



Breakthroughs in Immunotherapy



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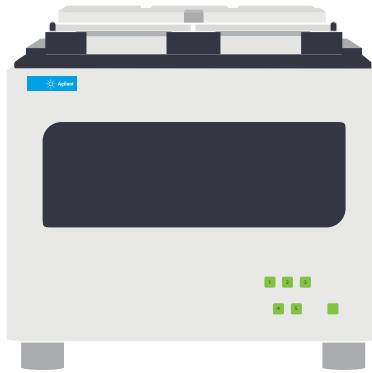
GEN Genetic Engineering
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Targeting the Tumor Microenvironment for Cancer Immunotherapy



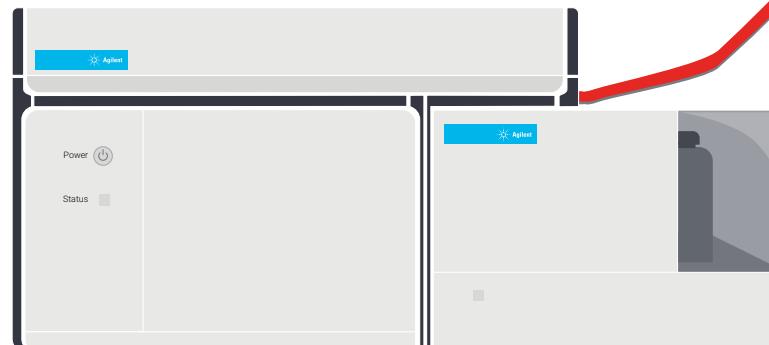
xCELLigence RTCA eSight

Real-time kinetic analysis of cell killing combined with live cell imaging



NovoCyte Quanteon flow cytometer

Multiparametric immunophenotyping by flow cytometry



Seahorse XF Analyzer

Kinetic analysis of immune cell fitness and fate



SureGuide CRISPR sgRNA
Stable, on-target gene editing



Accelerate your immune cell therapy development

Discover how the Agilent immune cell therapy tool bench can allow you to measure phenotype, potency, and persistence at all phases of development.

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Welcome

Immunotherapy is changing the landscape of cancer treatment, but most of the available tools are adapted and not purpose-built for this cell-centric workflow. A safe, potent, and persistent immune cell product depends on developers mustering the full repertoire of immune cell function; activation, proliferation, cell fate, cytotoxic killing, immunomodulation, and memory. All of which must be accomplished in the ever-changing, suppressive, and toxic tumor microenvironment.

Agilent is dedicated to supporting these next-generation cell therapies, providing key technologies to enable high-efficiency gene editing and assessment of real-time cell function, phenotype, fitness and fate. Used together, this tool bench provides the capability to measure and control immune cell function, thus enabling translational researchers and developers to achieve the necessary level of therapeutic potency and safety.

This e-book provides the latest recommendations from various leaders in immunotherapy research and their insights into exciting new developments. Their inspiration has guided and helped us compile these articles that showcase new analysis tools for scientists developing innovative cell-based solutions.

David Ferrick, Ph.D., senior director of the Cell Analysis Division at Agilent Technologies



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GEN Genetic Engineering & Biotechnology News

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The Agilent logo consists of a blue graphic element on the left, which is a stylized star or burst shape composed of numerous small circles or dots. To the right of this graphic, the word "Agilent" is written in a bold, black, sans-serif font.

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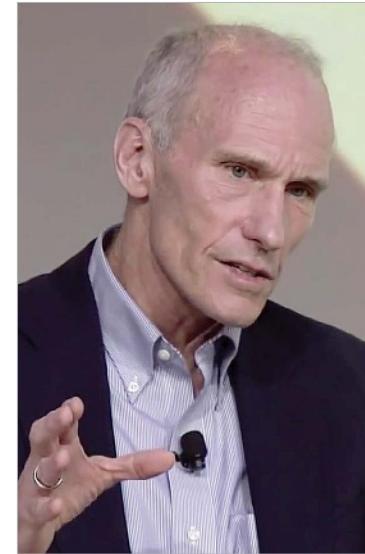
Breakthroughs in Immunotherapy

A Chat with Renowned American Immunologist and Oncologist, Dr. Carl June

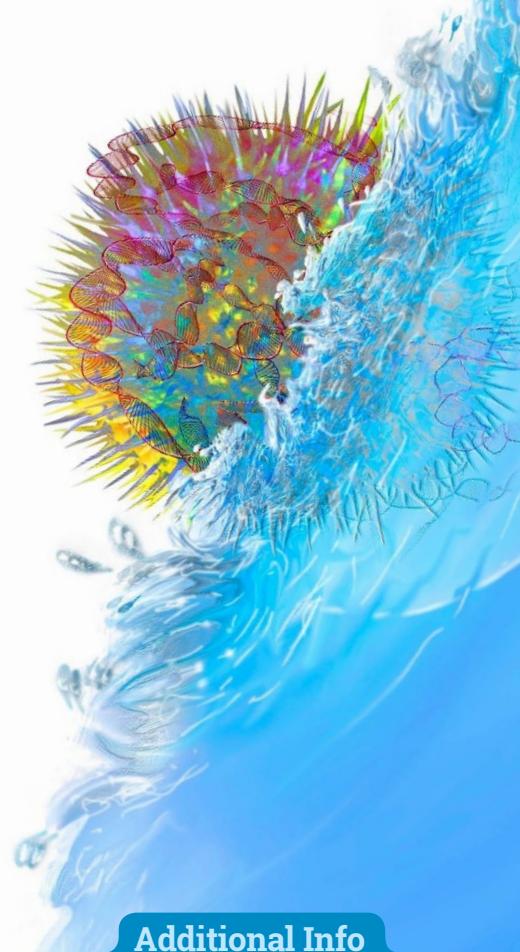
Agilent has steadily been developing innovative cell-based solutions designed to enable researchers and developers to overcome the challenges and capture the opportunities faced in the rapidly growing field of immunotherapy. Recently David Ferrick, an executive in the Cell Analysis Division at Agilent, caught up with Carl June, a professor at the University of Pennsylvania and Abramson Cancer Center, to discuss their perspectives on these unique challenges and opportunities. Dr. June has not served as a consultant for Agilent and has not received payments from Agilent.

David Ferrick: Carl, as you know, our relationship with you over the past few years has really helped us to advance several innovative solutions comprised of purpose-built tools in immunotherapy. Your guidance on the needs of this rapidly evolving field are invaluable. I'd like to focus on three areas: the engineering of human cells, how cell analysis tools are enabling cell therapy to reach its potential, and next steps in cell manufacturing QA/QC for cell-based therapies. As a pioneer in the engineering of human cells, how do you see the field evolving, and what do you think are the greatest challenges today?

Carl June: We're at an early stage where the proof of concept has been shown, and now accepted, that you can make synthetically engineered T cells that have enhanced performance characteristics in



Dr. Carl June



Additional Info

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of immune cell
persistence
in the tumor
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a variety of ways compared to the natural immune system. For a long time that was a question – could that be done? Can you improve upon a Darwinian, if you will, naturally evolved T cell, and if so can you do that safely?

I think we've had more than 1,000 patients treated with genetically engineered T cells, primarily with cancer, and there have not been any instances where the cells have transformed or had evidence of genotoxicity. This is a major turning point, where we have this orthogonal approach of combining the knowledge of the entire human genome and epigenome. We're using that to look at vulnerabilities in the tumor microenvironment and to make T cells that are designed and "purpose-built" to overcome the barriers that are in toxic tumor microenvironments.

David Ferrick: How do you see the potential for engineering other immune cells, such as natural killer cells (NKs), gamma deltas, or macrophages?

Carl June: That's one of the reasons it's so exciting to be in this field now. We

realize that the immune system is not just one instrument, to analogize, it's an orchestra. They have non-overlapping roles in the entire immune system. NK cells, for instance, kill and recognize targets in a way that is different from T cells. Similarly, there are subsets of T cells, gamma delta cells that are more like an innate immune cell, but they can also kill tumors. They have a different metabolism and can survive in different environments in a better way than alpha beta T cells. Because gamma delta cells don't have alpha beta T cell receptors, they won't cause graft versus host disease.

And recently macrophages have come to the forefront, as they kill and eliminate cells by phagocytosis rather than using a cytolytic mechanism like NK cells and T cells. In addition, we'll see engineered stem cells and their progeny, that after engraftment into patients can produce engineered cells of all the types we've just discussed.

David Ferrick: You've hit on an important point, that the immune system is like an orchestra, with homeostatic principles, and there are many cell types that cooperate

in both time and space to achieve that. One of the things we've focused on is the advancement of cell analysis tools that can generate information that is of a quality [time-resolved] that can help to understand this behavior along the timelines that occur *in vivo*. Could you comment on the value of real-time kinetic assays—let's call them the 'newer types' of cell-based solutions?

Carl June: That's a critical issue. The emerging data in basic immunology is showing that cells have major metabolic reprogramming steps. Acute effector T cells have, in general, dominant glycolysis for metabolism, whereas memory cells proliferate slower but live longer, and mostly use metabolism that's based on fatty acid oxidation, Krebs Cycle, and mitochondrial biogenesis. From what we've seen in mouse models of chronic infection and tumors, you want to have populations of both cells. Some cells that would be potent effector cells but are going to be short-lived and another set of cells that would be able to establish long-term cellular memory and function, for long-term immunosurveillance.

Metabolic assays such as Seahorse, are well poised to identify those cells, with those properties. I see in the future that there may be cell-based release assays for potency, and also predictive assays, as biomarkers of response in cell products.

David Ferrick: It's amazing what's happened so quickly. To think there would be, as you point out, this qualitative dichotomy between mitochondrial respiration and glycolysis that fits immune functionality so well—but hey, here we are. One question we hear regarding this 'newer type' of cell-based assay is about functional potency testing in terms of what the new product can do, and how long it will be able to perform. What do you see?

Carl June: One potential use of these new kinds of assays for cell analysis [Agilent ACEA xCELLigence, Agilent Seahorse XF] may be the ability to know which individuals could possibly make a curative product with current technologies and in others it would be futile. If you determine that an individual is not a good candidate, then that means you would go down the line of using third-party cells for instance,

so that's going to be a major change in the future. In addition, even in candidates where you determine you can manufacture a successful product, another application of this new "tool kit" may be finding, if you will, the heavy lifters. Some assays have shown, that when you do adoptive transfer, the T cells that carry out most of the tumor elimination are the progeny of just a few cells. If we can identify those T cells, up front, then potentially we can manufacture fewer cells, meaning the cost of manufacturing would go down, leading to a number of benefits in treatment.

David Ferrick: To follow up on that, how do you see some of the newer technologies like CRISPR being used to increase the fidelity and minimize the footprint of cell engineering so that we can get that protective immunity with a more natural approach which may be the key to durability and minimal side effects?

Carl June: There are a few genome-wide discovery approaches that identify 'targets of opportunity' in T cells that, in preclinical models, enhance their performance. It's a great time because of these technological

advances in genome editing using CRISPR, meganucleases, and so on, that make this possible.

The issues are somewhat different between solid tumors and hematologic malignancies. In hematologic malignancies, T cells, after infusion, generally traffic right to the bone marrow, that's a natural aspect to them. But in solid tumors, it may be rate limiting in many instances for T cells to enter the solid tumor. So, strategies that edit T cells to enhance their homing, penetration, and persistence of the solid tumor microenvironment are of great interest.

