Evaluating Functional Potency of Immunotherapies Targeting Tumors of B Cell Origin

Introduction

A growing understanding of the molecular interactions between immune effector cells and target tumor cells, coupled with refined gene therapy approaches, are giving rise to novel cancer immunotherapeutics with remarkable efficacy in the clinic against both solid and liquid tumors. To date, the most successful immunotherapies are those targeting blood-borne tumors. In recent years, CAR (Chimeric Antigen Receptor)-T Cell therapy has been one of the most prominent and breakthrough in cancer immunotherapy for relapsed and refractory hematopoietic malignancies. With the recent FDA approval of CD19 directed CAR-T for acute lymphoblastic leukemia, non-Hodgkin lymphoma, diffuse large B cell lymphoma, and the designation of Breakthrough Therapy for BCMA directed CAR-Ts for multiple myeloma, this technology has generated great excitement in the scientific community and has spun numerous basic, applied and clinical studies worldwide. ACEA therefore sought to adapt the xCELLigence Real-Time Cell Analysis (RTCA®) potency assay for *in vitro* assessment of immunotherapies targeting tumor cell lines originating from liquid tumors. To accomplish this, we undertook a tethering approach to immobilize liquid tumor cell lines of various origins on to the surface of gold biosensors embedded in the bottom of an electronic microtiter plate (E-Plate®). Using this approach, multiple common leukemic cell lines such as Raji can indeed be tethered to the E-Plate biosensors, giving rise to a robust impedance signal. After the tethered cells attained a certain level of growth and confluence, effector cells are added at different E:T ratios, resulting in a dose-proportional decrease in Cell Index. For the assay to be accurate, it is important that the tethering reagent is selective for the target cells, thereby precluding any impedance signal derived from the effector cells.

Potency Assay Principle Targeting Hematopoietic Tumors

The wells of the E-Plate are pre-coated with a tethering reagent specific for a cell surface marker expressed on the liquid cancer cell, enabling the suspension cells to be immobilized and adhere on the plate bottom embedded with biosensors **(Figure 1)**. As tethered cells proliferate, electric current flow between the biosensors is impeded. The magnitude of this impedance is dependent on the cell number, size, and attachment quality. Addition of immune effectors (such as NK cells, CAR-T, oncolytic viruses, checkpoint inhibitors, bispecific antibodies, etc.) results in time- and density-dependent target cell destruction, and the corresponding cytolytic activity is sensitively and continuously detected. This strategy allows assessment of the functional potency of immunotherapies targeting specific cancers of hematopoietic origin with much enhanced reproducibility and throughput using a simple workflow. **Table 1** below illustrates widely studied model liquid cancer cell lines that have been immunophenotyped and validated for selective tethering approaches and the RTCA potency assay.

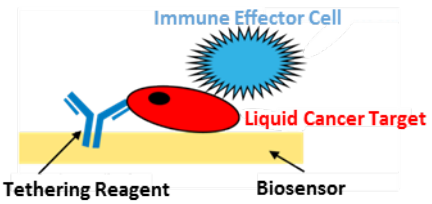

Figure 1. Pre-coating the wells of an E-Plate with a tethering reagent enables liquid tumor cells to proliferate on and be detected by the biosensors.

Cancer Type	Validated Cell Line	Selective Tethering Mechanism	Verified Target Expression by Flow Cytometry
Acute Lymphoblastic Leukemia (ALL)	NALM6	CD9	CD3-, CD9+, CD19+, CD20-, CD22+, CD38+, CD138+, CD269+, HLA-DR+
	RS4;11	CD9	CD3- , CD9+, CD14+, CD19+, CD20-, CD22+, CD38+, CD138+, CD269+, CD123+(low)
Chronic Myelogenous Leukemia (CML)	K562	CD29 or CD71	CD3-, CD14-, CD15+, CD19-, CD29+, CD33+, CD71+, CD235a+
Non-Hodgkin Lymphoma (NHL)	Daudi	CD40 or CD19	CD3-, CD19+, CD20+, CD40+
	Raji	CD40 or CD19	CD3-, CD19+, CD20+, CD40+
	Ramos	CD40 or CD19	CD3-, CD19+, CD20+, CD40+
Multiple Myeloma (MM)	RPMI 8226	CD9	CD3-, CD9+, CD19-, CD38+, CD138+, CD269+, HLA-DR+
	MM1R	CD9 and CD71	CD3-, CD9+, CD19-, CD38+, CD71+, CD138+, CD269+, HLA-DR+
Acute Myeloid Leukemia (AML)	HEL 92.1.7	CD29	CD13+, CD29+, CD33+, CD15+, CD123+
Chronic Lymphocytic Leukemia (CLL)	MEC2	CD40	CD3-, CD13+, CD19+, CD20+, CD40+, CD138+

