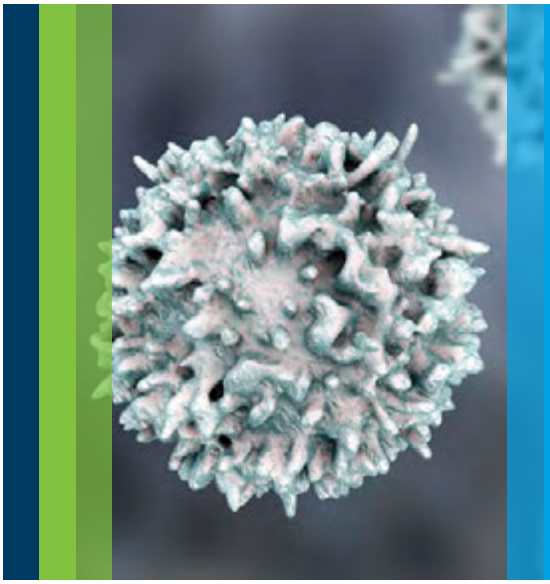


Article

Improving T-Cell Activation and Serial Killing Through Metabolic Modulation

Introduction

T cells are an essential part of the anti-cancer immune response, particularly cytotoxic $CD8^+$ T cells, which can recognize and destroy malignant cells. Harnessing the killing potential of T cells through immunotherapy has revolutionized cancer treatment and significantly improved patient survival outcomes.[1] T cell-based immunotherapies take several different forms, including checkpoint inhibitors (which block the binding of inhibitory proteins to T cell inhibitory receptors) and chimeric antigen receptor (CAR)-T cells (in which cells are engineered to express synthetic antigen receptors that direct and enhance cancer cell targeting).

Despite the successes of T cell immunotherapies, the tumor microenvironment (TME) poses a key barrier to efficient anti-tumor responses, limiting the success of CAR T cells against solid tumors. T cell metabolic fitness is essential to meet the demands of mounting an appropriate immune response, and to ensure survival, expansion and differentiation. While strong stimulation can promote fitness by enhancing survival and effector functions, over-stimulation can result in an exhausted, non-functional phenotype. The TME is highly hostile to immune cells and creates a nutrient-deficient environment that inhibits the metabolism, and therefore functions, of T cell-based immunotherapies.[2] Subsequently, T cells – and T cell-based immunotherapies – become exhausted, showing overexpression of inhibitory receptors, metabolic dysfunction, decreased cytokine production and reduced cytolytic activity.[3]

Recent research has shown that the inhibitory effects of the TME on immunotherapy effectiveness could be counteracted by engineering T cell metabolism to improve persistence and fitness. Strategies include adjusting cytokine profiles during manufacture and manipulation of T-cell differentiation to improve persistence in CAR T cells.[4,5] Combination of checkpoint therapies with other synergistic metabolic drugs, or with other immunotherapies such as CAR T cells or cancer vaccines, is also being attempted.[6]

If the efficacy of immunotherapies is to be improved through metabolic modulation, then highly accurate measuring of T cell metabolism and persistence is of the utmost importance. This editorial will discuss how T cell metabolism is being evaluated and manipulated for the improvement of T cell-based immunotherapies, and the platforms — both established and novel — involved in advancing this field.

Agilent

Trusted Answers

Boosting T cell activation, persistence and serial killing

In order to improve the efficiency of immunotherapies, it is essential to fully understand the complete and changing metabolic profiles of T cells as they develop from naïve to activated to memory phenotype. Naïve and resting memory T cells are maintained in a quiescent state. In this state, energetic demand is low and cells rely on the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS) for catabolic energy production. In this scenario, fatty acids, glucose and amino acids are used to generate adenosine triphosphate (ATP) for energy (Figure 1).[7,8] However, once T cells are activated, they switch to anabolic metabolism. In this state, glucose is fermented into lactate by aerobic glycolysis, instead of being oxidized in the mitochondria. Anabolism promotes cell growth and proliferation; ATP is produced by glycolysis, and glucose, fatty acids and amino acids are used to manufacture cellular components.[8]

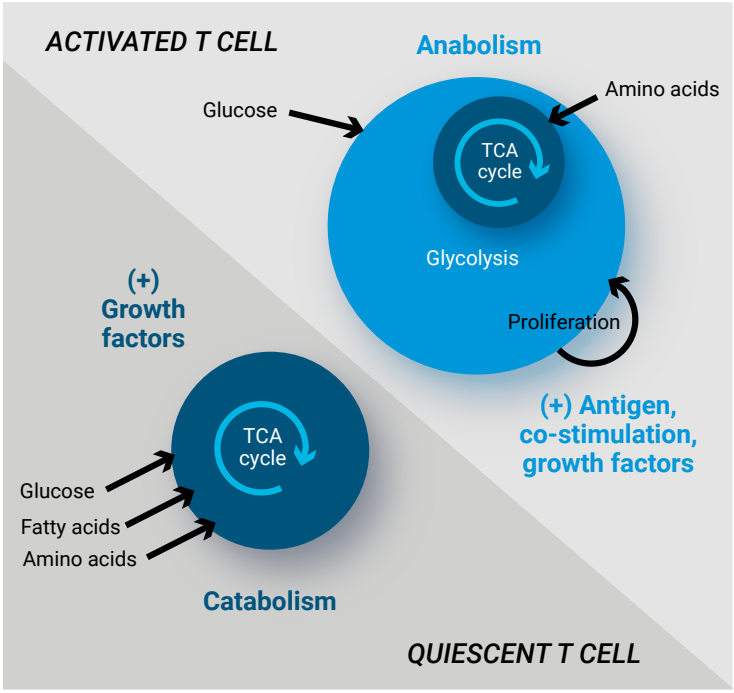


Figure 1. The metabolic phenotypes of active and quiescent T cells.

