Application Note

Agilent
Trusted Answers

A Novel Real-Time Co-Culture Assay Using xCelligence RTCA eSight for Immune Cell Invasion and Cytotoxicity

Authors

Jyothi T. Mony,
Alejandra Felix, Yama Abassi,
Brandon J. Lamarche,
Ryan Raver, and Xiaoyu Zhang
Agilent Technologies, Inc.

Introduction

Immune cells extravasate from blood vessels to infiltrate the tissues and perform effector functions that play a crucial role in tumor immune surveillance. These abilities are also harnessed by engineered cytotoxic natural killer (NKs) and Chimeric Antigen Receptor (CAR)-T cells used for cancer immunotherapy.

Although cellular immunotherapy has demonstrated effectiveness in hematological cancers, clinical responses in solid tumors need to be improved. The complex tumor microenvironment (TME) in solid tumors regulates lymphocyte recruitment and function. An important challenge faced by lymphocytes in solid tumors is to navigate the acellular space filled with structural scaffolding of the extracellular matrix (ECM) during migration/invasion. This ECM is heterogenous, and its components can modulate a wide array of cellular responses in both resident tumor cells and infiltrating lymphocytes.[1]

Traditionally, invasion, migration and cytotoxicity are evaluated in different endpoint assays using transwell assay systems to predict the in vivo function. The Boyden Chamber is a classical transwell arrangement that is widely used to evaluate migration and invasion.[2] It consists of a cylindrical cell culture insert with a porous membrane nested inside the well of a standard cell culture plate. Cell suspensions are added to the inner chamber of the insert and induced to migrate out through the pores of variable sizes (typically, 3 to 12 µm) using chemo-attractants in the outer chamber. The inner wells can be coated with ECM to evaluate invasion. The invaded/migrated cells are imaged and/or collected for quantification at predetermined time points. The Boyden Chamber can be modified for evaluating migration and cytotoxicity of effector cells in end-point assays.[3]

This application note describes a novel real-time co-culture assay using RTCA eSight for interrogating immune cell invasion and cytotoxicity that does not require the collection of embedded cells for end-point readouts (Figure 1). In this setup, the ECM layer is in direct contact with target tumor cells and can modulate response of both tumor targets and infiltrating lymphocytes, especially as the latter executes its cytotoxicity function. In the transwell-based approaches, cytotoxicity of the migrated immune cells is carried out in an outer chamber devoid of ECM.

The invasion and killing of target tumor cells by the NKs has been evaluated as proof of concept for the assay system. NKs are innate lymphocytes that kill infected, stressed, or transformed cells by identifying and distinguishing the "altered" cells from healthy cells using an array of activating and inhibiting receptors.[4] The NK92 cells were used as a model for studying invasion and cytotoxicity. The allogenic NKs derived from NK92 cell line are the first NK-based cellular immunotherapy to be granted the Investigational New Drug status by the US Food and Drug Administration.[5]

It is hypothesized that the Matrigel layer presents a challenge for the NK92 cells and increasing the distance for invasion would delay the killing of tumor cells. Also, NKs rely on proteases of the matrix-metalloproteinases (MMP) family to degrade various components in the ECM during invasion stage.

This application note demonstrates that the 96-well format of eSight can be easily used to systematically study the effects of different ECM constituents on tumor and immune cell interactions in the TME depending on user requirements.