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Protocol: Liquid Tumor Immunotherapy Potency Assay

This application note describes the experimental setup for assessing effector-mediated cytolysis of various liquid tumor cell lines. While NK-92 natural killer cell line is used as example, any type of effectors such as CAR-T or PBMC can be used. The protocol herein allows the identification of target cell killing kinetics as well as the optimal time point for cytotoxicity with different Effector:Target ratios.

This protocol has been developed for continuous monitoring of cell killing over the course of 4 days, allowing an initial day for target cells to attach to and proliferate in the E-Plate View 96 wells.

Day 1: Immobilization of liquid tumor cells

a. Coat E-Plate View 96 with diluted Tethering Reagent for 3 hours. Wash, add medium, and take background impedance (Cell Index) measurement.
b. Prepare liquid tumor target cells and add to the coated wells.
c. Leave the plate at room temperature for at least 30 minutes to allow the cells to settle.
d. Load the plate into xCELLigence RTCA instrument and start data acquisition to monitor target cell attachment and proliferation.

Day 2: Addition of immunotherapeutic effectors

e. Prepare NK-92 effector cells.
f. Pause xCELLigence data acquisition; remove the plate from instrument and place inside a laminar flow hood; remove nascent media from wells but avoid disturbing the attached cells. It is recommended to use a manual multichannel pipette to remove media rather than a vacuum aspirator. Add effector cells at different E:T ratios to the target cells.
g. Place the plate back into xCELLigence RTCA instrument and start data acquisition to monitor effector cell-mediated killing of immobilized target cells. We recommend collecting data at 30-minute intervals; however, the exact data collection frequency can be determined by the user.

Days 2-5: Assessment of cancer cell destruction

h. Continue data acquisition for as long as desired.
i. Analyze data using RTCA Software Pro.