Another opportunity is the tumor and T cell "tug-of-war" between metabolites, and where CRISPR engineering can help increase access to nutrients in the tumor microenvironment. CRISPR approaches can help develop cells that are resistant to that tug-of-war situation, so they can survive longer, and therefore proliferate better in a solid tumor microenvironment.

David Ferrick: That leads me to cell manufacturing and the QA/QC component of

cells as a therapeutic modality. Is there a role for cell-based assays of the kind that we're talking about, the 'newer type' that are being developed?

Carl June: Very much so. With autologous cell therapies, at least one issue has been that it's always going to be more expensive than having third-party cells that can be made in large batches. If you're going to make something expensive, you want to make sure it works. So, any [cell-based] assay that improves the probability that you're going to have an effective cell product will be something that everyone wants—patients, physicians, and third-party payors, etc.

Understanding the basis by which cells from candidates can manufacture an effective cell product is an important first

step. To find these answers flow cytometry approaches, kinetic measures of live-cell metabolism, and quantifying the ability of T cells to kill targets over time are all aspects that can be investigated now with tools that Agilent provides.

David Ferrick: To wrap up, what would you tell Agilent and others, who are working on building tools and trying to enable people in this field, about how they can contribute?

Carl June: Functional assays. For a long while, we only had flow cytometry. I believe that we can learn a lot from a weakness in the pharmaceutical industry, where many trials were done without much emphasis on studying the reason why some trials failed.

But when using engineered cells, you

can retrieve them back from the patient and study them and investigate more thoroughly the immunophenotyping and metabolic health of the cells and find out was this T cell exhaustion, had the cells senesced, or perhaps it never engrafted? I'm optimistic that we may find solutions through analysis of where it has failed. With that knowledge, I believe we're going to be able to make better "next generation" T cells that will overcome those vulnerabilities.

David Ferrick: Carl, it's a great note to end on. Thank you so much for helping us with this, it's been great.

Carl June: Well thanks David, it's been great to work with Agilent as well. ■

This interview has been edited for length and clarity.

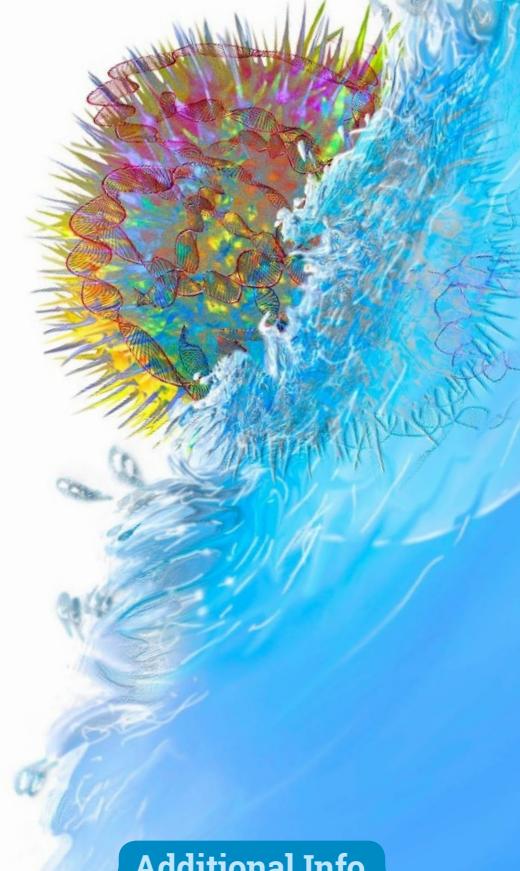
Combining High-Performance CRISPR Guides with Sensitive, Time-Resolved Cell-Based Assays

Cytotoxicity is not enough, *in vitro* assays must also assess persistence and durability

The great challenge of immune cell-based therapies is to safely engineer cells that have enhanced performance characteristics compared to native immune cells. Although we are still in the early stages of fully realizing this promise, proof of concept has been more than achieved with such hallmarks as the first approved CAR T cell therapies Kymriah and Yescarta^{1,2}.

CRISPR gene editing systems have emerged as highly effective surgical tools for modifying genomes in living cells with unprecedented ease. However, to reach their full potential increased activity, stability, and specificity is needed to achieve high fidelity edits with a minimal nuclear footprint. In addition, finding the most effective edits will require live cell analysis tools that deliver both functional relevance and assessment of potency and persistence. Live cell assays that are both quantitative and report out real-time kinetics can achieve these requirements. Based on a wealth of early learnings in immunotherapy, it is well appreciated that cytotoxicity is not enough. Engineered cells must be persistent in a hostile, immunosuppressive, and changing tumor microenvironment throughout the time course of tumor elimination and surveillance to achieve durable results³.

To address these challenges, Agilent has brought together an innovative set of solutions that



Additional Info

Download the
CRISPR Technical
Guide for a
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bring greater activity and accuracy to CRISPR editing. These solutions are combined with the ability to assess and validate the results on live-cell platforms that deliver relevant, time-resolved functional data.

Although the future remains bright for CRISPR gene editing, current challenges for wide-spread therapeutic use include better activity, specificity, and tunability for different situations. Guide RNAs that enable targeting of specific genomic loci can be made using enzymes to copy a DNA template into an RNA. However, direct chemical synthesis of guide RNAs such as used for Agilent SureGuide gRNAs (Agilent Technologies)⁴, has distinct advantages. Chemical synthesis provides a robust method for scalable manufacturing of highly pure sgRNAs, affords unique opportunities for sgRNA design, and precise installation of molecular functionality to augment CRISPR–Cas performance for research, industrial, and therapeutic applications. Figure 1 shows that chemical modifications added near the ends of guide RNAs can improve the stability and activity of the guide RNAs⁵. In a more recent study, improvements in CRISPR specificity were achieved by incorporating chemical modifications in guide RNAs at specific sites in their DNA recognition sequence and then systematically evaluating their on-target and

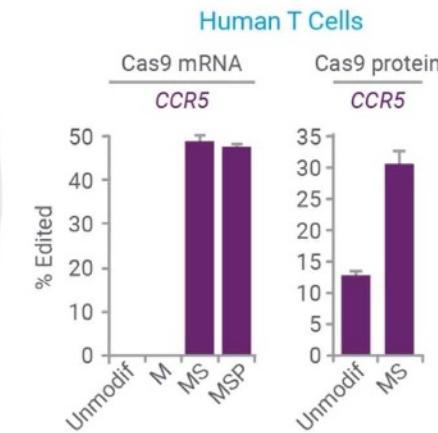
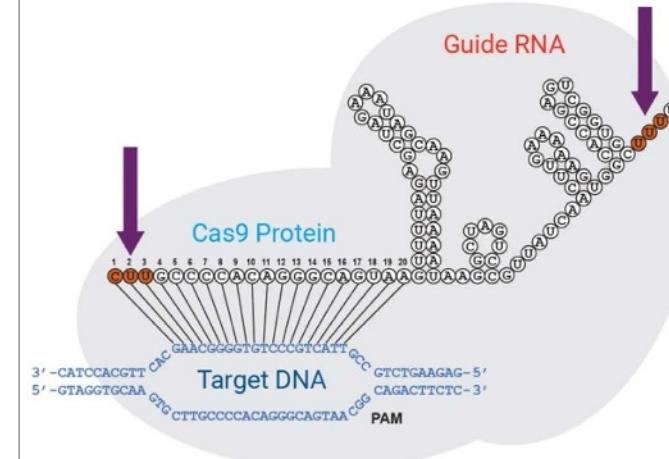
Figure 1

Figure 1. Chemical synthesis of guide RNAs enables incorporation of combinations of natural, modified natural, or unnatural nucleotides at defined positions that can impact CRISPR performance. Evaluation of modified single guide RNAs targeting the CCR5 gene containing three 2'-O-methyl (M), 2'-O-methyl 3'phosphorothioate (MS), or 2'-O-methyl 3'thiopACE (MSP) modified nucleotides at both the 5' and 3' ends were evaluated for activity in T cells using Cas9 delivered as mRNA or purified protein. MS and MSP modified guide RNAs had significantly higher activity than unmodified guide RNAs. Figure adapted from A. Hendel et al., Nat. Biotechnol. 33(9):985–989 (2015)

off-target activities⁶. This novel approach resulted in a dramatic reduction in off-target cleavage activities while maintaining high on-target performance (Figure 2).

Once you have edited your cells how will you assess and validate both the desired and undesired functional consequences? With the emergence of quantitative, time-resolved cell analysis platforms researchers can make more sensitive live-cell measures of function and simultaneously scan the temporal components of an ever-evolving immune response in terms of efficacy and persistence. For example, in a single experiment, one can now test gene edits for both killing efficiencies and serial killing at very low effector to target ratios. The results can then be related to inhibitory effects such as exhaustion, anergy, and other escape mechanisms due to interactions with a constantly evolving tumor and its microenvironment.

A great example of the value of combining functional sensitivity with time-resolution was recently published using an xCELLigence system (ACEA Biosciences now a part of Agilent). In this study, a CAR T cell strategy to address the heterogeneity of antigen expression within a cancer cell population was investigated⁷. A common obser-