The metabolic profiles of quiescence and activation

Maintenance of the quiescent state is an active process. It relies on retention of the cell cycle in the G0 stage with low mitochondrial, translational and transcriptional activity.[7] Signaling through molecules such as interleukin (IL)-7, is essential for maintaining homeostasis, as engagement of IL-7 with its receptor prevents atrophy of quiescent cells.[7,9,10] Activation of particular transcription factors – such as forkhead box class O (FOXO) family members – contributes to T cell quiescence too, which promotes expression of cellular activation inhibitors.[8]

Regulatory T (T_{reg}) cells are also essential in maintaining T cell quiescence through expression of co-inhibitory receptors such as cytotoxic T lymphocyte antigen 4 (CTLA-4), depleting proliferation-promoting cytokines and particular metabolic signaling.[7,11] One of the main T_{reg} coordinators of metabolic control of quiescence is FOXP3, which inhibits glycolysis and upregulates OXPHOS, even in nutrient- or oxygen-restricted environments.[12]

The switch from T cell quiescence to activation and differentiation requires a complete shift in metabolic activity that begins when an antigen (or a CD3/CD28 activator) is presented to the T cell receptor (TCR) and CD28 co-receptor (Figure 2).[13,14] The signaling pathways stimulated by receptor binding induce the expression of transcription factors that promote proliferation, initiation of the cell cycle, differentiation and a robust increase in glycolytic activity. Of these, one of the most crucial regulators is the mammalian target of rapamycin (mTOR) pathway.[15] In addition to the internal

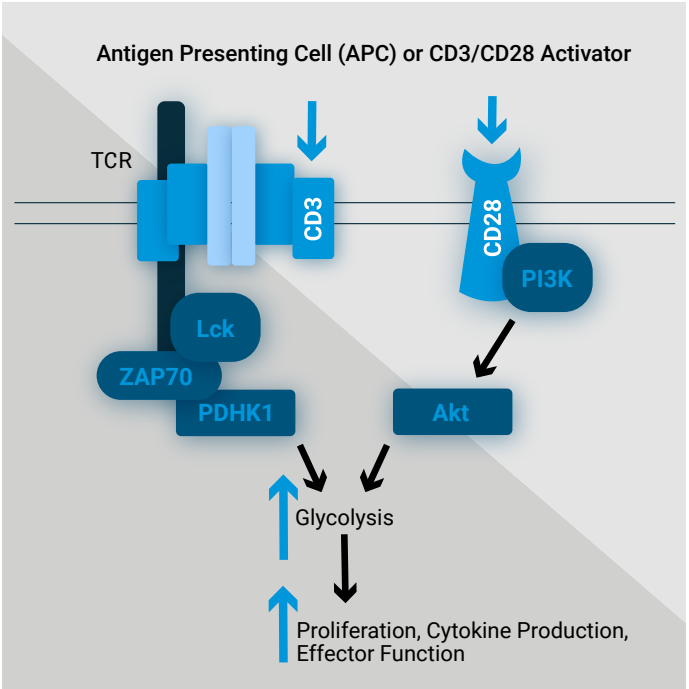


Figure 2. APC or CD3/CD28 activators induce quiescence exit and initiate a metabolic shift in T cells.

cellular processes, external metabolic cues also support T cell activation, including the presence of certain cytokines (e.g., IL-2), the availability of nutrients and redox and oxygen sensing.[7]

The signaling cascades initiated by TCR engagement lead to metabolic reprogramming by changing the structure and function of the cell's mitochondria. Early on, following quiescence exit, activation of pyruvate dehydrogenase kinase 1 (PDHK1) inhibits movement of pyruvate into the mitochondria, pushing the cell towards glycolysis.[16] In activated effector T (T_E) cells, fission of cristae in the mitochondria leads to reduced efficiency of the electron transport chain and promotion of aerobic glycolysis, as determined by measuring the extracellular acidification rate (ECAR).[17]

Reprogramming metabolism

Analysis of T cell metabolism is essential in understanding the dynamics of T cell activation and effector functions. Levels of glycolysis can be measured in real time as a signature of efficient T cell activation, as the switch to glycolysis as an energy source is directly linked to effector function.[16] As a consequence, assessment of glycolysis and mitochondrial activity by an instrument such as the Agilent Seahorse XF can give real-time insights into the efficacy of T cell-based immunotherapies, and the effects of reprogrammed metabolism to enhance immune responses.[18,19]

During normal immune function, T cells undergo metabolic reprogramming to shift from oxidative functions that support immune surveillance, to anabolic metabolism to support rapid growth and proliferation. This metabolic fitness, and the ability to use surrounding nutrients are modulated by immune checkpoints.[20] The TME is extremely disruptive to T-cell metabolism. Rapidly proliferating tumor cells also engage in aerobic glycolysis, depleting nutrients and consequently limiting the metabolism and function of activated T cells.[21] This depletion of glucose can also affect T-cell differentiation, favoring T_{reg} cells over cytotoxic T cells, and leading to an immunosuppressive environment.[22] Over time, repeated stimulation of the TCR on cytolytic $CD8^+$ T cells leads to T cell exhaustion, and a loss of function.[22] For CAR T cells, this can result in inefficient or transient effects, an inability to infiltrate solid tumors or failure to prevent relapses.

Studies have shown that assessing metabolic capacity in real time by measuring the oxygen consumption rate (OCR) and the ECAR of CAR T cells can predict their therapeutic potential.[23,24] Several methods of metabolic reprogramming have shown improved persistence and effector function in T cells, including transient glucose restriction during later rounds of proliferation to induce a shift to a more anabolic profile, enhancing tumor clearance.[25] In a recently published Agilent AppNote, it has been shown that arginine preconditioning enhances T cell cytotoxicity and mitochondrial respiration.[26] In addition, engineering receptor pathways can facilitate increased persistence; for example, blocking PPAR gamma coactivator 1α (PGC-1α) can also prevent T cell exhaustion, by limiting the mitochondrial defects that eventually lead to loss of function and self-renewal.[27]

Further insights into improving CAR T cell persistence have been gained by examining the metabolic profiles of T cells in other states, such as resting memory T (T_M) cells. Once T_M cells are established, they can persist for extremely long periods of time, up to the entire lifetime of the individual.[28] T_M cells also exhibit greater anti-tumor responses than fully differentiated T_E cells, leading to the hypothesis that CAR T cells would be more effective with less differentiated, more T_M-like phenotypes.[5] Much like naïve T cells, T_M cells display aerobic metabolism, with a similar mitochondria structure that favours OXPHOS.[17]

Efforts to direct CAR T cells towards a T_M-like phenotype have shown considerable success via several methods. For example, CAR T cells engineered to express decreased levels of coinhibitory receptors show a more memory-like phenotype and prolonged anti-tumor activity.[29] In addition, strategies that induce an increase in mitochondrial metabolism also increase a stem-like phenotypic state, known as stem cell-like memory T cells (T_{SCM}). These long-lived memory cells show enhanced self-renewal and multipotentcy, as well as enhanced anti-tumor activity compared to conventional T_M cells.[30] Pharmacological manipulation of metabolism has also shown positive results, as cells cultured with pharmacologic inhibitors of Akt (a serine/threonine kinase involved in transcriptional control of glucose metabolism), exhibit improved anti-tumor immunity with memory-like qualities.[31] Expanding CAR T cells in IL-15 rich media inhibits mTORC pathway activity, resulting in less differentiated cells that express reduced exhaustion markers, increased anti-apoptotic qualities and increased proliferation when activated.[32]