Materials and methods

Cells

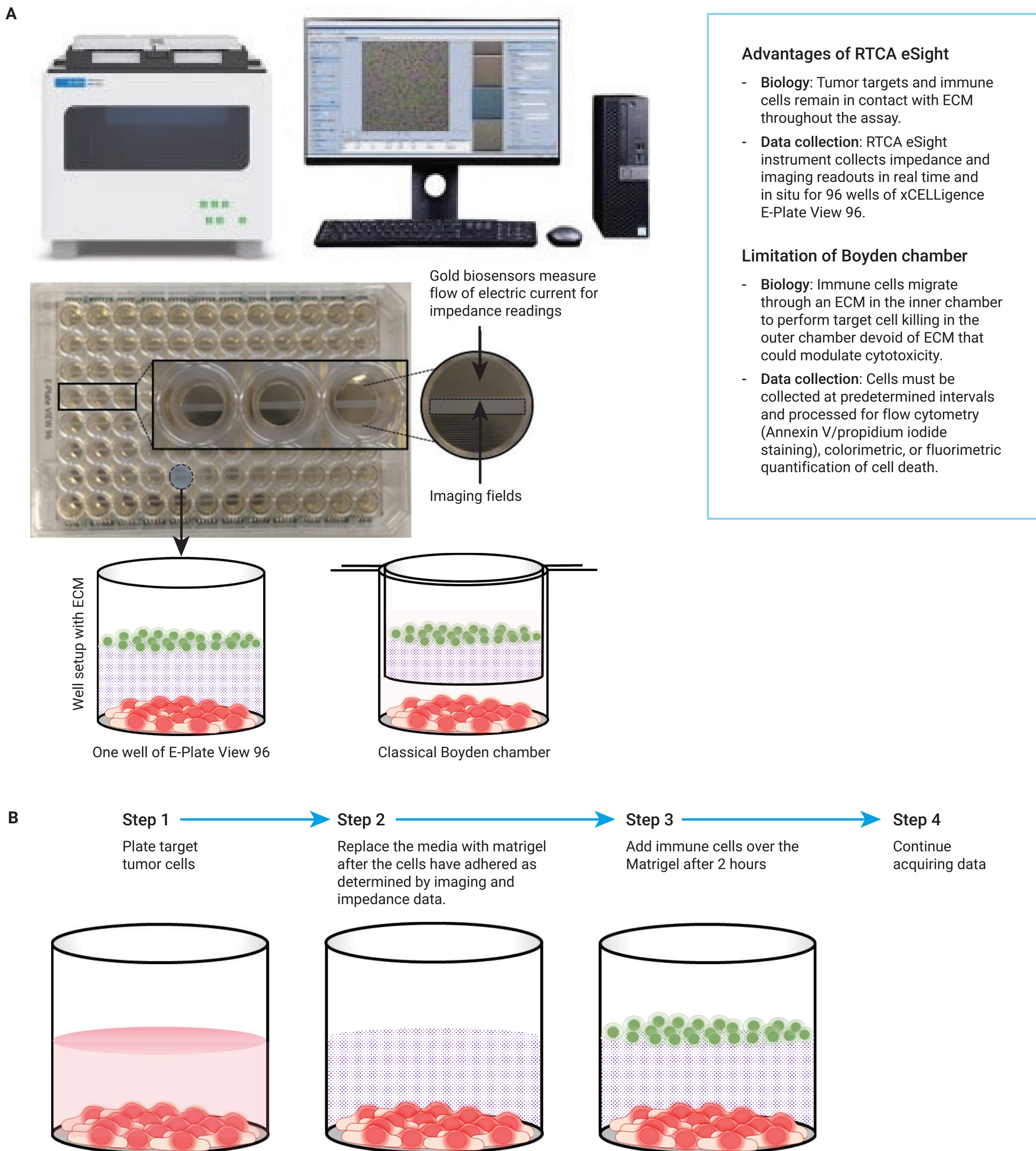
MCF7 human breast adenocarcinoma cell line (ATCC, part number HTB-22) was transduced with eLenti Red (Agilent Technologies, part number 8711011) at a multiplicity of infection of 1 and cultured in the presence of 2 µg/mL Puromycin (InvivoGen, part number ant-pr-1) for 14 days to select for MCF7-red cells stably expressing nuclear-localized red fluorescent protein (RFP). MCF7 and MCF7-red cells were cultured in EMEM media (ATCC, 30-2003) supplemented with 10% heat inactivated FBS (Sigma, part number 12106C-500ML) and 1% Pen/Strep (Hyclone, part number SV30010).

NK92 cells (Creative Bioarray, CSC-C0499) were grown in MyeloCult H5100 media (Stemcell Technologies, part number 05150) supplemented with 30 mL of horse serum (Gibco, part number 16050-122), 600 IU/mL of rhIL-2 (Stemcell Technologies, part number 78036) and 1% Pen/Strep (Hyclone, part number SV30010).

eGFP-NK92 cells (BPS Bioscience, part number 78399) were grown in X-VIVO 15 (Lonza Ltd, part number 04-418Q) supplemented with 5% human serum (Sigma, H4522), 500 IU/mL of rhIL-2 (Stemcell Technologies, part number 78036) and 0.5 µg/mL Puromycin.