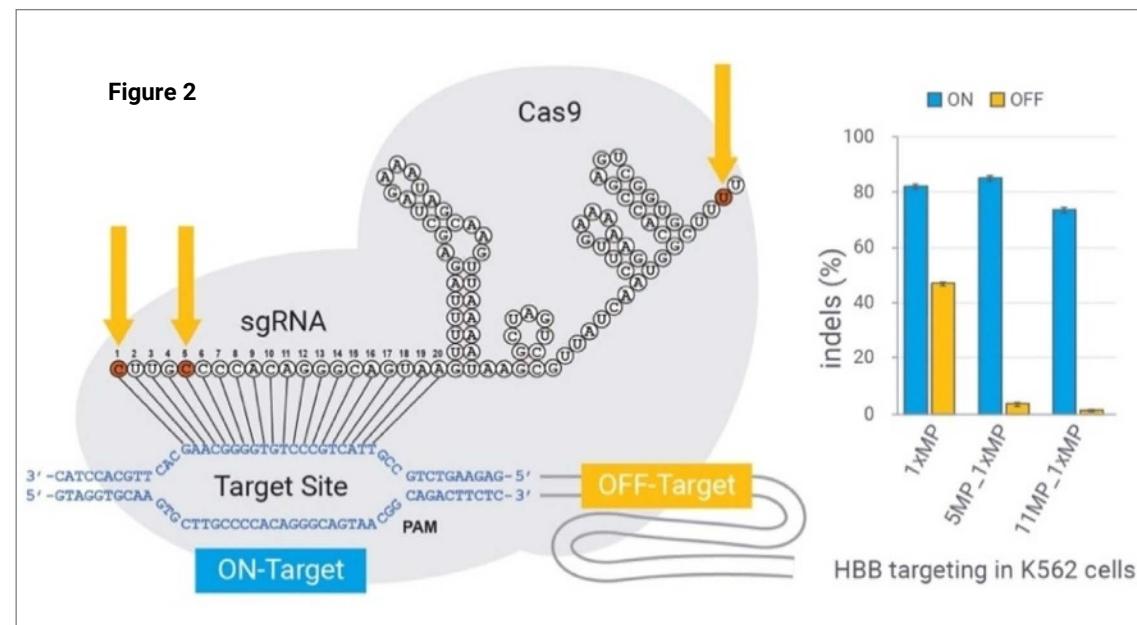
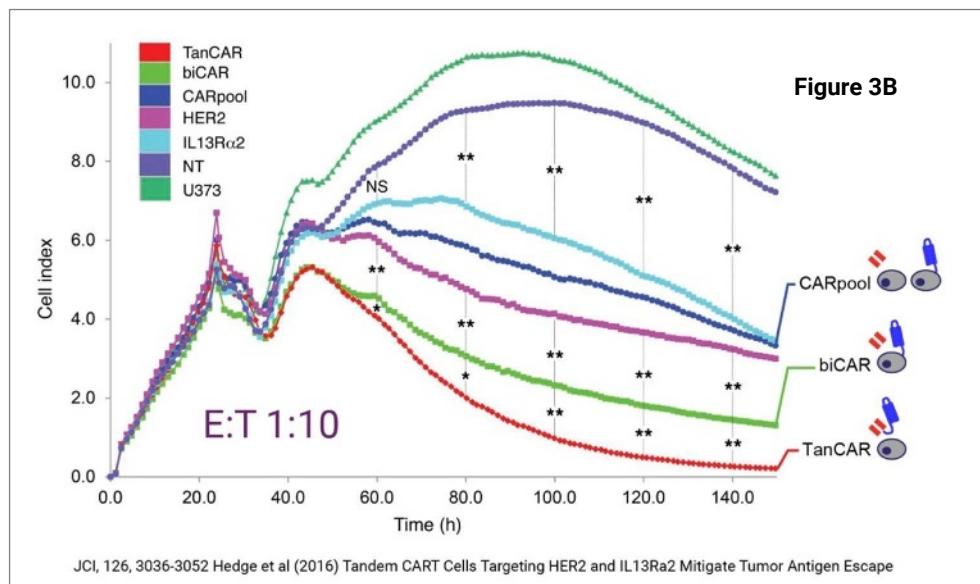
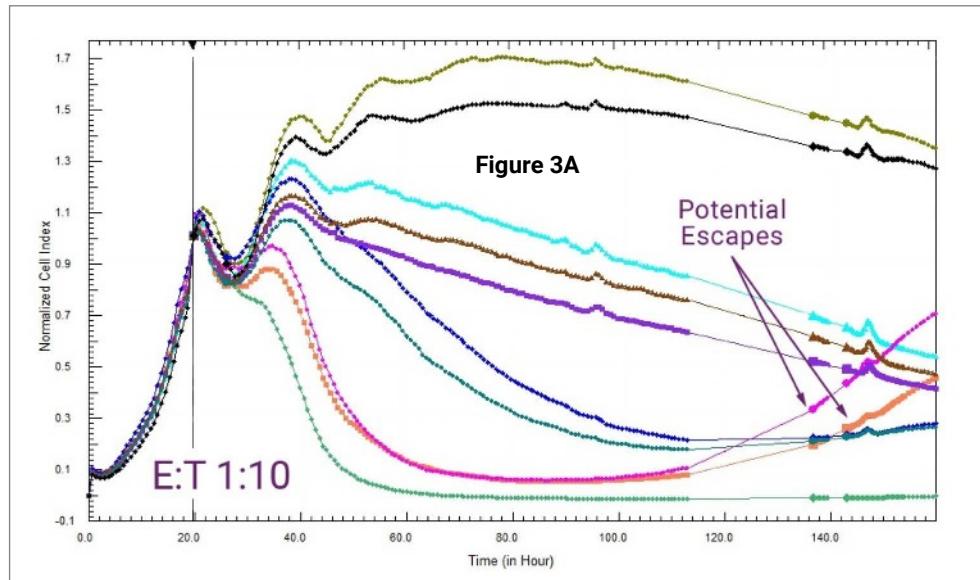


Figure 2. The HBB gene, which contains the mutation that causes sickle cell disease was used as an important, but challenging test to improve CRISPR specificity. It turns out that the favorite guide RNA for editing the sickle cell mutation in the HBB gene yields quite a high level of off-target activity at another genomic location. Adding a single modification at either position 5 or 11 of the HBB guide RNA sequence dramatically reduced the level of off-target activity as shown in the gold bars on this graph while maintaining a high level of activity at the on-target site. Figure adapted from Ryan et al., Nucleic Acids Res. 46(2):792–803 (2018)

vation with adoptive cell therapies is that when cancer cells expressing the targeted antigen are killed off, cells that lack the antigen continue propagating undeterred. To minimize this phenomenon, the authors targeted two tumor cell antigens simultaneously and used xCELLigence to assess both function and persistence. The results shown in Figure 3a enabled the authors to choose the optimal engineering constructs based on a very sensitive quantification of the killing efficacy using low effector to target ratios of various combinations of costimulatory domains with a constant antigen binding domain (N. Ahmed, unpublished results). Simultaneously, they were also able to identify potential escape phenotypes due to the time resolution of the assay. In Figure 3b, key results are shown for different scenarios where CARs targeting the HER2 and IL13Ra2 antigens were

Figure 3a. Various combinations of proprietary costimulatory domains with a constant antigen binding domain were quantified for killing efficacy using a low target to effect ratio (1:10) and simultaneously for identification of potential escape phenotypes (pink and orange lines) based on time resolution of the data (N. Ahmed, unpublished results, April 2, 2019).

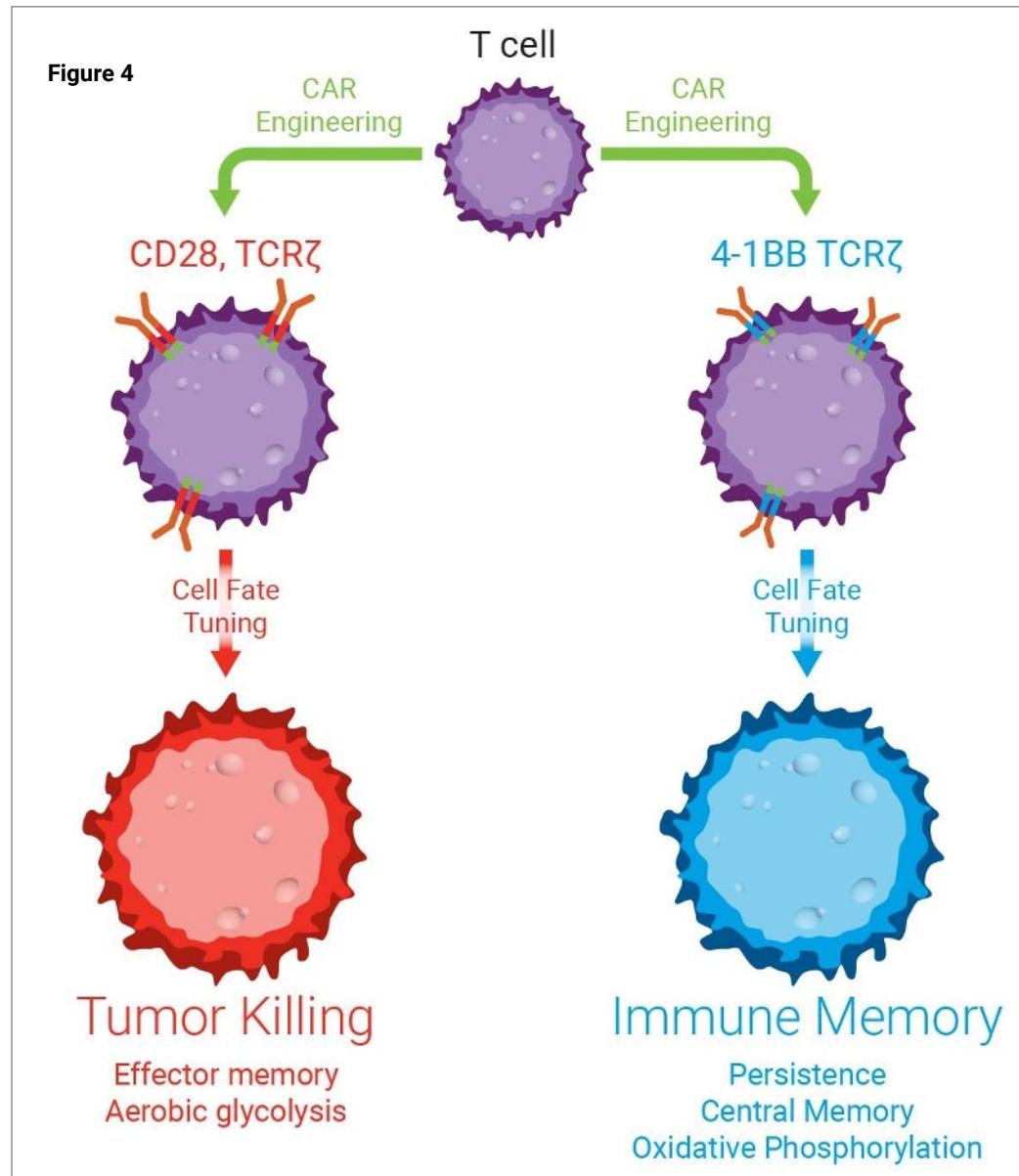
Figure 3b. Using xCelligence to monitor the killing of the glioblastoma cell line U373 by CAR-T cells targeting either one or both of the antigens HER2 and IL13Ra2. In the figure legend: U373 = target cell line alone; NT = target cells treated with nontransfected T cells (i.e. not expressing a CAR); IL13Ra2 = target cells treated with T cells expressing a single CAR targeting IL13Ra2; Her2 = target cells treated with T cells expressing a single CAR targeting Her2; see text for descriptions of CARpool, biCAR, and TanCAR. 1:10 E:T ratio. Figure adapted from Hegde et al., J Clin Invest. 2016 Aug 1;126(8):3036–52.



expressed in separate T cells (CARpool), as distinct proteins within the same T cell (biCAR), or as a single fusion protein within T cells (TanCAR). When incubated with glioblastoma target cells each of these CAR T approaches displayed differential killing capacity and kinetics. These nuances in serial killing behavior are readily elucidated by continuous impedance monitoring but would go undetected in traditional end-point assays.

Another sensitive, time-resolved platform that can deliver rich, functional data is the Agilent Seahorse XF Analyzer (Agilent Technologies). A pivotal study published by the Carl June group showed how different CAR T engineering constructs can have dramatically different effects on cell fate and function by driving different metabolic programs⁸. It reveals how one might optimize tumor elimination, persistence in the microenvironment, and durability. In Figure 4, a summary of their findings reveals how CAR T cells containing the coreceptor signaling domain, 4-1BB,

Figure 4. Choice of T cell signaling domains impacts metabolic programming differentially, introducing the possibility of fine-tuning cell-based therapies. 4-1BB containing CAR T cells are more aerobic with increased mitochondria resulting in enhanced *in vitro* persistence and central memory cell fate. In contrast, CD28 enforces a more glycolytic program leading to increased effector memory.
Figure adapted from Kawalekar et al., Immunity 44, 380–390 (2016).
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elicit an aerobic program consistent with central memory formation and persistence. In contrast, engineering with the CD28 coreceptor signaling domain reprograms metabolism towards aerobic glycolysis resulting in enhanced effector memory cell fate. This study was a powerful proof of concept for engineering metabolic traits to establish the right balance of effector and memory cells to address immune cell exhaustion and persistence in hostile tumor microenvironments paving the way for durable memory and immunosurveillance. ■

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Application Note

The Role of Potency Assays in Cell Therapy Manufacturing

Although cell therapies have shown great promise in clinical studies, challenges with the large scale manufacturing of these therapies still need to be addressed. Stringent quality control is necessary to ensure the therapy's potency before a patient receives treatment. Typically, a potency assay measures the ability of a drug to elicit a particular response at a certain dose in a relevant biological system. Although the preferred method for determining potency is a single, quantitative biological assay, due to the complex and personalized nature of cell therapies, this approach may not always be straightforward.