Postponing T cell exhaustion

Following the peak of effector function, T cells may become exhausted and nonresponsive. This occurs through chronic stimulation with antigens, for example from chronic viral

infection, or prolonged stimulation with tumour antigens coupled with the inibitory TME in cancer (Figure 3). Exhausted T cells show metabolic insufficiency, which results in the inability to respond to further antigen appearances, reduced cytokine production and loss of self-renewal.[33,34] As with the other stages of the T cell life cycle, this too comes with its own metabolic profile, namely a reduction in glycolysis, impaired mitochondrial dynamics and a greatly reduced spare respiratory capacity (SRC) – the amount of extra ATP that can be produced by OXPHOS to meet sudden demand.[18] The status of T cells can be assessed by measuring metabolic activity, or by examining markers on the T cell surface using flow cytometry, as coinhibitory receptors are upregulated as T cells move into exhaustion.[35]

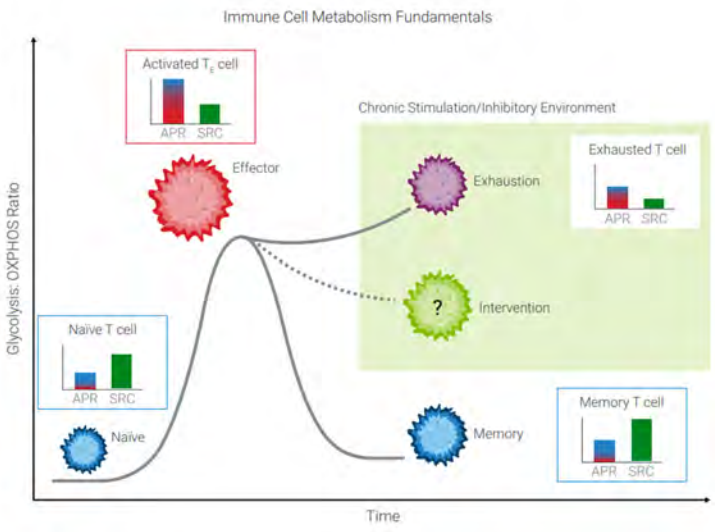


Figure 3. The life cycle of a T cell and corresponding metabolic profiles.

In the pursuit of improved efficacy and prolonged survival for CAR T cells, T cell exhaustion is an essential factor to consider. However, since exhaustion protects T_E cells from overstimulation-induced death, impairing exhaustion may also limit persistence, so a balance must be struck to optimize immunotherapies.[36] Several methods have been explored to stave off T cell exhaustion, including use of co-stimulatory domains in the CAR molecule and culturing cells with different cytokines.[4] One of the most widely used co-stimulatory domains is 4-1BB, which is highly expressed on exhausted T cells. Use of 4-1BB co-stimulation has been shown to increase mitochondrial capacity and provide a strong anti-tumor response when combined with immune checkpoint inhibitors.[37] When 4-1BB domains are incorporated into CAR molecules, CAR T-cell persistence is increased in populations of central memory T cells, with significantly enhanced respiratory capacity and mitochondrial biogenesis, as measured using a Seahorse XF analyzer.[38]

Cytokines including IL-2, IL-7, IL-15 and IL-21 have been explored for the enrichment of CAR T cell culture medium.[4] CAR T cell populations must proliferate to large numbers in order to mount a full anti-tumor response. Currently, *ex vivo* proliferation strategies are based around IL-2, but this cytokine also drives the cells towards terminal differentiation and imminent exhaustion, limiting their effectiveness. Alternative approaches are being explored, such as the use of IL-21, which promotes a more quiescent-like state, using OXPHOS for energy and prolonging cell survival.[39]

The importance of CAR T cell serial killing

Cytoxic T lymphocytes (CTLS) kill target cells through cell-to-cell interaction and the delivery of cytolytic enzymes, and are able to engage with multiple target cells in a serial manner. Highly motile *in vivo* CTLs have been estimated to kill up to 20 target cells per day, requiring persistence and prolonged metabolic fitness.[40] The most efficient and effective CAR T cells must not only be capable of persisting for prolonged periods of time and avoiding exhaustion, but they must also be capable of continued serial killing. Cytolytic kinetics and the rate of serial killing can be assessed using real-time cell analysis systems, such as the xCELLigence RTCA technology. For example, a study performed using solid tumor cells showed that engagement of CARs by antigens showed equal rates of serial killing when compared to TCR engagement. However, after 20 hours, this killing became limited as CARs were downregulated, suggesting that serial killing needs to be combined with improved CAR persistence to boost therapeutic efficacy.[41] One such method could be the use of co-stimulatory domains. In addition to 4-1BB co-stimulatory domains being incorporated into CAR molecules, research has also shown that expressing 4-1BB ligand (4-1BBL) on the surface of CAR T cells increases their ability for serial tumor cell killing in a cellular impedance-based cytotoxicity assay.[42]

Previous research has shown that long-term effector functions can also be supported by arming activated T cells with bispecific antibodies.[43,44] This strategy of arming effector cells with tumor-specific antibodies can be extended to CAR T cells. A study in which CAR T cells were loaded with bispecific antibodies targeting two common tumor antigens showed that the CAR T cells were able to successfully carry out serial killing of target cells for up to 19 days and 4 rounds of killing, without becoming exhausted.[45]

Circumventing the immunosuppressive TME

The immunosuppressive, hypoxic environment of the TME severely inhibits the fitness and function of *in vivo* T cells, through a number of mechanisms, including nutrient limiting and boosting T_{reg} function. This effect extends to CAR T cell therapies, and despite their successes for hematological cancers, the TME continues to limit progress of CAR T cell therapies for solid tumors. Early onset T cell exhaustion can occur in solid tumors due to overstimulation coupled with a nutrient-depleted environment, though several avenues are being explored in an effort to counteract the effects of the TME.[3] For example, real-time cell killing assays have shown that synthetic cytokine receptor signaling can push T cells towards a more persistent, stem-like phenotype, resulting in improved anti-tumor responses in difficult-to-treat solid tumor models.[46] Again, expression of co-stimulatory domain 4-1BB on CAR T cells improves tumor infiltration, and the mitochondrial changes induced by the domain support the resistance of CAR T cells to the TME, particularly when combined with immune checkpoint inhibitors.[37]

The TME plays host to a wide range of immunomodulatory factors which, along with the lack of nutrients and oxygen, downregulate T cell effector functions. T_{reg} cells play a substantial role in this, by secreting immunosuppressive cytokines such as IL-10 and transforming growth factor β (TGF-β) and expressing inhibitory cell-surface proteins such as CTLA-4. Production of these T_{reg} cells is promoted by factors such as the metabolic enzyme indoleamine 2,3-dioxygenase (IDO), which is highly expressed in the TME.[47] Limiting these immunosuppressive factors could be an alternative method for enhancing immunotherapy effectiveness in solid tumor cancers. So far, the use of CRISPR/Cas9 to knock out the TGF-β receptor gene on CAR T cells has shown success. Knockout cells continued to show long-term, serial cytotoxic effects in a real-time cellular impedance-based assay even when exposed to high levels of TGF-β, outperforming CAR T cells still expressing the receptor.[48]

Across the spectrum of research to support the optimization of T-cell based immunotherapies, the key themes are supporting T-cell activation, remodelling metabolic capabilities and preventing exhaustion in order to prolong persistence and serial killing. With such a diverse body of research, a versatile and comprehensive range of instruments is required to support the ongoing improvement of cancer immunotherapies and patient survival.