ECM invasion and cytotoxicity assay

The ability of NK92 cells to invade the Matrigel and kill the MCF7-red target cells was evaluated by concurrent impedance and imaging readouts on an xCELLigence RTCA eSight (Agilent Technologies) as outlined in Figure 1. Background impedance signal was measured with 50 µL of EMEM media in the wells of E-plate VIEW microplate (Agilent Technologies, part number 0030060101030). MCF7-red target cells (30,000 in 100 µL) were added to the wells and the plate was placed at room temperature for 30 min to facilitate an even distribution at the bottom. The plate was returned to its cradle in the eSight to acquire data. Impedance was read every 15 minutes and images were taken every 60 minutes. Images from four fields of view were acquired in each well in brightfield, red, and green fluorescence channels. Exposure time was set at default in brightfield, 150 ms in red, and 300 ms in green channels. Matrigel (Corning, part number 356234, 354234) was thawed overnight at 4 °C, diluted with DMEM and supplemented with 10% FBS to a final total protein concentration of 6 mg/mL. After 24 hours the data collection was paused. Media in the wells were aspirated and varying volumes of Matrigel (50, 75, and 100 µL, data shown here) was layered over the MCF7-red cells and incubated for 30 minutes at room temperature, followed by 30 minutes at 37 °C/5% CO_2 for polymerization of the Matrigel. Impedance and imaging data were collected for an hour during which the NK92 cells were suspended in EMEM media and the cell numbers were adjusted to achieve E:T of 3:1 in 100 µL. NK92 cell suspension (100 µL) was layered over the Matrigel and the total volume in all wells was adjusted to 200 µL. The plate was loaded back into the eSight cradle and data acquisition was resumed. Percent cytolysis was calculated from normalized cell impedance readings using the formula [(1 – (treated/untreated) × 100].



Advantages of RTCA eSight

- **Biology**: Tumor targets and immune cells remain in contact with ECM throughout the assay.
- **Data collection**: RTCA eSight instrument collects impedance and imaging readouts in real time and in situ for 96 wells of xCELLigence E-Plate View 96.

Limitation of Boyden chamber

- **Biology**: Immune cells migrate through an ECM in the inner chamber to perform target cell killing in the outer chamber devoid of ECM that could modulate cytotoxicity.
- **Data collection**: Cells must be collected at predetermined intervals and processed for flow cytometry (Annexin V/propidium iodide staining), colorimetric, or fluorimetric quantification of cell death.

Figure 1. Invasion-cytotoxicity assay setup. (A) The RTCA eSight instrument, E-plate View 96 and comparison of the setup in one of the 96 wells with a Boyden chamber. Advantages of this setup and limitations in data collection of the classical approaches are outlined in the box. (B) Outline of the steps in the assay.

MMP inhibition and kinetics of NK invasion

Ilomastat (GM6001, Selleck Chemicals, part number S7157), a broad spectrum MMP inhibitor, was dissolved in the Matrigel and media at a final concentration of 2 and 10 μM to assess the role of MMP-dependent NK92 invasion. Percent cytolysis was calculated from normalized cell impedance readings and red object count data using the formula [(1 – (NK92 + MMPi/MMPi treated) × 100].

Results and discussion

Target cell killing is delayed with increasing invasion distance for NK cells

Impedance increases with time and stabilizes as the MCF7-red target cells adhere and proliferate to reach confluence (Figure 2A). The addition of Matrigel (50 μL) modulates the impedance signature of MCF7 cells and importantly, delays the cytolysis by NK92 cells added at 24 hours. The drop in impedance due to cytolysis is further delayed with increasing volumes of Matrigel (Figure 2B, representative data for 50, 75 and 100 μL). The KT60 (Figure 2C, time to achieve 60% killing with respect to controls) was 67, 76, and 89 hours, respectively.