The US FDA does not define potency tests for cell and gene therapy products because potency measurements are designed specifically for a particular product. They also do not make recommendations regarding specific assay types, nor propose acceptance criteria for product release.

In the past, decades-old standard release assays for endotoxin and sterility were mandated for cell therapies. A potency assay was viewed as a required secondary assay. "Many groups chose the cheapest and technically least complicated assay just to check off a box on a certificate of release," says Carl June, M.D., professor, Perelman School of Medicine, University of Pennsylvania and Abramson Cancer Center.

But now, third-party payers, patients and physicians are expecting more convincing data to better predict clinical outcomes for expensive therapies. Functional potency assays are going to assume a greater role to assess the cell therapy's ability to engraft, proliferate and kill tumors.



Additional Info

Read this xCELLigence RTCA application note to learn more about potency evaluation: 

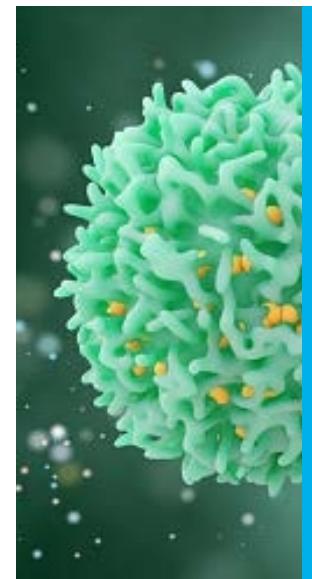
When designing a potency assay for cell therapy manufacturing, the first step is to define potency in the context of the specific therapy.

"Keep it simple. Often, fancy ideas about a potency assay can be almost impossible to establish in a reproducible and reliable fashion," states Mark Lowdell, Ph.D., director of Centre for Cell, Gene & Tissue Therapeutics, Royal Free Hospital, professor of Cell & Tissue Therapy, University College London.

"In the context of manufacturing, maintaining a balance between mimicking the human disease, complexity, and cost is critical," adds Krishnendu Roy, Ph.D., director, Center for ImmunoEngineering, Georgia Institute of Technology.

Currently, a potency assay for CAR-T cells could simply measure the expression of the desired CAR on the cell surface. However, it should be noted that this does not directly measure function; and there is no proof that the tumor cells expressing the relevant antigen for the CAR will be killed. If the projected activity *in vivo* is not taken into account, this type of potency may not be sufficiently predictive.

In a UK clinical trial Dr. Lowdell is involved with, a bank of allogeneic mesenchymal stromal cells (MSCs) is being transduced to express tumour necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL). Engineered MSCs expressing TRAIL may be used as an efficient form of cell therapy due to the MSC's non-immunogenic nature and ability to infiltrate the tumor microenvironment. To meet quality control standards, the product is tested based on the proportion of cells that express TRAIL and by the amount of TRAIL expressed by the positive cells.



"In the context of manufacturing, maintaining a balance between mimicking the human disease, complexity, and cost is critical."

Krishnendu Roy, Ph.D., director,
Center for ImmunoEngineering,
Georgia Institute of Technology.

Often, functional potency assays are designed without considering other cells in the tumor microenvironment or the tissues the disease affects. Considering these factors during assay creation would help better predict potency or efficacy in the human patient.

"Ultimately, personalized medicine is the objective," says Dr. Roy. "The goal is to eventually design something that is better than just taking into account the general average conditions of patients. Using a patient's biopsy for potency assays in cancer cases would take into consideration patient-specific conditions, if not the whole microenvironment. In other disease that may not be possible."

In addition, studying a large cohort of patients during assay development would help scientists better understand patient variability.

Validation Considerations

Reproducibility, robustness and ease of performance are critical to ensure assay consistency across samples from multiple

facilities and laboratories. Assay readouts should have a low coefficient of variation (CV) and a high signal-to-noise ratio. Variability attributed to the cells, reagents, instruments, platform devices if applicable, such as microchips, and operators should be minimized as much as possible.

"For example, in current methods, one takes a tumor cell line and co-cultures the CAR-T cells with it to determine that the CAR-T cells recognize the tumor, express the same molecule and kill the tumor cells. Although this may be considered a good functional assay, it does not reflect the patient condition or the tumor microenvironment, which is especially relevant in solid tumors," discusses Dr. Roy.

These conditions can be incorporated into the potency assay by co-culturing with other cells from the tumor microenvironment or by creating a 3D culture. The additional variables may provide a more accurate *in vivo* behavioral prediction but also leads to complexity when translating to manufacturing. While the current method is simpler, it does not correlate exactly with functionality.

According to Dr. Lowdell, the potency assay for an allogeneic cell product called INKmune™ measures the activation of NK cells. Since it is an off-the-shelf product, the manufacturing process does not take into account individual patients; therefore, a prescreened donor pool of normal NK cells is used to show activation. For autologous products, the only way to reduce or control variability is to use standardized targets or molecules.

Building in positive and negative controls should also be part of assay qualification. In autologous situations, variability within the same product can be donor related. For example, internal antibody-binding bead standards can be used in select flow cytometric assays. The antibodies bind both to the beads and the cells. The beads must fall into a defined region indicating that the appropriate antibody and amount were added, and that the operator performed the assay correctly. With internal controls, any irregularities between samples can be attributed to biological variation rather than technical assay failure.

The Role of Real-Time Cell-Based In Vitro Assays

The cell therapy manufacturing process can be quite long, ranging from several days to weeks. "Changes in cell behavior and phenotype can happen during the process," says Dr. Roy. "Real-time assays would be very beneficial to generate continuous feedback during that process, which would help us verify the quality of the batch and reduce manufacturing costs."

In the R&D phase, real-time feedback is critical to understand the cell properties that are predictive of functional potency. The real-time measurement of these quality attributes would then ensure the production of highly potent cells.

Researchers are still working to define which quality attributes need to be measured to predict potency. "Some engineered cells are compared to the wild type to make sure the desired changes took place. For CAR-T cells, the cell's viability, surface markers, and cell killing activity are measured to evaluate the end product. But this set of measurements does not really indicate that these cells are going to be effective in this particular patient," continues Dr. Roy.

The xCELLigence Real Time Cell Analysis (RTCA) instrument by Agilent Technologies allows for longer duration real-time assays. Real-time assays can more accurately evaluate the biology, efficacy or potency of the product, especially when the cell activity can be monitored over many hours or days with multiple readouts. While endpoint assays provide valuable information, they may fail to predict activity *in vivo* if the cells kill later than the measurement time.

Additionally, killing assays with high background noise may fail to identify robust candidates for cell therapy. With the highly sensitive xCELLigence



"Keep it simple. Often, fancy ideas about a potency assay can be almost impossible to establish in a reproducible and reliable fashion."

Mark Lowdell, Ph.D., director of Centre for Cell, Gene & Tissue Therapeutics, Royal Free Hospital, professor of Cell & Tissue Therapy, University College London.

RTCA assay, target cell proliferation and killing by effector cells can be monitored at lower effector to target ratios. These low ratios more closely mimic physiological conditions, which may better predict patient response. Researchers can also use reduced concentrations of the precious cells in the assays, allowing them to simultaneously test more conditions.

"The high throughput xCELLigence RTCA assay can measure cell killing continuously, which makes it an excellent functional potency assay. For INKmune, target cells resistant to NK killing are grown in the xCELLigence E-plate, then pooled donor NK cells are added to show nothing happens. Twenty-four hours later, INKmune is added, and, if it is functional, the NK cells get switched on and a change occurs in the target cell cytotoxicity in the next five days," explains Dr. Lowdell.

Cell-Based Assays versus Animal Studies

Animal studies are often complex, lengthy, and expensive. They may not be reflective or predictive of the human patient or disease

process, and there simply may be no good animal model. "In the manufacturing of cell therapies, potency assays cannot be animal studies," indicates Dr. Roy. "Cell-based *in vitro* assays are a surrogate to determine cell function. The more the human disease can be mimicked, the better the *in vivo* predictions. As better *in vitro* assays are developed they will play a significant role during the manufacturing process."

A critical fundamental issue with animal studies is the difference in homology between human and other mammalian cells. When human effector cells are injected into the bloodstream of a patient, they bind to specific molecules in the tumor microenvironment to activate an immune response, which leads to target tumor cell killing. When these human effector cells are injected into a rodent used for animal studies, the binding between human effector cells and rodent ligands may not be predictive of the killing activity in a human tumor microenvironment.

Another challenge with animal studies is the enhanced immune response observed

when a tumor is artificially established in an animal model. Typically, a rapidly dividing tumor cell line is injected into the animal, creating a lump. Subsequently, this tumor creates an acute inflammatory response. In patients, a tumor is part of a chronic immune response due to the localized tumor microenvironment and its immunosuppressive nature. Acute and chronic inflammatory responses lead to different immunological outputs, so the immune response observed in an animal model may not be indicative of the product potency and efficacy in patients.

According to Dr. Lowdell, "the UK regulatory board was excited to see a dynamic model of a tumor growing without a local inflammatory response using the xCELLigence RTCA instrument. I believe real-time assays are going to be increasingly used both in drug development and manufacturing as a potency or release assay."

Screening for Safety

Screening for contamination and sterility is routine but other safety screens are challenging; the understanding of how a

cell therapy may adversely affect a patient is still progressing.

The product's risk profile dictates what safety requirements need to be assessed. Allogeneic products pose a graft versus host disease (GvHD) risk. Another risk is cytokine release syndrome (CRS), a systemic inflammatory response syndrome that arises as a complication due to infections, which may also occur when administering adoptive T cell therapies. These are difficult to predict due to the inherent variation between patient immune responses.

Since cell therapies are being used to treat life threatening illnesses such as cancer, the risk of toxicity is often outweighed by the potential for improved patient survival. There is a difference between toxicity that has no benefit to the patient and therapeutic toxicity, which can be an indication that the cells are actually killing the tumor cells. "What we really are controlling for is unexpected side effects," states

Dr. Lowdell. "Indeed, you might end up screening out the effective agent because part of the adverse effect may be the therapeutic response."

"When we look at potency in more patient-like *in vitro* models, we are looking for predictive signals that can be correlated with clinical trial observations," says Dr. Roy. "In terms of CAR-T cells, we are beginning to understand why some cases of neurotoxicity and CRS happen, but do not yet have the detailed understanding to predict the outcomes of these therapies."