The complete Agilent solution for CAR T cell optimization

Cell-based immunotherapies such as CAR T cells are "living drugs" with complex, multifaceted mechanisms of action. Throughout research and process development, they are required to meet defined safety, purity and potency requirements, making the collection of robust, accurate data essential. Analysis and modulation of T-cell metabolism and persistence can help to reach these requirements and produce more efficacious immunotherapies. However, numerous investigations resulting in multiple data sets can be complicated, and tax available resources. Agilent offers a complete range of solutions to simplify and enhance workflows for T cell-based immunotherapy research and process development. From essential instruments in immunometabolism with unquestionable pedigree such as the Seahorse XF analyzers, to innovative xCELLigence Real-Time Cell Analyzers, NovoCyte flow cytometers and Cytation Imaging Readers, Agilent instruments can help accelerate and advance discoveries, process development, manufacturing, QC release and meet approval criteria.

Seahorse XF analyzers

The metabolic profiles of T cells change significantly over the course of quiescence, activation, killing and exhaustion. Analysis and modulation of these changes can provide insights into the cells' ability to proliferate, differentiate and carry out effector functions. The Seahorse XF technology is an all-in-one, live cell metabolic assay platform that can perform automatic, real-time measurements of oxygen consumption rate (OCR), proton efflux rate (PER) and ECAR, as well as calculate the rates of ATP production from mitochondrial respiration and glycolysis, thereby providing a complete assessment of cell bioenergetics. The extremely high sensitivity of the Seahorse XF technology allows the measurement of OCR even at very low levels, enabling accurate assessment of even quiescent cell types. Since its launch in 2006, the Seahorse has been used in over 17,000 peer-reviewed publications and is is a key contributor to the current understanding of immunometabolism and the recognition of the fundamental role of the metabolic changes that occur during T cell activation and differentiation.[49,50]

Seahorse XF technology and assay kits enable straightforward and precise measurement of metabolic activity in T cell and CAR T cell function, offering profound insights into their metabolic roles. For instance, the Seahorse

XF T Cell Metabolic Profiling kit facilitates comprehensive and robust analysis of T cell metabolism by simultaneously measuring glycolytic and mitochondrial activity in real time under basal and stressed conditions (Figure 4). The XF Human T Cell Activation Assay kit provides a rapid and standardized method to assess early metabolic responses associated with activation in human T cells, aiding in the understanding of the initial activation dynamics and the impact of test compounds on both naïve and activated T cells.[18]

xCELLigence RTCA analyzers

Persistence and survival alone are not sole determinants of an efficient for an efficient CAR T cell therapy. Cells must be able to enact serial killing and maintain effector functions long term without entering an exhausted state. In addition, accurate potency data is essential to meet approval criteria.

Cellular impedance assays are an increasingly popular method of measuring potency, and overcome many of the challenges of other traditional endpoint assays, as they do not require radiation, are label-free and non-invasive. The Agilent xCELLigence Real-Time Cell Analysis (RTCA) platform offers a robust, simple and automated solution for cellular impedance assays. The system measures quantitative, real-time kinetics, allowing the continuous measurement of both proliferation and long-term potency of CAR T cells, generating a more comprehensive insight into T cell function and serial killing than endpoint assays (Figure 5). The 21 CFR Part 11 compliant RTCA Software Pro is intuitive and easy-to-use, and its Immunotherapy module enables measurement of cytolysis of target tumor cells in a highly automated fashion.

To help tackle the barriers to effective immunotherapy posed by solid tumors, an extravasation assay workflow has been developed using the xCELLigence RCTA platform.