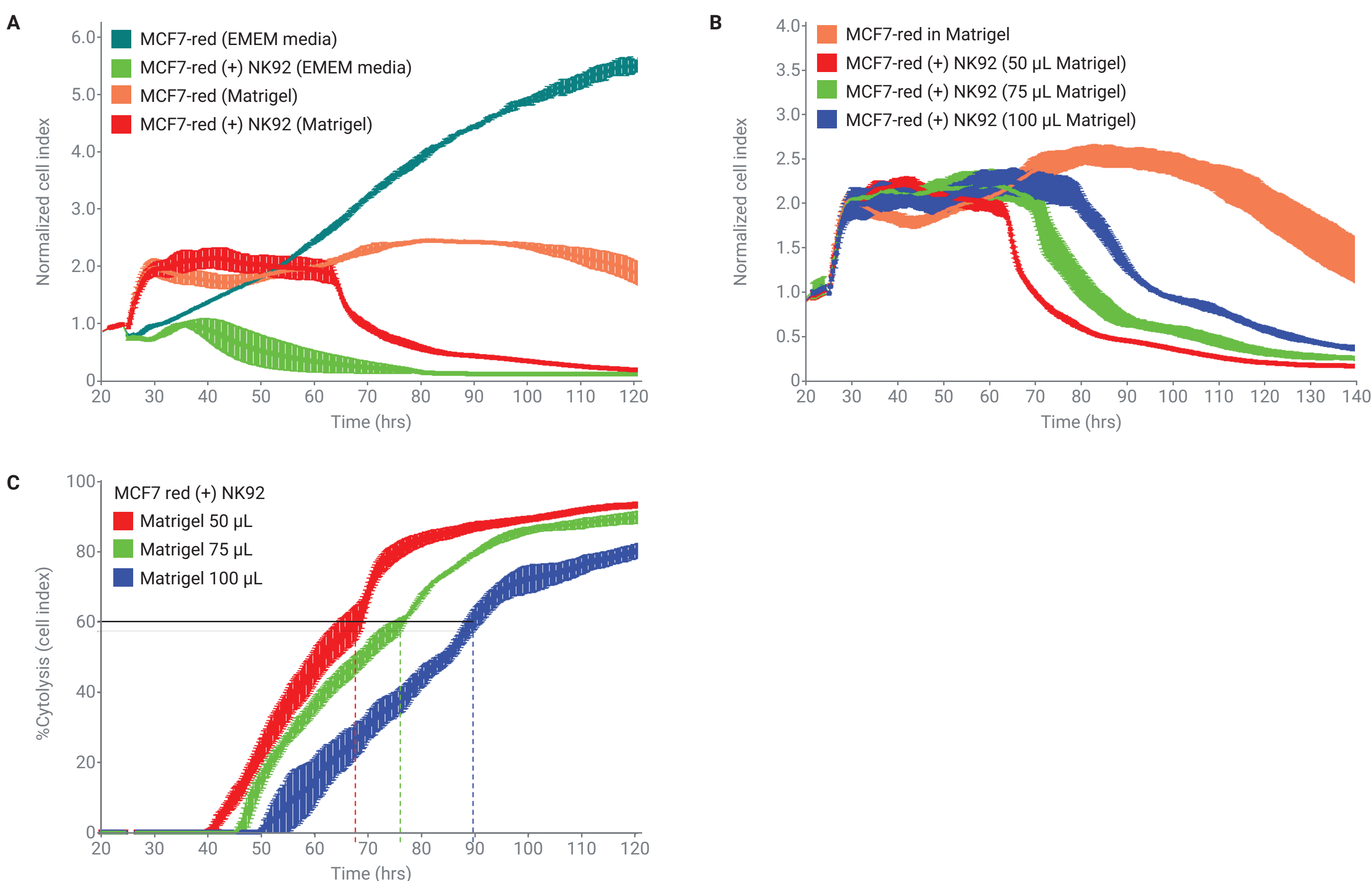


Figure 2. Cytotoxicity of NK92 is delayed with increasing invasion distance. (A) The addition of NK92 at 24 hours (E:T =3:1) results in a drop in impedance (CI) due to cytolysis of targets and it is delayed in Matrigel. (B) Cytolysis is further delayed in increasing volumes of Matrigel. (C) Percent cytolysis was calculated with reference to untreated MCF7 growing in Matrigel.

MMP inhibition delays target cell killing by NK cells

As cytolysis was delayed with increasing invasion distance for the NKs, it is hypothesized that MMPs would play an important role in lymphocyte invasion. Consistent with the function of MMPs in invasion, percent cytolysis computed from normalized impedance readings (Figure 3A) and live cell imaging (red fluorescence, Figure 3B) confirmed both delayed and reduced target cell killing by GFP-NK92 cells in the presence of Ilomastat (2 and 10 µM). Image analysis concurs with impedance measurements, confirming the kinetics of cell killing.

Image analysis corroborates delayed invasion and killing with increasing invasion distance

Representative images of MCF7 clusters reveal increased clumping and cell death in response to GFP-NK92 (Figure 3C). Although there is increased tumor cell death, only a few GFP-NK92 were detected in the imaging fields. Interestingly, highly active NK cells were detected that made multiple contacts with different MCF7 red targets in clusters suggesting serial killing activity.

