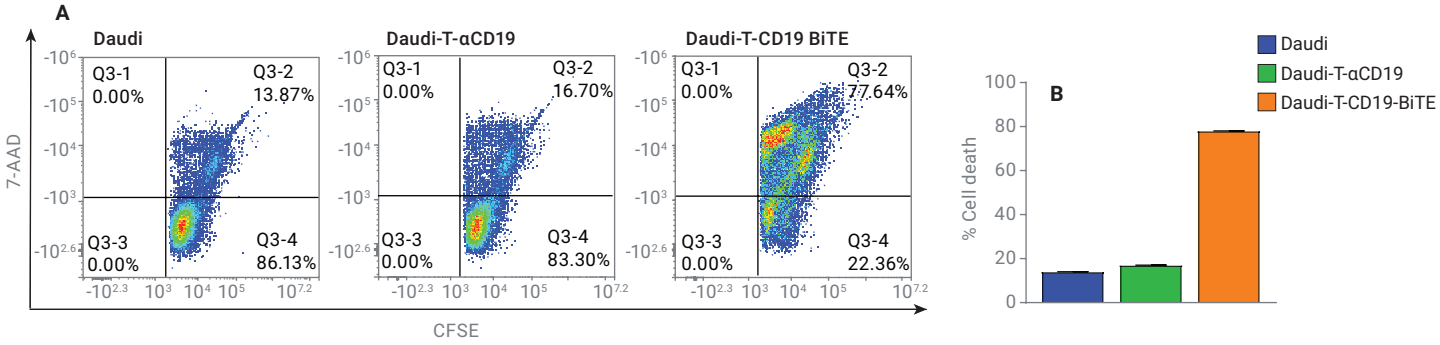
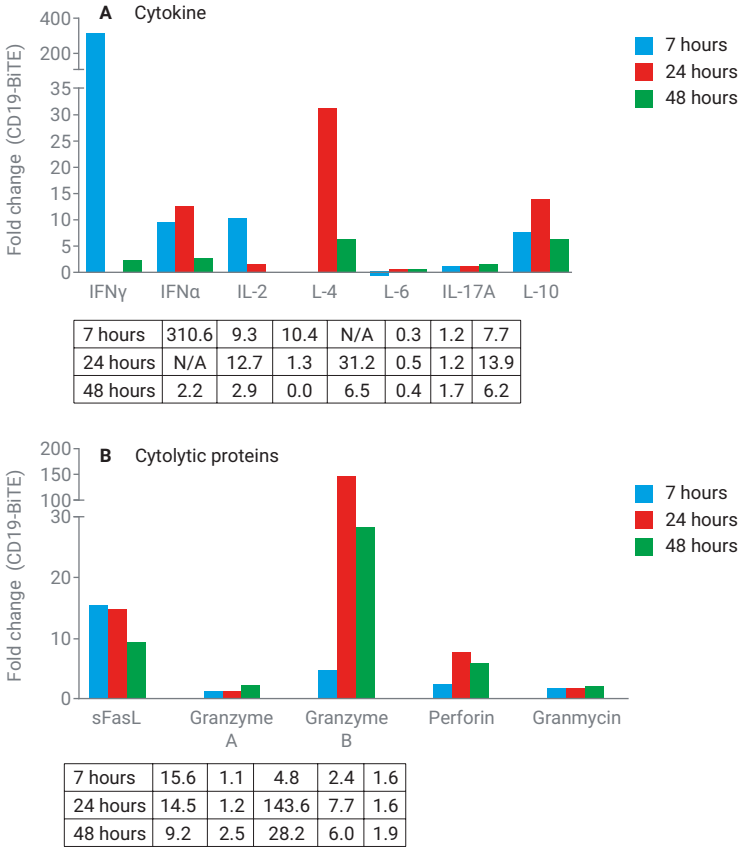


Figure 3. CD19-BiTE enhances T cell-mediated B cell killing measured by flow cytometry staining. Daudi cells were labeled with CFSE, and seeded at 100,000 cells/well. At 18 hours after Daudi cell seeding, enriched T cells were added with CD19-BiTE or an anti-CD19 antibody. At 48 hours after T cell addition, 7-AAD was added to identify dead cells by flow cytometry. A) Represents FCM plots. B) Frequency of CFSE+ 7-AAD+ dead Daudi cells.

CD19-BiTE enhances cytokine and cytolytic protein secretion by T cells in a bead-based immunoassay

To further study the effects of CD19-BiTE on T cell activation and function, cytokine and cytolytic protein secretion were measured. Cells were cultured as described in Figure 2, and a supernatant was taken 7, 24, and 48 hours after the addition of T cells. This was done to measure 13 human proteins known to affect T cell function with a bead-based multiplex assay (LEGENDplex Human CD8/NK panel, Biolegend) (Figure 4). Consistent with our findings from RTCA and flow-based cytolysis assays, an increased expression of CTL-associated proteins was observed. These data demonstrate that the presence of CD19-BiTE significantly enhances production of cytokines and effector molecules that mediate and sustain target cell killing.

At 7 hours after the addition of effector T cells, cytokines associated with CTL response, such as IFNγ, TNFα, and IL-2, are increased 300-, 9-, and 10-fold, respectively. Secretion of cytolytic proteins, such as FasL, Granzyme B, and perforin, are also dramatically increased by 24 hours, consistent with the CTL killing response seen. These data demonstrate that CD19-BiTE enhances T cell-mediated B cell killing by increasing the production of cytokines and cytolytic proteins essential for a robust CTL response.



7 hours	310.6	9.3	10.4	N/A	0.3	1.2	7.7
24 hours	N/A	12.7	1.3	31.2	0.5	1.2	13.9
48 hours	2.2	2.9	0.0	6.5	0.4	1.7	6.2

7 hours	15.6	1.1	4.8	2.4	1.6
24 hours	14.5	1.2	143.6	7.7	1.6
48 hours	9.2	2.5	28.2	6.0	1.9

Figure 4. CD19-BiTE enhances the cytotoxic activity of T cells in a bead-based multiplex immunoassay. Daudi target cells were seeded at 50,000 cells/well in an Agilent 96-well E-Plate coated with αCD40 antibody. At 18 hours after Daudi cell seeding, enriched human effector T cells from primary PBMCs were added at a T:Daudi cells ratio of 10:1. Supernatant was collected at 7, 24, and 48 hours after the addition of T cells for bead-based multiplex immunoassay (LEGENDplex Human CD8/NK Panel). The relative fold change between protein expression of Daudi + T + CD19BiTE to Daudi + T + αCD19 was determined for cytokines (A) and cytolytic proteins (B).

Conclusion

We have coupled quantitative cell killing assays with biomarker quantitation to provide an in-depth view of how CD19-BiTE affects T-mediated killing of B cells in a single workflow. The continuous monitoring of cell number, size, and attachment quality using RTCA enables the quantitative and kinetic assessment of the killing process. This is simultaneously corroborated by flow cytometry. Linking this cell killing data with quantitative analysis of cytokine and effector protein production allows simultaneous analysis of T cell activation and function. This workflow, which integrates both cellular and molecular phenomena, advances current methods of analysis for both basic and applied research of cancer immunotherapy.

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Published in the USA, September 1, 2022
5994-5245EN