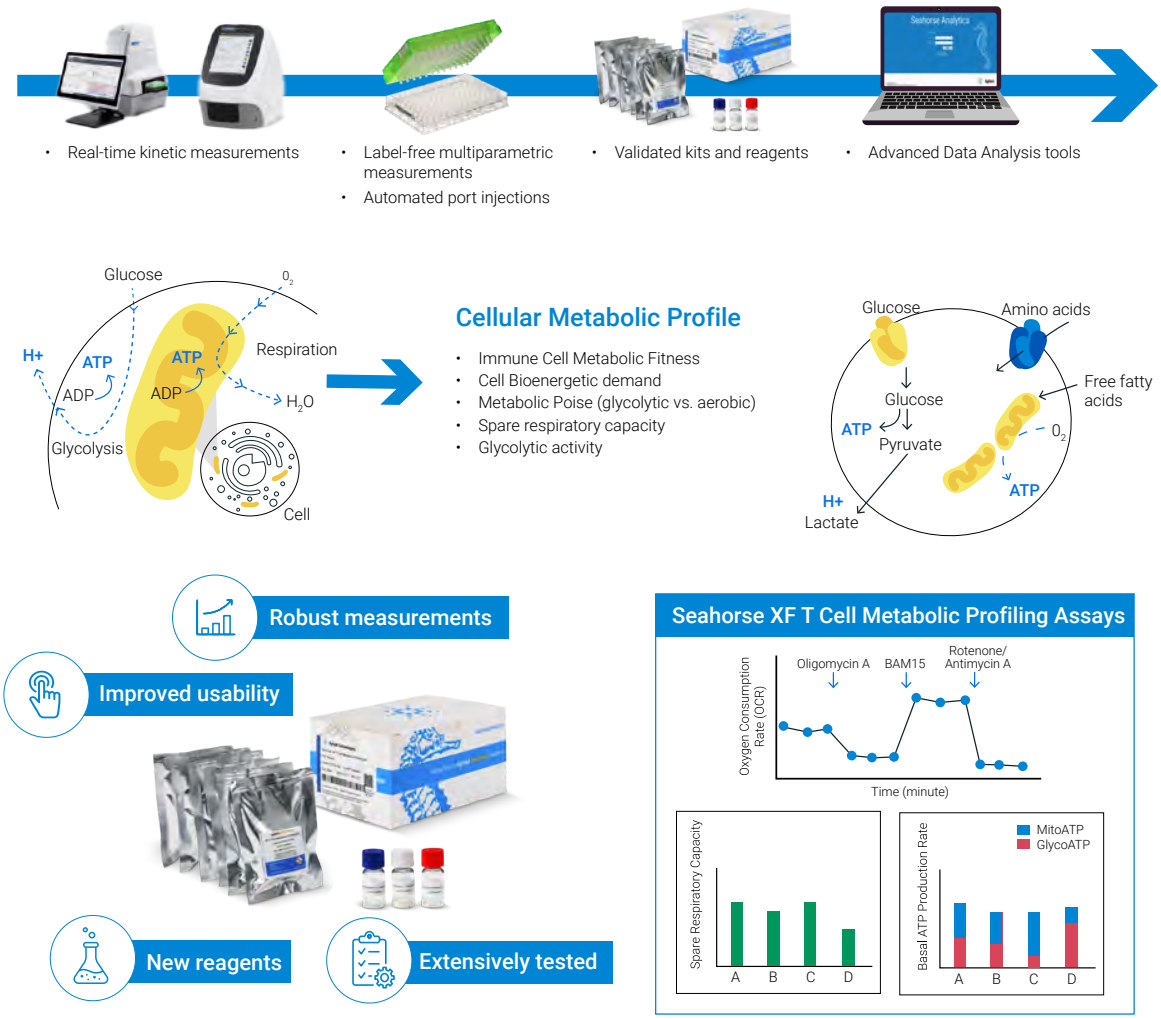
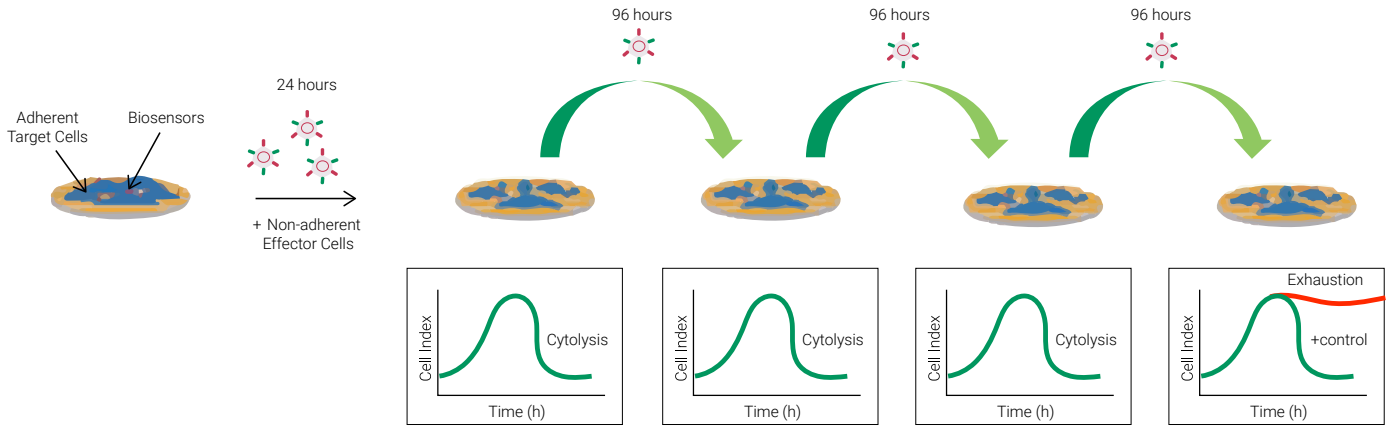


Figure 4. The Agilent Seahorse XF T Cell Metabolic Profiling Kit: Designed for T cells.

Workflow

xCELLigence RTCA Immune Cell-Mediated Killing Assay (Serial Killing)



Step 1

Plate target cells

Step 2

At 24 hours add effector T cells

Step 3

At 96 hours remove media and T cells, plate on fresh cells. Cytolysis is assessed until >50% of effector cells stop killing.

Figure 5. The xCELLigence RTCA immune cell-mediated serial killing assay workflow.

This simulates an extracellular membrane layer, successfully replicating conditions for invasion and cytotoxicity of CAR T cells in solid tumors.[51] In addition, the latest xCELLigence RTCA eSight combines impedance technology with live cell imaging, allowing continuous, label-free visualization of T cell anti-tumor activity. The eSight can automatically recognize markers of immune cell activation such as clustering, allowing researchers to track cells for real-time monitoring and analysis.[52]

NovoCyte flow cytometers

Over a decade of development in flow cytometry has given rise to the NovoCyte range of flow cytometers, including the NovoCyte Penteon, NovoCyte Quanteon, NovoCyte Advanteon and Novocyte Opteon. These instruments offer a highly sensitive, highly flexible method of analyzing T cell activation, proliferation and differentiation. With up to 5 lasers accommodating up to 73 different fluorescence channels, NovoCyte flow cytometers can process both FACS tubes and up to 384-well plates. The accompanying 21 CFR Part 11 compliant software, NovoExpress, is intuitive and industry-leading, providing an easy-to-use interface and exceptional data acquisition, analysis and reporting.

NovoCyte flow cytometers offer a range of capabilities to enhance T cell research and immunotherapeutic development, such as automated cell cycle analysis. The fluorescent dye carboxyfluorescein succinimidyl ester (CFSE) is transferred to daughter cells, and enables tracking of cell proliferation and survival, which is automatically calculated using the NovoExpress software.[53] Flow cytometry assays can be performed to analyze T cell activation, differentiation and exhaustion markers, providing insight into the status of T cells and the effects of metabolic manipulation.[54]

Automated live-cell imaging microplate reader systems

Effective immunotherapies cannot be developed without robust orthogonal approaches that support an in-depth understanding of the cellular mechanisms of immune cells and their interactions with tumor cells. Agilent microplate readers provide intuitive, well-established platforms for quantifying immune cell activation via ELISA and other assay formats, while Agilent liquid handlers and automation solutions support increased throughput and reliability. Agilent's automated imaging systems, including the BioTek Lionheart FX automated microscope and the Cytation live-cell imaging systems, offer high-throughput solutions to address the needs of both routine experiments and sophisticated, mechanistic experimental design. These imaging systems are able to efficiently capture a large sample field of view, including entire well areas in 384-well microplates, in a single

image. Flexible kinetic imaging protocols allow user-defined intervals and duration to generate complete profiles of T cell activation and efficacy. The accompanying software automatically calculates and reports key metrics for immune cell activation such as clustering and target cell killing. In addition, brightfield and widefield imaging are supported for 3D cell culture models to give more detailed insight into cell behavior and survival.