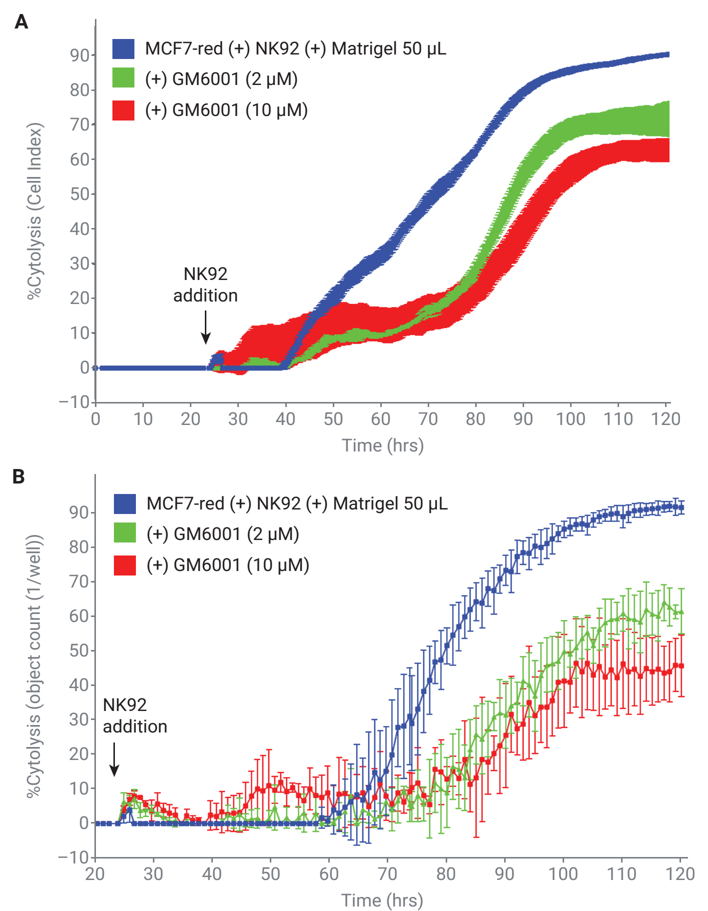


Figure 3. MMPs play an important role in invasion, and its inhibition results in delayed cytotoxicity. (A) Cytolysis is reduced GFP-NK92 at 24 hours (E:T = 3:1) results in a drop in impedance (CI) due to the killing of target cells. (B) Live cell imaging confirms the reduced loss of target cells (red fluorescence) in the visual fields in the presence of MMP inhibitor (2 µM and 10 µM).