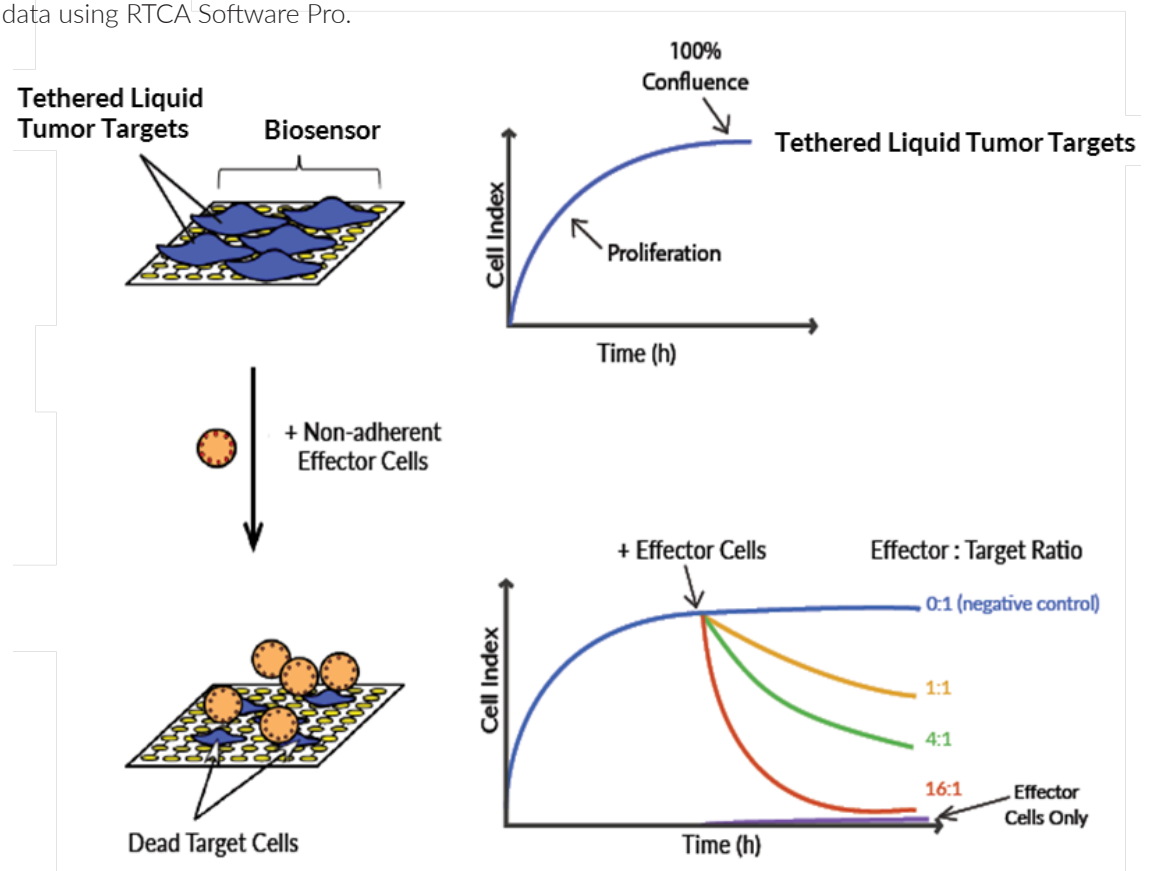
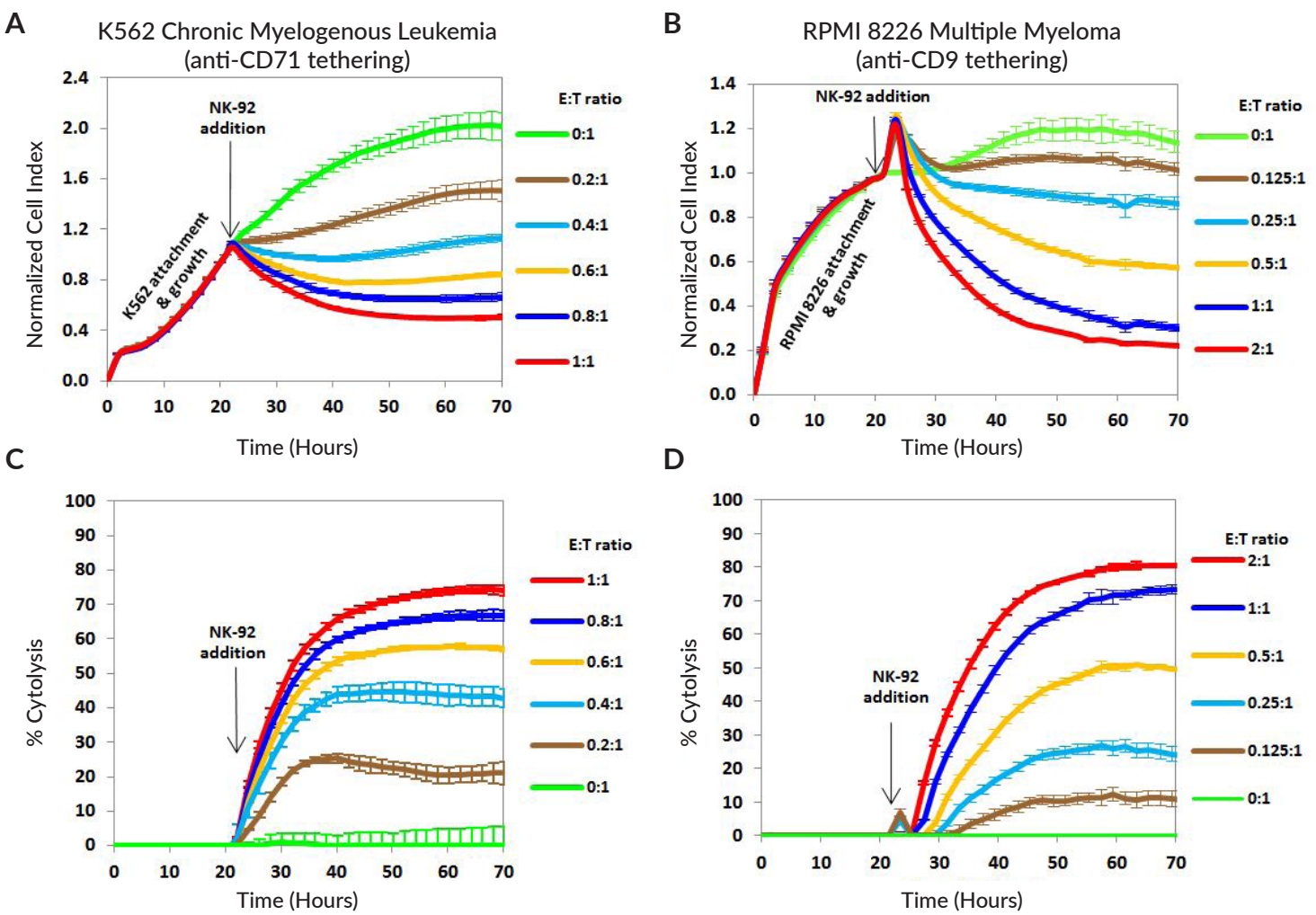