Conclusion

T-cell based immunotherapies have shown great success, but still have challenges to overcome, such as treatments for solid tumors, and prolonging effective killing and persistence. As CAR T-cell therapies continue to develop further, metabolic manipulation could be the key to overcoming these limitations, supported by rapid, automated and highly accurate analysis of metabolism and serial killing. One such example is the recent development of metabolically enhanced CAR T cells armed with bispecific antibodies, developed and evaluated using a workflow incorporating the Agilent Seahorse XF, the xCELLigence RCTA and the NovoCyte flow cytometer. The study showed that these cells were safe, effective serial killers of solid tumor targets, and demonstrated the ability of the Agilent portfolio to support research breakthroughs and engineer improved therapies.[55]

Agilent offers end-to-end solutions for T cell immunotherapies, ranging from foundational research into T cell metabolism, through process development, to potency assays to meet approval criteria for clinical release (Figure 6). Both as a whole and individually, these instruments can support a comprehensive understanding of T cell functionality and the creation of life-saving novel therapeutics.

Explore the Agilent portfolio to optimize your CAR T cell development

References

1. Want MY, Bashir Z, Najar RA. T Cell Based Immunotherapy for Cancer: Approaches and Strategies. *Vaccines*. 2023;11(4):835. doi: 10.3390/vaccines11040835

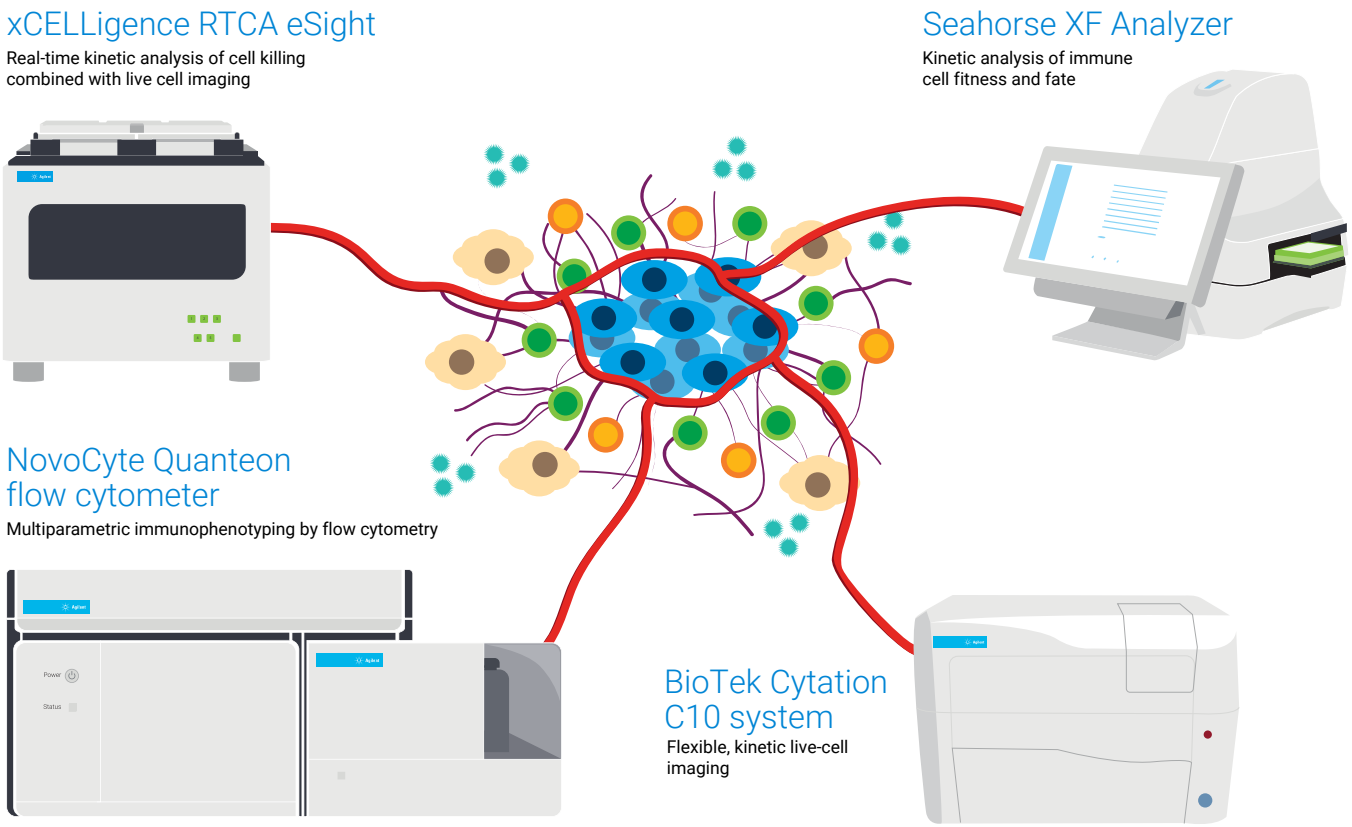


Figure 6. End-to-end solutions from Agilent.