"Twenty to thirty percent of patients are not helped by CAR-T therapy. Our center and others are starting to look into the many unanswered questions. It goes back to quality attributes," continues Dr. Roy. "What are the properties of the most effective, potent and safe cell therapies? We have learned a lot from the biologics era. As a field, we need to think about the correlative, predictive parameters of potency assays early on." ■

Application Note

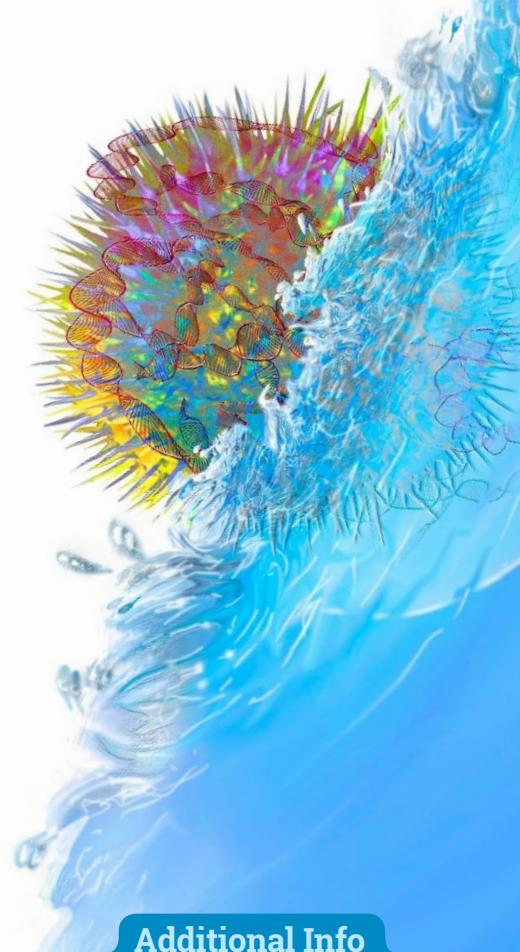
Immunophenotyping, Above and Beyond

Targeting the immune system has dramatically increased the need for tracking the native and more recently engineered phenotypes of multiple cell lineages.

Flow cytometry has developed from being a quantitative tool for identifying and classifying mixtures of cells using fluorochrome-labeled monoclonal antibodies, to becoming an essential-workhorse for cell analysis research and development. The last decade has brought a series of advancements in instrumentation and software along with an exponential expansion of antibodies and fluorochromes. This has dramatically expanded the applications and cell phenotypes that can be accurately measured with the blink of an eye¹. It is no longer a characterization tool but a comprehensive phenotyping system with blazing speed and a growing rainbow of fluorophores to maximize the number of parameters that can be measured per cell.

In human testing, cytometric immunophenotyping is now employed routinely to diagnose, classify, stage and monitor therapy in blood cancers and a growing list of age-related diseases.² It can be used to determine the origin and stage of differentiation of leukemias and lymphomas.³ Sampling is nominally invasive, making it an ideal method for monitoring treatment and detecting recurrence, especially in cell-based immunotherapies.⁴

The deep roots and recent successes of immunotherapy in cancer treatment is rapidly changing the



Additional Info

Read this Novocyte Quanteon application note to learn more: 

research and development paradigms for how to intervene in disease. However, immune parameters capable of sufficiently characterizing antitumor responses across various immune networks are underdeveloped. Converting the recent successes of immunotherapy into broadly applicable clinical research strategies with improved response rates necessitates improving our ability to monitor immunity at all stages of research, development, diagnosis, and treatment.

Flow cytometry holds great potential to address this gap as it is more compact, adaptable and accessible than ever. Customizable and compatible with auto-amplifying and high-throughput technology, increasingly sensitive to a growing array of fluorophores, parallel processing multiple inputs, cytometers allow us to collect vast amounts of information from easily obtained and processed samples. "We engineered a new generation of cytometry to ensure that every increment of enhanced performance was accessible at the benchtop, with software that is easy to use. The result is that investigators would be better able to keep pace with the demand for the discovery and implementation of novel immunotherapies", explained Xiaobo Wang, (Figure 1). Dr Wang, former President and Chief Technology Officer at ACEA Biosciences, joined Agilent from ACEA, as General Manager of the Flow Cytometry and Real-Time Cell Analysis

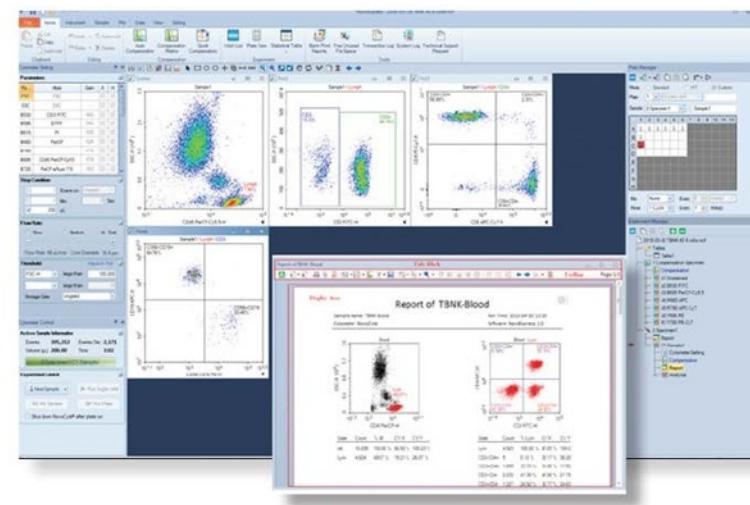


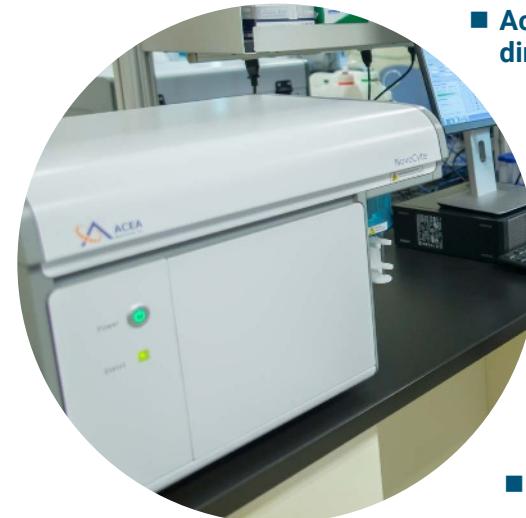
Figure 1. NovoExpress has a user-friendly interface for easy access to settings, analysis, reports and plates/sample layout.

business. "Our goal is to bring the best possible performance and experience to the benchtop. We will know we have succeeded when you get the same answer, again and again, no matter who is operating the cytometer."

As inferred by Dr. Wang , the resolution wars in cytometry are drawing to a close. Fluorescence resolution in most instruments is comparable and sufficient. The competition is, therefore, rightfully shifting to usability features that are dramatically improving accessibility, efficiency, and decision making. Many experts in the field predict software experience and automation to be the top differentiators among cytometers going forward, especially at the bench, where everyone wants them to be.

As a cell engineering tool, flow cytometry is employed from screening through validation of accurate targeting to comprehensive phenotypic characterization. A great example is Precision BioSciences. This company is applying its ARCUS genome editing technology to develop allogeneic cell-based immunotherapies. Their immunotherapies are derived from select, healthy subjects as opposed to compromised cancer patients to overcome inherent problems of consistent and scalable manufacturing of autologous cells. "Cytometry is the key tool in our work. This is how we analyze our cells before, during and after the manufacturing, including how we trace our cells in patients and analyze the cellular component of their immune system during treatment", explained Vladimir Senyukov, the Director

NovoCyte Advantages



- Wider dynamic range for detecting leads to a reduction in time and mistakes
- Accurate volumetric-based, direct cell counting system
- NovoExpress drag-and drop function, makes data analysis quick and efficient
- NovoExpress can generate PDF reports with one click
- Innovative fluidics deliver higher reproducibility and exceptionally low CV's
- Fully automated wash and shut down; ready-to-go for the next user
- Walk-away automation can be achieved with the NovoSampler Q™

of BioAnalytical Development at Precision BioSciences. He went on to explain, "What I like about the NovoCyte Quanteon is that it's compact, fast and it has accurate volumetric-based cell counting. It is a robust and stable instrument."

In summary, the Agilent line of NovoCyte cytometers is delivering in an industry that was already considered too crowded for new entrants by pushing the boundaries of accessibility and performance. Democratization is at the roots of its appeal and adoption, especially in the fields of immuno-oncology and immunotherapy. As flow cytometry continues to drive new applications, especially in nontraditional workflows such as cell engineering and manufacturing, it must continue to become less complex. The solution is to make the platforms smarter by being able to configure to the customer's on-demand expectations and preferences. This is where Agilent NovoCyte excels. ■

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Table 1.

The following are common features cited by users when speaking about the NovoCyte line of cytometers:

1. The wider dynamic range for detecting signals eliminates the need to adjust PMT voltage. This feature makes data acquisition simple, especially for new users. Consequentially, this leads to a reduction in time and mistakes.
2. The NovoCyte has an accurate volumetric-based cell counting system that performs direct absolute cell counting during data acquisition without the need for reference counting beads or extra steps.
3. The NovoExpress software is straightforward and easy to use for sample acquisition and analysis. Samples can be analyzed with the same setting by a simple drag-and-drop function, making the process of data analysis quick and efficient.
4. The report function on the NovoExpress software can generate PDF reports with one click, making data-sharing simple.
5. The groundbreaking fluidics provide pulse-free sample delivery resulting in higher reproducibility and exceptionally low CVs.
6. The fully automated flow cell wash and shut down features mitigate flow cell clogging. The system is always ready-to-go for the next user.
7. Walk-away automation can be achieved with the NovoSampler Q. This provides superior shaking, even at ultralow volumes, and accommodates the most popular plate formats, bar coding, and built-in APIs for lab automation systems.⁵

Roundup

Quantifying Immune-Cell Mediated Killing

At present, most of the commercially available cancer immunotherapies employ monoclonal antibodies or cancer vaccines, but a different kind of cancer immunotherapy, adoptive cell transfer (ACT), is progressing rapidly. ACT usually involves the harvesting of patients' own immune cells, expanding these cells (or engineered versions of them), and then reintroducing them to patients.

ACT-based treatments pose serious developmental challenges, not the least of which is the need to evaluate the cancer-killing abilities of the immune cells. In addition to being robust and simple, the ideal *in vitro* assay closely mimics activity *in vivo*. The ideal *in vitro* assay must also predict long term behavior in animal models, and ultimately, in human patients. To facilitate the study of immune cell-mediated killing, ACEA Biosciences – now a part of Agilent Technologies – has developed the xCELLigence® Real-Time Cell Analysis (RTCA) platform.

The RTCA platform is described below, the main text of which is devoted to showing how the RTCA platform is helping advance real-world projects. In this article, several key opinion leaders tell GEN how they are using the RTCA platform.

GEN: Why is there such a great disparity in the potency of current cancer immunotherapies between patients or between patient subpopulations?

Dr. Anderson: Two factors, genetics and the tumor microenvironment, are major focuses of investigation. In addition, adaptive resistance is not very well understood yet, but it's going to be a problem for many types of immunotherapy. Cancers have adapted to evade treatment strategies in the past,



Additional Info

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and we're now seeing that this is true for immunotherapy as well. We're trying to predict how tumors might evade our therapies so we can develop strategies to head that off.

Dr. Bamdad: I believe the underlying problem is how the cells are being handled *in vitro* before they're given back to the patients. It's looking more like the longer they're cultured *in vitro*, the worse it is for the patient, because as these cells mature, the distribution of the different subpopulations of T cells changes. This has a negative impact on persistence (that is, how long they will be effective in the patient) and also on what is called the cytokine storm, or neurotoxicity. Our understanding of this topic is increasing exponentially.