Figure 2. Monitoring immune cell-mediated killing of immobilized liquid cancer cells in real-time using xCELLigence.

Results: Real-time Leukemia and Multiple Myeloma Killing by NK-92 cells

To evaluate the potency of a cytotoxic NK-92 cell treatment of liquid cancers as a function of time using the RTCA potency assay, target tumor cells were immobilized onto the plate bottom by the indicated selective tethering mechanisms. Various doses of NK-92 effector cells were subsequently added to the tumor cells to achieve different effector:target (E:T) ratios. The destruction of target cells were hence detected by the biosensors and reported as a decrease of impedance (Cell Index, CI), which was monitored by the RTCA instrument over the next 1-2 days. **Figures 3A** and **3B** illustrate the liquid tumor cell attachment through cell surface marker-specific tethering on the biosensor-embedded wells. As expected, the efficiency with which the tethered target cells are killed is dependent on the ratio between NK-92 effector cells and the target cancer cells. Target cells alone (E:T=0:1) are used as negative controls for cytolysis.

While the CI decrease after effector addition directly correlates with cell viability, it can be readily converted to percent cytolysis through mathematical calculations that take into account the signal from the target cells alone control. As shown in **Figures 3C** and **3D**, percent cytolysis increases in a time- and E:T ratio-dependent manner. For the two liquid tumor cell lines shown below, at several E:T ratios tested, the % cytolysis reaches a plateau after 40-45 hours that is less than 100%, indicating incomplete lysis by NK92. We speculate that at low E:T ratios, the effectors cells are a limiting factor and cannot kill all the target cells which continue to proliferate. It is possible that after a certain period of co-culturing, the two cell populations eventually reach an equilibrium in cell number that is reflected by the plateauing of the signal. **Figures 3E** and **3F** show the extent of NK-92-mediated cytolysis of target cells at different E:T ratio at either 4 or 24 hours after NK-92 addition.



(Figure 3 continues on the next page)