2. Rangel Rivera GO, Knochelmann HM, Dwyer CJ, et al. Fundamentals of T Cell Metabolism and Strategies to Enhance Cancer Immunotherapy. *Front Immunol*. 2021;12:645242. doi: 10.3389/fimmu.2021.645242
3. Jiang W, He Y, He W, et al. Exhausted CD8+T cells in the tumor immune microenvironment: New pathways to therapy. *Front Immunol*. 2021;11. doi:10.3389/fimmu.2020.622509
4. Huang Y, Si X, Shao M, Teng X, Xiao G, Huang H. Rewiring mitochondrial metabolism to counteract exhaustion of CAR-T cells. *J Hematol Oncol*. 2022;15:38. doi: 10.1186/s13045-022-01255-x
5. López-Cantillo G, Urueña C, Camacho BA. CAR-T Cell Performance: How to Improve Their Persistence? *Front Immunol*. 2022;13:878209. doi: 10.3389/fimmu.2022.878209
6. Luby A, Alves-Guerra M-C. Targeting Metabolism to Control Immune Responses in Cancer and Improve Checkpoint Blockade Immunotherapy. *Cancers*. 2021;13(23):5912. doi: 10.3390/cancers13235912
7. Chapman NM, Boothby MR, Chi H. Metabolic coordination of T cell quiescence and activation. *Nat Rev Immunol*. 2020;20(1):55–70. doi: 10.1038/s41577-019-0203-y
8. Pearce EL. Metabolism in T cell activation and differentiation. *Curr Opin Immunol*. 2010;22(3):314–320. doi: 10.1016/j.coi.2010.01.018
9. Jacobs SR, Michalek RD, Rathmell JC. IL-7 is essential for homeostatic control of T cell metabolism *in vivo*. *J Immunol*. 2010;184(7):3461–3469. doi: 10.4049/jimmunol.0902593
10. Kimura MY, Pobezinsky LA, Guinter TI, et al. IL-7 signaling must be intermittent, not continuous, during CD8+ T cell homeostasis to promote cell survival instead of cell death. *Nature Immunol*. 2013;14:143–151. doi: 10.1038/ni.2494
11. Chinen T, Kannan AK, Levine AG, et al. An essential role for the IL-2 receptor in T_{reg} cell function. *Nat Immunol*. 2016;17(11):1322–1333. doi: 10.1038/ni.3540
12. Angelin A, Gil-de-Gómez L, Dahiya S, et al. Foxp3 Reprograms T Cell Metabolism to Function in Low-Glucose, High-Lactate Environments. 2017;25(6):1282–1293. doi: 10.1016/j.cmet.2016.12.018
13. Sauer S, Bruno L, Hertweck A, et al. T cell receptor signaling controls Foxp3 expression via PI3K, Akt, and mTOR. *PNAS*. 2008;105(22):7797–7802. doi: 10.1073/pnas.0800928105
14. Klein Geltink RI, O'Sullivan D, Corrado M, et al. Mitochondrial Priming by CD28. *Cell*. 2017;171(2):385–397. doi: 10.1016/j.cell.2017.08.018
15. Yang K, Shrestha S, Zeng H, et al. T Cell Exit from Quiescence and Differentiation into Th2 Cells Depend on Raptor-mTORC1-Mediated Metabolic Reprogramming. *Immunity*. 2013;39(6):1043–1056. doi: 10.1016/j.immuni.2013.09.015
16. Menk AV, Scharping NE, Moreci RS, et al. Early TCR Signaling Induces Rapid Aerobic Glycolysis Enabling Distinct Acute T Cell Effector Functions. *Cell Rep*. 2018;22(6):1509–1521. doi: 10.1016/j.celrep.2018.01.040
17. Buck MD, O'Sullivan D, Klein Geltink RI, et al. Mitochondrial Dynamics Controls T Cell Fate through Metabolic Programming. *Cell*. 2016;166(1):63–76. doi: 10.1016/j.cell.2016.05.035
18. Walls J, Romero N. Assessing T Cell Bioenergetic Poise and Spare Respiratory Capacity Using Extracellular Flux Analysis. Agilent Technologies. https://www.agilent.com/cs/library/applications/an-xf-tcell-metabolic-profiling-kit-5994-4494en-agilent.pdf. Published March 15, 2022. Accessed April 3, 2024.
19. Buck MD, Sowell RT, Kaech SM, Pearce EL. Metabolic Instruction of Immunity. *Cell*. 2017;169(4):570–586. doi: 10.1016/j.cell.2017.04.004
20. Siska PJ, Rathmell JC. T cell metabolic fitness in antitumor immunity. *Trends Immunol*. 2015;36(4):257-264. doi:10.1016/j.it.2015.02.007
21. Pavlova NN, Thompson CB. The Emerging Hallmarks of Cancer Metabolism. *Cell Metab*. 2016;23(1):27–47. doi: 10.1016/j.cmet.2015.12.006
22. Beckermann KE, Dudzinski SO, Rathmell JC. Dysfunctional T cell metabolism in the tumor microenvironment. *Cytokine Growth Factor Rev*. 2017;35:7–14. doi: 10.1016/j.cytogfr.2017.04.003
23. Joaquina S, Forcados C, Caulier B, Inderberg EM, Wälchli S. Determination of CAR T cell metabolism in an optimized protocol. *Front Bioeng Biotechnol*. 2023;11:1207576. doi: 10.3389/fbioe.2023.1207576
24. Scharping NE, Menk AV, Moreci RS, et al. The Tumor Microenvironment Represses T Cell Mitochondrial Biogenesis to Drive Intratumoral T Cell Metabolic Insufficiency and Dysfunction. *Immunity*. 2016;45(2):374–388. doi: 10.1016/j.immuni.2016.07.009
25. Klein Geltink RI, Edwards-Hicks J, Apostolova P, et al. Metabolic conditioning of CD8+ effector T cells for adoptive cell therapy. *Nature Metab*. 2020;2:703–716. doi: 10.1038/s42255-020-0256-z
26. Pillai RR, Gonsalves C, Romero N, et al. Metabolic preconditioning improves engineered T cell fitness and function. Agilent Technologies. https://www.agilent.com/cs/library/applications/an-metabolic-preconditioning-5994-5451en-agilent.pdf Published January 12, 2024. Accessed April 3,2024.
27. Lontos K, Wang Y, Joshi SK, et al. Metabolic reprogramming via an engineered PGC-1α improves human chimeric antigen receptor T-cell therapy against solid tumors. *J Immunother Cancer*. 2023;11:e006522. doi: 10.1136/jitc-2022-006522

28. Kedzierska K, Valkenburg SA, Doherty PC, Davenport MP, Venturi V. Use it or lose it: establishment and persistence of T cell memory. *Front Immunol*. 2012;3:357. doi: 10.3389/fimmu.2012.00357

29. Nakamura K, Yagyu S, Hirota S, et al. Autologous antigen-presenting cells efficiently expand *piggyBac* transposon CAR-T cells with predominant memory phenotype. *Mol Ther Methods Clin Dev*. 2021;21:315–324. doi: 10.1016/j.omtm.2021.03.011

30. Li W, Pan X, Chen L, et al. Cell metabolism-based optimization strategy of CAR-T cell function in cancer therapy. *Front Immunol*. 2023;14. doi:10.3389/fimmu.2023.1186383

31. Crompton JG, Sukumar M, Roychoudhuri R, et al. Akt Inhibition Enhances Expansion of Potent Tumor-Specific Lymphocytes with Memory Cell Characteristics. *Cancer Res*. 2015;75(2):296–305. doi: 10.1158/0008-5472.CAN-14-2277

32. Alizadeh D, Wong RA, Yang X, et al. IL15 Enhances CAR-T Cell Antitumor Activity by Reducing mTORC1 Activity and Preserving Their Stem Cell Memory Phenotype. *Cancer Immunol Res*. 2019;7(5):759–772. doi: 10.1158/2326-6066.CIR-18-0466

33. Blank CU, Haining WN, Held W, et al. Defining 'T cell exhaustion'. *Nat Rev Immunol*. 2019;19:665–674. doi: 10.1038/s41577-019-0221-9

34. MacIver NJ, Michalek RD, Rathmell JC. Metabolic Regulation of T Lymphocytes. *Annu Rev Immunol*. 2013;31:259–283. doi: 10.1146/annurev-immunol-032712-095956

35. Jachimowicz L, Lei M, Ye P, Lu Y, Guenther G. Comprehensive Analysis of T Cell Status Following Activation Using a 16-Color Immunophenotyping Panel on the NovoCyte Advanteon. Agilent Technologies. https://www.agilent.com/cs/library/applications/application-tcell-cell-analysis-5994-1455en-agilent.pdf. Published October 5, 2020. Accessed April 3, 2024.