Dr. MacLeod: In CAR T cell– and other T cell–mediated therapies, one of the big reasons for variability is the use of a patient's own cells. Instead of taking an autologous approach, at Precision Biosciences, we are engineering healthy donor cells to transfer into unrelated patients, and this allogeneic approach allows us to make something more defined, more consistent. This is evident when we use assays to evaluate the function of these products such as the cytotoxicity assays developed by ACEA Biosciences for the xCELLigence instrument.

Dr. Golubovskaya: Patients differ in their immune profiles, and tumors differ in the biomarkers they present. We cannot predict these differences, and we rely on analyzing these biomarkers in each patient to help us predict their response to immunotherapy.



Kristin Anderson, Ph.D.

Postdoctoral Research Fellow
Fred Hutchinson Cancer Research Center
University of Washington

“Adaptive resistance is not very well understood yet, but it’s going to be a problem for many types of immunotherapy.”

GEN: What are the main challenges in assessing and monitoring the potency and efficiency of cytotoxic immunotherapies targeting cancer?

Dr. Anderson: The challenges differ for each tumor type. Most experiments that work with human samples have to be done in a Petri dish, which does not replicate the complexities of the tumor microenvironment. In addition, we have had problems looking at ovarian cancer cells using some of the “gold standard” assays, such as chromium release and flow cytometry killing assays. The cancer cells we use often do not take up or retain the chromium label well, resulting in high background readings.

With flow-based assays, which also require labeling, if you put in too many cell types, which are often necessary as controls, overcrowding can occur and result in nonspecific tumor cell death. In these scenarios, background cell death makes it difficult to evaluate true T cell-mediated killing.

Dr. Overstreet: One of the biggest challenges is to improve our ability to model and predict what will work in humans. Many of these pathways are understood in the mouse, and while some of them have provided good translation to humans, others have not. We have been able to build *in vitro* systems on the xCELLigence platform in which we can study the cognate interactions between human T cells and human tumor cells in a way that might be more reflective of what you would see in a patient’s tumor.

Dr. Bamdad: Timing, accuracy, and flexibility of your instrumentation are paramount. You can use fluorescence-activated cell



Cynthia C. Bamdad, Ph.D.

Founder and CEO
Minerva Biotechnologies

“Timing, accuracy, and flexibility of your instrumentation are paramount.”

sorting (FACS) to look at CAR T-cell killing, but it gives a very indirect measure, and it lets you look only at a snapshot in time. You can't see how your CAR T cells or cancer cells are evolving over time.

You need a technology that allows you to look at the co-culture of your cancer cells and CAR T cells over time to be able to identify the correct distribution of naïve or central memory to effector memory T cells. You need to be able to determine how long it takes these cells to mature so you get to see when cell killing occurs, on the opposite end of the spectrum, when CAR T cells become exhausted and the cancer cell population starts to grow again. Being able to see what happens in real time is critical to your CAR design and how you culture your CAR T cells *in vitro* to get the proper subtype distribution.

Dr. MacLeod: One of the main challenges is being able to translate the *in vitro* results to how the therapy will actually perform *in vivo*, either in animal models or in patients. The traditional assays for measuring cytotoxicity are usually short, sometimes 3 or 4 hours, and to see a response, you use very high effector-to-target cell ratios. This will show activity, but it doesn't reflect the entire mechanism of action.

These are living products—they don't just kill cells, they also proliferate, and that's an important part of the mechanism. When you are able to look at killing of target cells over longer periods of time at lower effector-to-target ratios, that allows the effector cells to proliferate and kill multiple targets—what is called serial killing—and that type of longer assessment is a much more stringent way to evaluate T-cell activity.



Dan MacLeod, Ph.D.

Associate Director, Cell Therapy Discovery
Precision BioSciences

"These are living products—they don't just kill cells, they also proliferate, and that's an important part of the mechanism. When you are able to look at killing of target cells over longer periods of time at lower effector-to-target ratios, that allows the effector cells to proliferate and kill multiple targets—what is called serial killing—and that type of longer assessment is a much more stringent way to evaluate T-cell activity."

GEN: Why is it important to measure target cell killing directly rather than by quantifying related factors such as levels of secreted compounds or the activation of effector cells?

Dr. Anderson: Both are important, but it's especially important to measure target cell death because every tumor is a little different. Some tumor cells may respond to one killing mechanism and not to another, and because they are also so readily able to adapt to evade T cell-mediated killing, we need to be able to see that the T cells can actually kill the targets.

It is important to note that the xCELLigence assay isn't technically a direct readout of cell killing, it's a measure of impedance, which reflects the ability of the target cells to adhere to the bottom of the plate. Since dead cells do not impede the signal, this is an indirect readout of cell killing. For the purposes of our experiments, it has yielded the most reproducible results and allowed us to study physiological effector-to-target ratios that we couldn't evaluate using the other assays.

Dr. Overstreet: If what you ultimately want to see is how a T cell interacts with a tumor cell and kills it, then that's what you should be measuring. T cells have an array of overlapping/semiredundant ways of killing a target cell, and by choosing to measure a single analyte, you could be missing the critical limiting factor in the particular interaction. But if you look at how a tumor cell is dying in real time, then you can see that dynamic interaction that is the net result of all the effector functions, and you can observe how your therapeutic can alter that interaction.



Michael Overstreet, Ph.D.

Scientist, MedImmune
(a unit of AstraZeneca)

“One advantage of xCELLigence assays compared to traditional means of evaluating T-cell toxicity is the greater specificity.”

GEN: In the design and optimization of a CAR T cell– and other T cell–mediated therapies, what are the most crucial characteristics of an assay method used to analyze immune cell–mediated killing kinetics?

Dr. Anderson: Sensitivity, accuracy, and reproducibility are critical. One advantage of a high-throughput technology is the ability to include many controls, which are necessary to ensure that what we are seeing is actually T cell–mediated killing. The xCELLigence instrument also allows us to monitor the health of the target cells, and the software corrects for nonspecific background killing.

Dr. Overstreet: One advantage of xCELLigence assays compared to traditional means of evaluating T-cell toxicity is the greater specificity. Given that we're able to monitor in real time over 3–4 days with these assays, instead of capturing a single time point over 4–6 hours, we can lower our effector-to-target cell ratios down to 0.5–2:1—approaching a 1:1 interaction between T cells and tumor cells. You see slower, steadier killing, which allows for the back-and-forth communication between a tumor cell and a T cell that might be more reflective of what you see in a tumor. At those lower effector-to-target ratios, the specificity of the killing is also improved—dramatically increasing dynamic range.

Dr. Bamdad: The ability to simultaneously test a number of different conditions is important. Using FACS, which is a sequential method, it would have taken us 6–9 months to evaluate our 60 different CARs against various types of cancer cells under different conditions. Using our 6 × 96–plate xCELLigence platform, we were able to analyze 576 conditions in parallel,

“The [xCELLigence] platform's nondestructive analysis allows you to perform any kind of orthogonal readouts to assess the antitumor responsiveness of your T cells, such as flow cytometry or cytokine analysis.”

Michael Overstreet, Ph.D.

enabling us to complete this work in less than a month. We are also able to add reagents to the experiments while they're in progress and assess the effects on cancer killing in real time.

Dr. Golubovskaya: Being label-free, real-time, dose-dependent, and time-dependent are the most important characteristics for immune cell-mediated killing assays. The xCELLigence technology is very good because it is measuring the cytotoxicity of the T cells and the death of the target cancer cells in real time.

GEN: What are the specific advantages of the xCELLigence instrument and the benefits of a label-free assay method? How would you describe its role in early-stage research and commercial product development?

Dr. Overstreet: I've had requests from scientists throughout our organization interested in using the instrument because it is so data rich with its real-time analysis. The label-free aspect of the xCELLigence platform streamlines your workflow. Also, the platform's nondestructive analysis allows you to perform any kind of orthogonal readouts to assess the antitumor responsiveness of your T cells, such as flow cytometry or cytokine analysis.

Combining this instrument with some of the scientific infrastructure and human immunology model development that we've done here, a lot of people are really excited about our approach and our ability to test molecules in a relevant human cell-based system. We hope this will ultimately improve our preclinical modeling and strengthen our rationale for taking molecules into the clinic.

Dr. Bamdad: We are working with the Fred Hutchison Cancer



Vita Golubovskaya, Ph.D.

Director of Research and Development
ProMab Biotechnologies

“The xCELLigence technology is very good because it is measuring the cytotoxicity of the T cells and the death of the target cancer cells in real time.”

Center, and we expect to start human trials later this year. As you near clinical trials, you have to bridge the gap from working on the benchtop and in animals to treating patients. Every CAR T cell and tumor cell we put into animals we look at in parallel on the xCEL-Ligence instrument. So far, the results have perfectly mirrored the results we're getting in animals. That gives us more confidence as we're modifying what we do to move toward human testing.

Dr. Golubovskaya: The advantages and benefits of using a label-free assay are as follows: fewer steps, fewer variables, and a more direct assay. We have the xCELLigence system with 6×96 -well plates and are measuring the cytotoxicity of CAR T cells against cancer cells in real time. We plate different types of cancer cells on day 1, and the next day we add our effector CAR T cells at different effector-to-target ratios, allowing us to analyze cell killing at different time points and at different doses. The system's software allows you to quantify the assay at any time point, so you can see the kinetics of killing. It's a very visual, convenient assay.

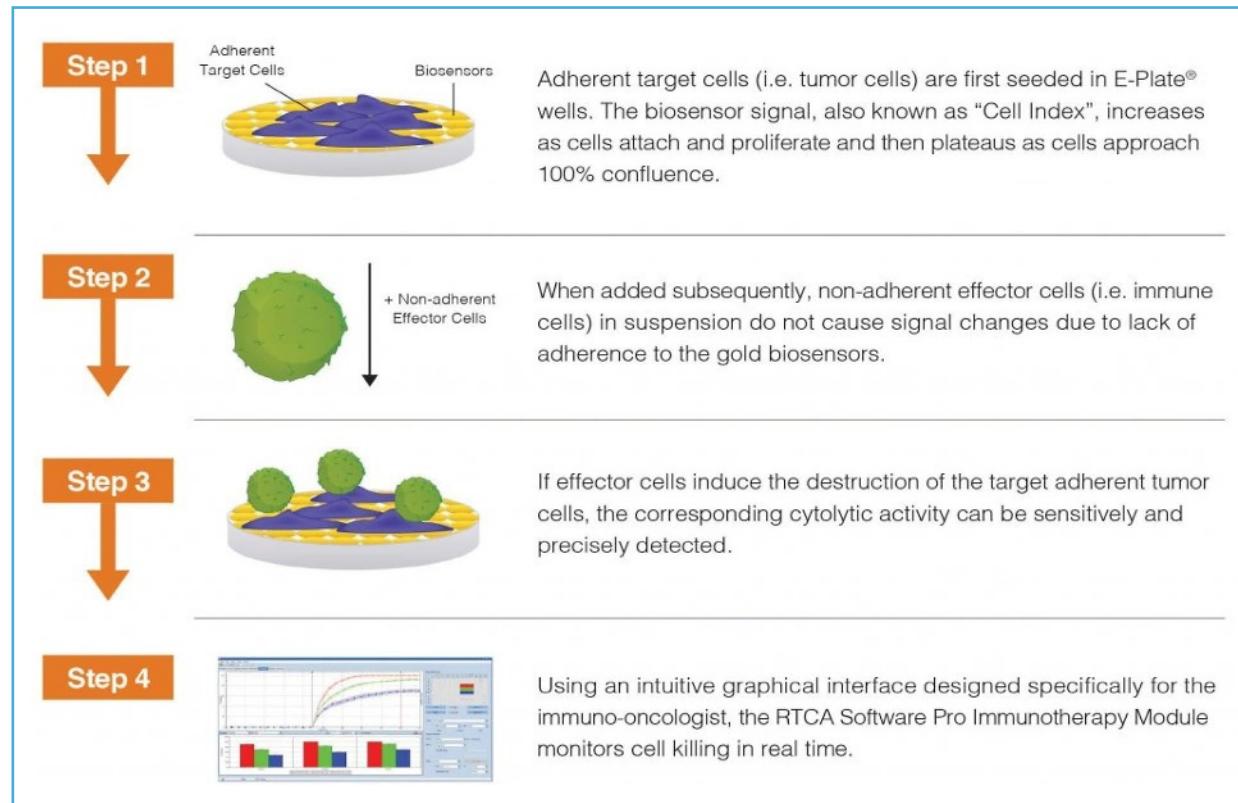
Dr. MacLeod: The biggest benefit is simplicity, which makes the assay easier to run, a little more consistent, and easier to teach to new users. We've mainly been using the instrument at a very early stage, when we're just testing out a large number of different CAR T constructs. The sensitivity of the assay allows us to use very few cells, and because we can detect killing at low effector-to-target cell ratios, we are able to do a lot more screening. ■