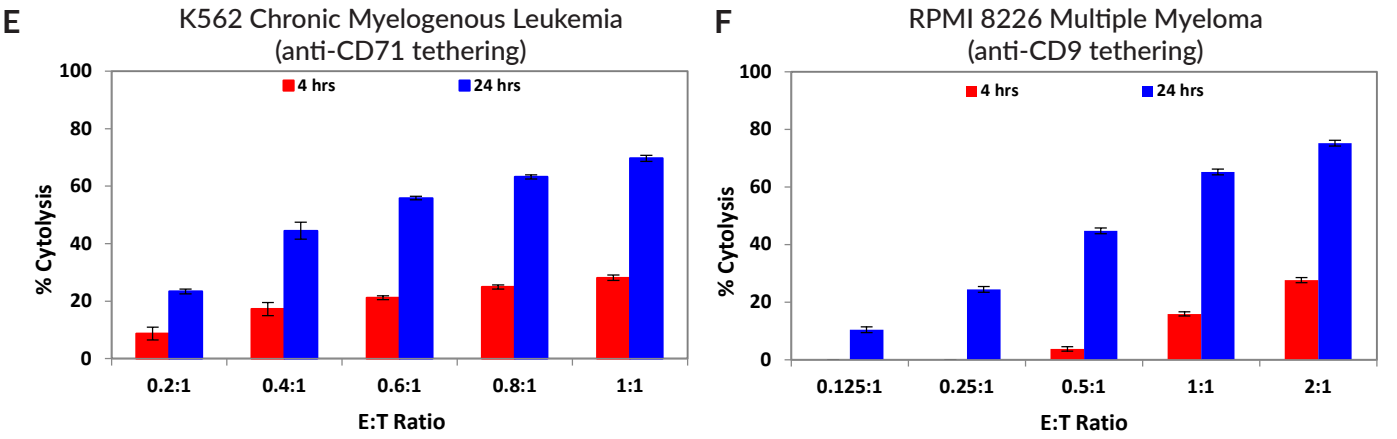


Figure 3. Liquid Tumor Potency Assay. (A, C, E) K562 cells, tethered by anti-CD71 antibody, and **(B, D, F)** RPMI 8226 cells, tethered by anti-CD9 antibody, were seeded at 30,000 and 60,000 cells/well, respectively. **(A, B)** When left untreated, the immobilized K562 and RPMI 8226 cells proliferate to the point of confluence. However, upon addition of increasing quantities of effector NK-92 cells, the impedance signal decreases in a dose dependent manner. Samples have been internally normalized for the Cell Index value measured before NK92 addition (Normalized Cell Index). **(C, D)** The Cell Index plot is converted to a % Cytolysis plot by the xCELLigence Immunotherapy Software. **(E, F)** % Cytolysis measured at 4 and 24 hours after NK92 addition for different E:T ratios.

One major advantage of continuous impedance-based monitoring is that the time dependency of cytolysis is captured at high frequency of measurements defined by the user (e.g. every 10 seconds) which can be challenging with traditional end-point approaches. Consequently, kinetic parameters that encompass such temporal information can be effectively derived. One example is the KT_{50} parameter, which represents the time required to achieve 50% cytolysis at a given E:T ratio. A lower KT_{50} value signifies a more efficient cytolytic kinetic (representitive data,**Table 2**). As expected, at a constant E:T ratio of 1:1, there is a wide range of NK92 killing efficiency against broad spectrum of liquid cancer types. While the % Cytolysis parameter shows the potency of a specific E:T ratio at a given time point, the KT_{50} parameter allows the temporal dimension and provides insights for the rate of cell killing based on the target cell type.

Cancer Type	Cell Line	E:T ratio	% Cytolysis			KT_{50}
			4 hrs.	24 hrs.	48 hrs.	
Acute Lymphoblastic Leukemia (ALL)	NALM6	1:1	9	86	87	17.5
	RS4;11	1:1	5	77	79	15.4
Chronic Myelogenous Leukemia (CML)	K562	1:1	28	70	74	9.5
Non-Hodgkin Lymphoma (NHL)	Daudi	1:1	7	74	82	13
	Raji	1:1	58	88	84	3.5
	Ramos	1:1	0	45	60	29
Multiple Myeloma (MM)	RPMI 8226	1:1	28	75	81	17.2
	MM1R	1:1	63	97	97	3
Acute Myeloid Leukemia (AML)	HEL 92.1.7	1:1	0	60	75	17
Chronic Lymphocytic Leukemia (CLL)	MEC2	1:1	0	53	63	20.9

Conclusion

Herein, in a simple work flow, we have demonstrated the utility of xCELLigence RTCA to evaluate the potency of an immunotherapy against a broad spectrum of liquid tumors and to monitor the destruction kinetics of liquid cancers at physiologically relevant E:T ratios. The protocol described here involves substantially less work than traditional assays: target liquid tumor cells are simply seeded and tethered into a pre-coated E-Plate, after which effector cells were added, and the kinetics of cancer cells destruction is non-invasively monitored over the course of days or even weeks. Data acquisition is continuous and automatic. The real-time and quantitative nature of the impedance data makes it easy to make potency comparisons between immunotherapy treatments and dosages.

Utilizing this surface-tethering approach, several effector cells, i.e., PBMC, NK, CAR-T, as well as biological molecules such as Bi-specific T cell Engagers (BiTEs) targeting the EpCAM protein expressed on tumor cells and blocking antibodies against the immune checkpoint inhibitor PD-1 (Cerignoli et al., 2018). The xCELLigence platform is well-suited for liquid cancer potency assessments, providing quantitative evaluation with high reproducibility and greatly simplified workflow for manufactured immuno-oncology therapies.

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