36. Chow A, Perica K, Klebanoff CA, Wolchok JD. Clinical implications of T cell exhaustion for cancer immunotherapy. *Nat Rev Clin Oncol*. 2022;19:775–790. doi: 10.1038/s41571-022-00689-z

37. Menk AV, Scharping NE, Rivadeneira DB, et al. 4-1BB costimulation induces T cell mitochondrial function and biogenesis enabling cancer immunotherapeutic responses. *J Exp Med*. 2018;215(4):1091–1100. doi: 10.1084/jem.20171068

38. Kawalekar OU, O'Connor RS, Fraietta JA, et al. Distinct Signaling of Coreceptors Regulates Specific Metabolism Pathways and Impacts Memory Development in CAR T Cells. *Immunity*. 2016;44(2):380–390. doi: 10.1016/j.immuni.2016.01.021

39. Hermans D, Gautam S, García-Cañaveras JC, et al. Lactate dehydrogenase inhibition synergizes with IL-21 to promote $CD8^+$ T cell stemness and antitumor immunity. *PNAS*. 2020;117(11):6047–6055. doi: 10.1073/pnas.1920413117

40. Weigelin B, den Boer ATh, Wagena E, et al. Cytotoxic T cells are able to efficiently eliminate cancer cells by additive cytotoxicity. *Nature Comms*. 2021;12(1). doi:10.1038/s41467-021-25282-3

41. Davenport AJ, Jenkins MR, Cross RS, et al. CAR-T Cells Inflict Sequential Killing of Multiple Tumor Target Cells. *Cancer Immunol Res*. 2015;3(5):483–494. doi: 10.1158/2326-6066.CIR-15-0048

42. Nguyen P, Okeke E, Clay M, et al. Route of 41BB/41BBL Costimulation Determines Effector Function of B7-H3-CAR. CD28ζ T Cells. *Mol Ther Oncol*. 2020;18:202–214. doi: 10.1016/j.omto.2020.06.018

43. Sen M, Wankowski DM, Garlie NK, et al. Use of Anti-CD3 × Anti-HER2/neu Bispecific Antibody for Redirecting Cytotoxicity of Activated T Cells Toward HER2/neu^+ Tumors. *J Hematother Stem Cell Res*. 2004;10(2). doi: 10.1089/15258160151134944

44. Lum LG, Thakur A, Al-Kadhimi Z, et al. Targeted T-cell Therapy in Stage IV Breast Cancer: A Phase I Clinical Trial. *Clin Cancer Res*. 2015;21(10):2305–2314. doi: 10.1158/1078-0432.CCR-14-2280

45. Thakur A, Scholler J, Schalk DL, June CH, Lum LG. Enhanced cytotoxicity against solid tumors by bispecific antibody-armed CD19 CAR T cells: a proof-of-concept study. *J Cancer Res Clin Oncol*. 2020;146:2007–2016. doi: 10.1007/s00432-020-03260-4

46. Kalbasi A, Siurala M, Su LL, et al. Potentiating adoptive cell therapy using synthetic IL-9 receptors. *Nature*. 2022;607:360–365. doi: 10.1038/s41586-022-04801-2

47. Tormoen GW, Crittenden MR, Gough MJ. Role of the immunosuppressive microenvironment in immunotherapy. *Adv Radiat Oncol*. 2018;3(4):520–526. doi: 10.1016/j.adro.2018.08.018

48. Alishah K, Birtel M, Masoumi E, et al. CRISPR/Cas9-mediated TGFβRII disruption enhances anti-tumor efficacy of human chimeric antigen receptor T cells *in vitro*. *J Transl Med*. 2021;19:482. doi: 10.1186/s12967-021-03146-0

49. Wei Y, Ding J, Li J, et al. Metabolic reprogramming of immune cells at the maternal-fetal interface and the development of techniques for immunometabolism. *Front Immunol*. 2021;12. doi:10.3389/fimmu.2021.717014

50. Voss K, Hong HS, Bader JE, Sugiura A, Lyssiotis CA, Rathmell JC. A guide to interrogating immunometabolism. *Nat Rev Immunol*. 2021;21(10):637-652. doi:10.1038/s41577-021-00529-8

51. Mony JT, Felix A, Abassi Y, Lamarche BJ, Raver R, Zhang X. A Novel Real-Time Co-Culture Assay Using xCelligence RTCA eSight for Immune Cell Invasion and Cytotoxicity. Agilent Technologies. https://www.agilent.com/cs/library/applications/an-immune-cell-invasion-cytotox-esight-5994-6888en-agilent.pdf. Published November 29, 2023. Accessed April 3, 2024.

52. Wang T, Yang G, Ye P, et al. Continuous, Label-Free Monitoring of Immune Cell Clustering and Proliferation Using Agilent xCELLigence RTCA eSight. Agilent Technologies. https://www.agilent.com/cs/library/applications/an-immune-cell-clustering-activation-esight-5994-5767en-agilent.pdf. Published March 1, 2023. Accessed April 3, 2024.

53. Jachimowicz L, Guenther G. Proliferation and Cell Cycle Analysis Using the NovoCyte Flow Cytometer. Agilent Technologies. https://www.agilent.com/cs/library/applications/application-proliferation-cell-cycle-analysis-novocyte-5994-2120en-agilent.pdf. Published February 2, 2021. Accessed April 3, 2024.

54. Jachimowicz L, Lei M, Ye P, et al. *Ex Vivo* Phenotyping and Potency Monitoring of CD19 CAR T Cells. Agilent Technologies. https://www.agilent.com/cs/library/applications/5994-2377EN-D6J_CART.pdf. Published October 27, 2020. Accessed April 3, 2024.

55. Thakur A. Bispecific Antibody Armed Metabolically Enhanced Headless CAR-T Cells - Safe, Effective Serial Killers of Solid Tumors. Agilent Technologies. https://www.agilent.com/en/video/bispecific-antibody-armed. Accessed April 3, 2024.

www.agilent.com

Published in the USA, November 4, 2024
5994-7739EN

Agilent
Trusted Answers