Game-Changing Platform Speeds Discovery and Development

Critical to the design and development of effective immunotherapies such as chimeric antigen receptor (CAR) T cells, checkpoint inhibitors, and oncolytic viruses, is the ability to monitor the potency of treatments against target tumor cells *in vitro*. In addition to being robust and simple, the ideal *in vitro* assay should also be highly predictive of how the therapy will behave in animal models and, ultimately, in human patients.

To more closely mimic activity *in vivo*, ACEA Biosciences – now a part of Agilent Technologies – has developed the xCELLigence® Real-Time Cell Analysis (RTCA) platform to quantitatively monitor cancer cell killing over extended time periods. This label-free and noninvasive technology captures the response of adherent tumor cells to different treatments through proprietary biosensors embedded into custom microtiter plates.

The workflow is remarkably simple: plate and incubate target tumor cells, add effector cells to be tested, then let the xCELLigence platform monitor tumor cell killing without further human intervention. Although liquid tumors (that is, blood cancers) are not naturally adherent, they can also be monitored by the xCELLigence



platform through ACEA Biosciences' antibody-mediated tethering kits for use with these custom plates.

The straightforward workflow makes it easy to analyze multiple parameters simultaneously, such as different doses of checkpoint inhibi-

tors or variations of CAR constructs. The high sensitivity of this assay also enables low, physiologically relevant effector-to-target ratios to be examined, and its continuous data monitoring yields a complete and nuanced view of cancer cell killing that is missed by endpoint data. ■

Application Note

Multifunctional Potency Assays for Cancer Immunotherapy

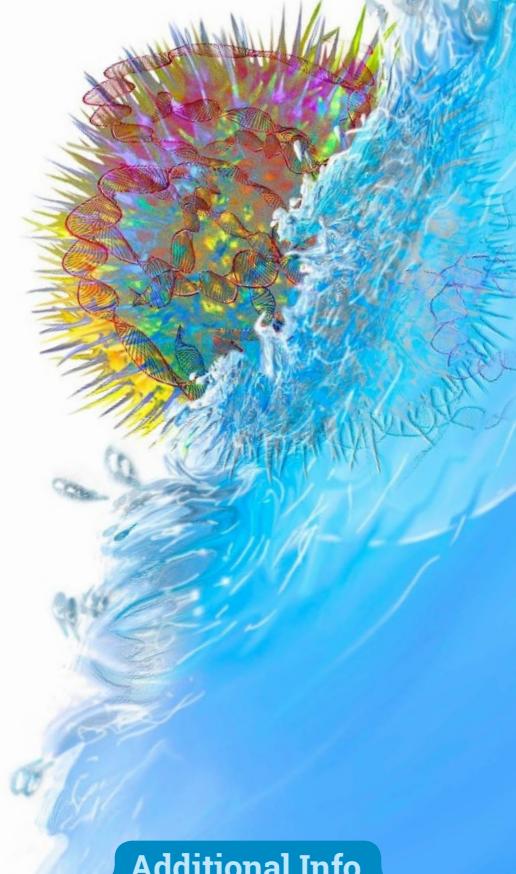
ACEA Biosciences Describes New Tools to Quantify Tumor Cell Killing Enhanced by Bispecific T-cell Engagers

By Lauren Jachimowicz, PhD, Aimee Chiavario, Peifang Ye,
Garret Guenther, PhD, Kenneth Chan, PhD, Jeff Shurong Xue, PhD

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+ cytotoxic T lymphocytes (CTLs), which constitute 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 for tumor immunology studies.

Moving this research from the bench to the clinic is critically important, but reliable tools are needed to design *in vitro* assays that closely mimic activity *in vivo*. In this study, we use an impedance-based technology in combination with a bead-based multiplex flow cytometry assay to evaluate both the target and effector cells in a T-cell-mediated B-cell-killing potency assay (Figure 1). Real-time cell analysis (RTCA) technology with ACEA Biosciences' xCELLigence® system provides us with a continuous readout of target cell viability, while flow cytometry enables the study of T-cell activation and



Additional Info

Download the
xCELLigence
RTCA Cancer
Immunotherapy
Handbook:



functions by measuring cytokine and cytolytic protein secretion.

Representing a promising new class of therapeutics, bispecific T-cell engagers (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, thus enhancing CTL effector function against various B-cell-derived tumors.

Here, the capability of CD19-BiTEs to enhance the cytotoxic effects of T lymphocytes on a B-cell lymphoma cell line (Daudi cells), was evaluated using two distinct assays. Target cell death was monitored using an real-time cell analysis impedance assay while secretion of cytokines and cytolytic proteins was qualified in a bead-based multiplex flow cytometry assay to evaluate the potency of the over multifunctional T-cell response.

Enhancing T-cell-mediated target cell killing

To demonstrate T-cell-mediated target-cell cytotoxicity on the xCELLigence platform, human

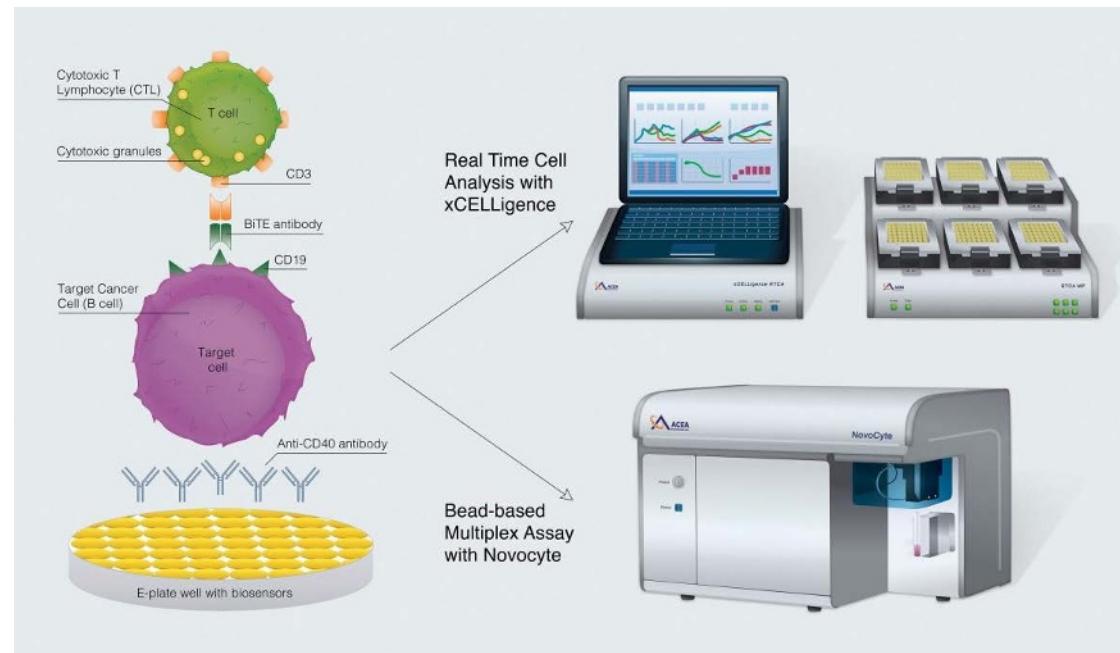


Figure 1. Target cell death was monitored using the xCELLigence RTCA impedance assay while secretion of cytokines and cytolytic proteins was qualified in a bead-based multiplex assay on the NovoCyte flow cytometer to evaluate the T-cell response.

T-cell killing of a B-cell lymphoma cell line (Daudi cells) was assessed. B-cell cancers are prominent immunotherapy targets because they are readily accessible within the bloodstream and are not affected by the microenvironment complexities or mixed tissue types associated with solid tumors. To help accelerate research in this area, ACEA Biosciences developed an xCELLigence Immunotherapy Kit for studying the cell killing of liquid cancers.

In this study, Daudi cancer B cells were immobilized on an xCELLigence E-Plate® precoated with an anti-CD40 tethering antibody. After the Daudi cells were seeded, T cells enriched from primary peripheral blood mononuclear cells (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 anti-CD19 antibody control was also added.

The xCELLigence technology utilizes proprietary E-plates which contain gold biosensors that are able to monitor cell behavior through impedance. The impedance signal of the Daudi cell monolayer was recorded every 15 minutes, and was reported using a unitless parameter called 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

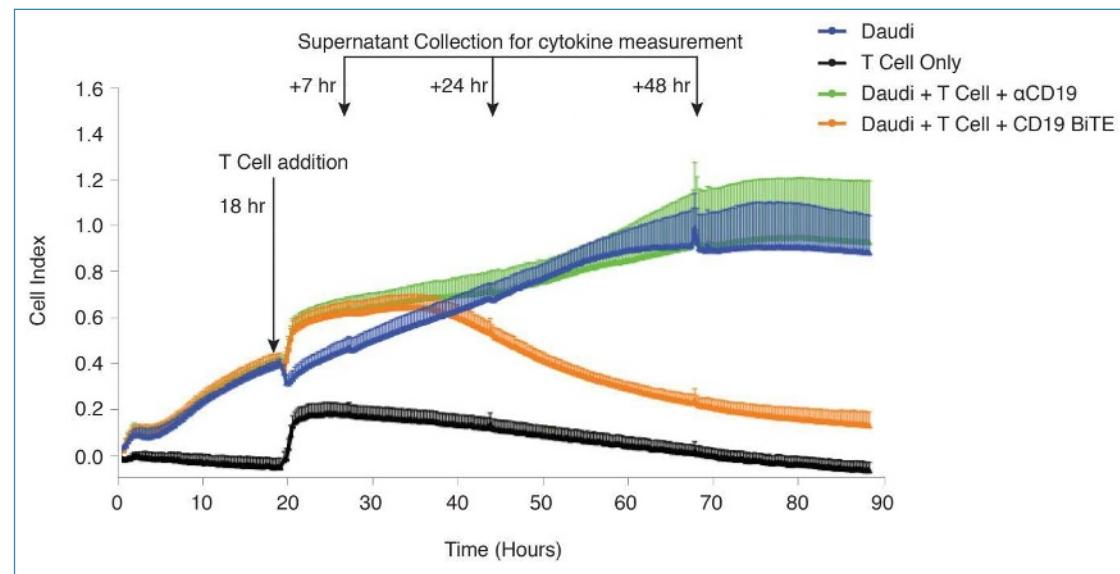


Figure 2. CD19 BiTE enhances T-cell-mediated B-cell killing measured by the xCELLigence RTCA impedance assay. Daudi target cells were seeded at 50,000 cells/well in a 96-well E-Plate® coated with anti-CD40 antibody. Eighteen hours post Daudi cell seeding, enriched human effector T cells from primary PBMCs were added at a ratio of T:Daudi cells of 10:1. At the same time, CD19-BiTE (0.33 µg/mL) or an anti-CD19 antibody (0.33 µg/mL) was added. Supernatant was collected at 7, 24, and 48 hours after the addition of T cells for the protein measurement that followed the experiment. Interaction of cells with gold biosensors is measured through cellular impedance. This impedance value is plotted as the unitless parameter called Cell Index and correlates with cell number, size, and cell-substrate attachment quality. An increase in Cell Index corresponds to target cell proliferation, while a decrease represents target cell killing. The impedance signal was recorded every 15 minutes.

is generated from T cells alone, which can be subtracted as background (Figure 2, black line). Daudi cell growth is undisturbed by the addition of T cells and anti-CD19 antibody as a control (Figure 2, green line). However, a rapid decrease in Cell Index is observed with the addition of the CD19 BiTE in the presence of T cells, indicating that the Daudi target cells are being killed (Figure 2, orange line). This data demonstrates the capability of the CD19 BiTE to enhance T-cell-mediated cytotoxicity of B cancer cells.

Boosting cytokine and cytolytic protein secretion

To further study the effect of CD19-BiTE on T-cell activation and function, cytokine and cytolytic protein secretion was measured. Cells were cultured as described in Figure 2, and supernatant was taken at 7, 24, and 48 hours after the addition of T cells to measure 13 human proteins known to affect T-cell function with a bead-based multiplex assay on the NovoCyte flow cytometer (Figure 3). Consistent with our findings from RTCA, an increased secretion of CTL-associated proteins was observed. This data demonstrates that the presence of CD19-BiTE significantly enhances the production of cytokines and effector molecules that mediate and sustain target cell destruction.

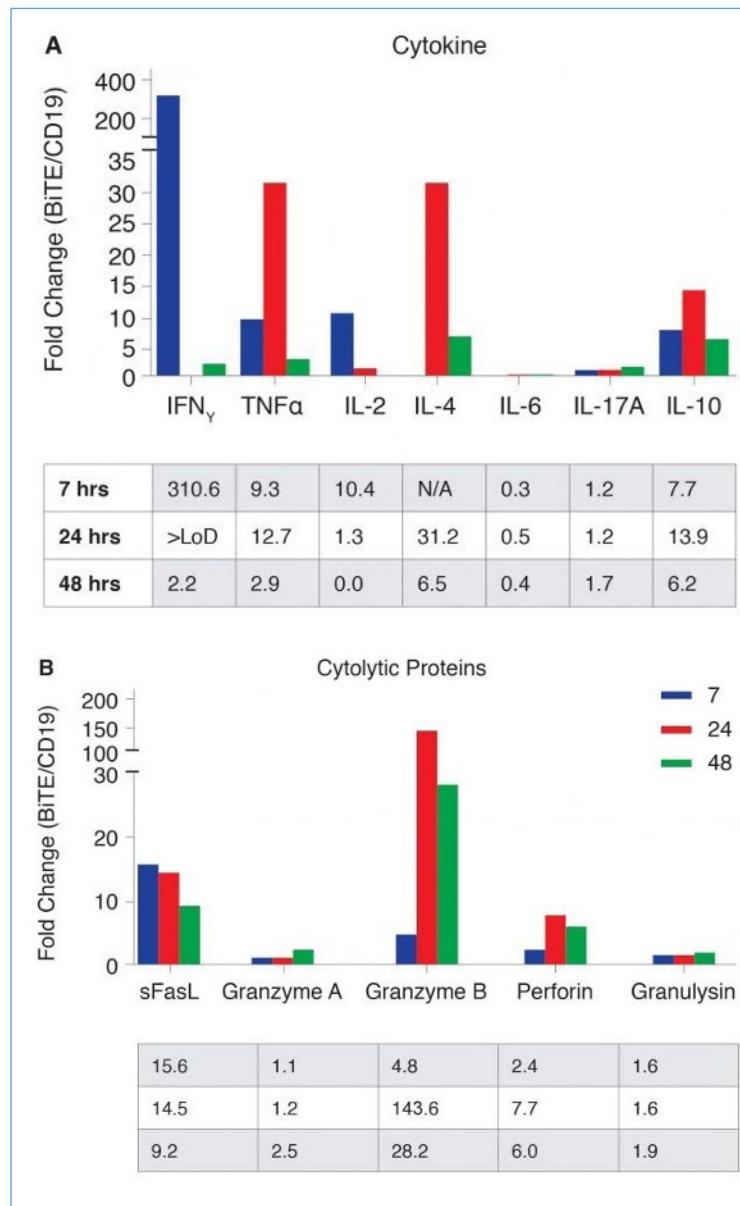


Figure 3. CD19-BiTE enhances the cytotoxic activity of T cells in a bead-based multiplex immunoassay. Daudi target cells were seeded at 50,000 cells/well in a 96-well E-Plate coated with anti-CD40 antibody. Eighteen hours post Daudi cell seeding, enriched human effector T cells from primary PBMCs were added at a ratio of T:Daudi cells of 10:1. At the same time, CD19-BiTE (0.33 μ g/mL) or an anti-CD19 antibody (0.33 μ g/mL) was added. Supernatant was collected at 7, 24, and 48 hours after the addition of T cells for the bead-based multiplex immunoassay. The relative fold change between protein expression of Daudi + T + CD19-BiTE to Daudi + T + α CD19 was determined for cytokines (A) and cytolytic proteins (B).

Seven hours after addition of effector T cells, cytokines specifically associated with a CTL response such as IFNy, TNFa, and IL-2 increased 300-, 9-, and 10-fold, respectively.

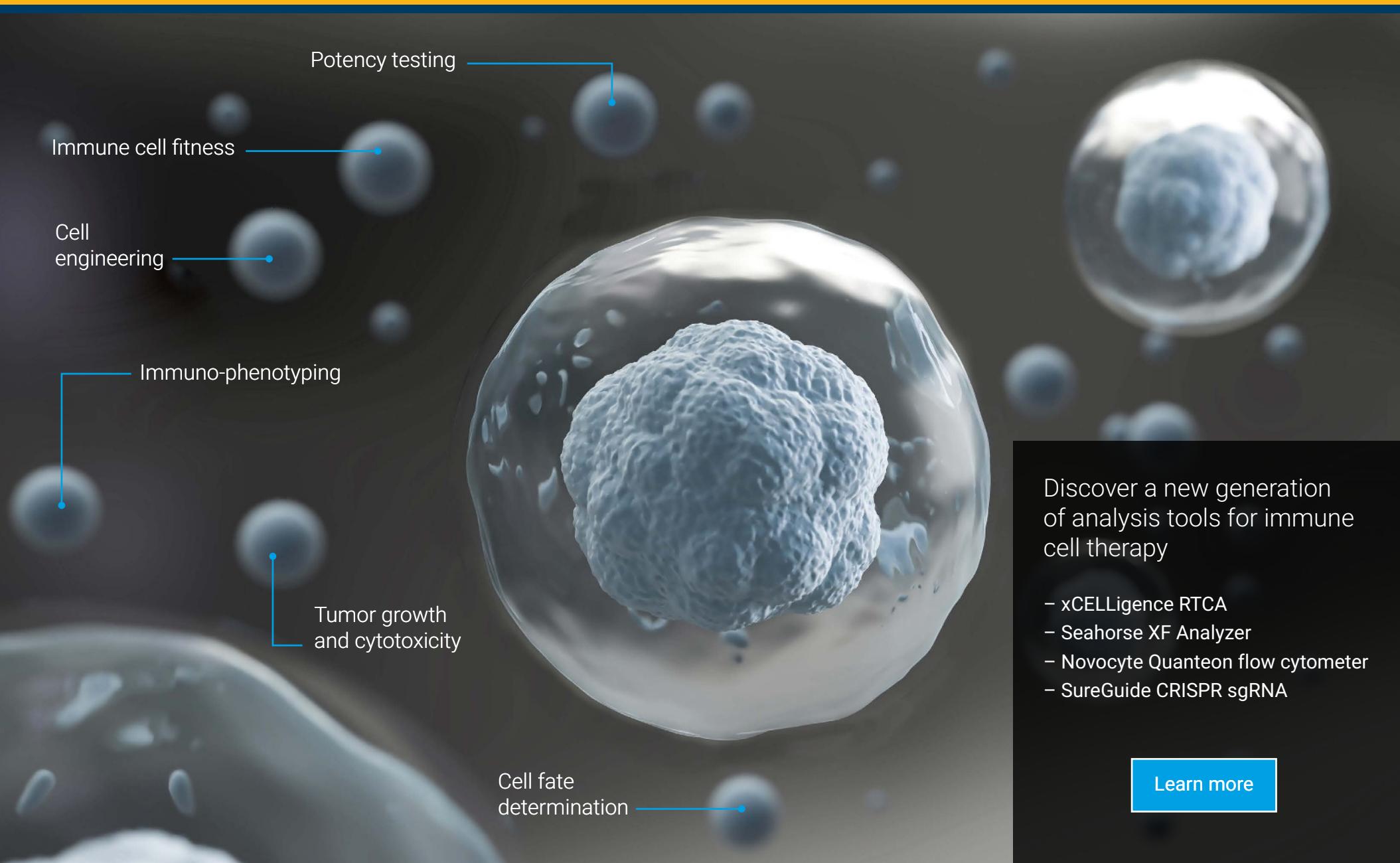
Secretion of cytolytic proteins such as sFasL, granzyme B, and perforin also dramatically increased by 24 hours after the addition of effector T cells, consistent with the CTL-killing response observed. This data demonstrates that CD19-BiT^E enhances T-cell-mediated B-cell killing by increasing the production of cytokines and cytolytic proteins essential for a robust CTL response.

Summary

Here, we have coupled quantitative cell-killing assays with biomarker quantitation to provide an in-depth view of how CD19-BiT^E affects T-cell-mediated killing of B cancer cells in a single workflow. The continuous monitoring of cell number, size, and attachment quality using RTCA enables quantitative and kinetic assessment of the killing process. Linking this cytotoxicity data with quantitative analysis of cytokine and effector protein production allows for simultaneous analysis of T-cell activation and function. This workflow, which integrates both cellular and protein analyses, advances current methods for research of cancer immunotherapy. ■

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