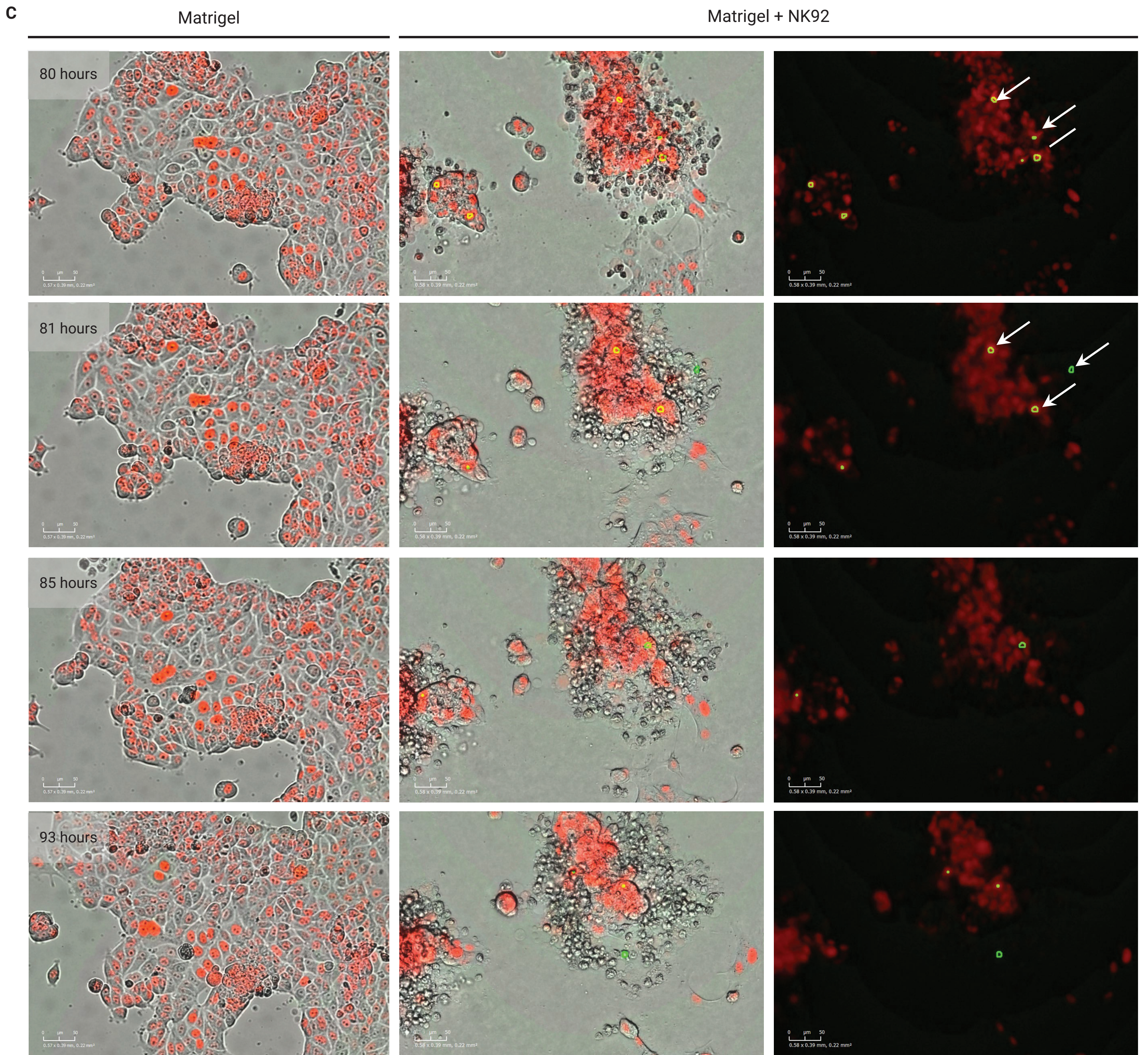


Figure 3C. Representative images for clustering of MCF7 and progressive loss red fluorescence associated with cytotoxicity. A few green GFP-NK92 cells (yellow outline) make multiple contacts with MCF7-red target cells in the clusters, resulting in cell death over the course of the assay.

Conclusion

Recapitulating the features of the tumor microenvironment in solid tumors could play a pivotal part in formulating and refining the strategies for cellular immunotherapy. The dysregulation of ECM in tumor microenvironment of solid tumors has been widely investigated for its ability to modulate diverse cellular responses. In this regard, there is a need for efficient in vitro assays that evaluate the responses of tumor and immune cells in the presence of ECM.

The ability of NKs and other immune cells to invade the extracellular space to reach the target cells could depend on the ability to degrade ECM.[6] Various constituents of ECM can regulate the function of NK cells.[7] In this application note, the ability of NK92 cells to invade a Matrigel layer and kill the target cells has been evaluated. The cytotoxicity outcome measured by loss in impedance and live cell imaging serves as a surrogate for the migratory/invasion potential of the NK92 cells. As a proof of concept, it was established that this assay format allows simultaneous evaluation of invasion and cytotoxicity in the presence of an ECM by demonstrating that the killing of target cells can be delayed by varying the invasion distance through the Matrigel.

NKs can express multiple MMPs and transmigration assays have shown that NK92 invasion in Matrigel is reduced in the presence of MMP inhibitor GM6001.[6] Typically, studies evaluate invasion/migration potential and cytotoxicity functions separately in complex workflows as is the case with the study that evaluated the effect of MMP inhibitor. Alternatively, some studies have evaluated both migration and cytotoxicity using complex cumbersome experimental setups.[8] Live cell imaging and real-time impedance readouts have been used to demonstrate the contribution of MMPs in invasion-cytotoxicity assays with NK92. Consistent with the role of MMPs in facilitating invasion, the killing of target cells is delayed by the broad spectrum MMP inhibitor.

Although imaging and impedance data were consistent in the invasion-cytotoxicity assay, relying on imaging data can be challenging. Tumor cells respond differently to the Matrigel and as highlighted in the representative images the MCF7 target cells form irregular and messy clumps as they undergo cell death. Surprisingly, rather than finding a swarm of invading NK cells, only a few NK cells in and around the MCF clusters were detected. Nonetheless, images and videos were generated of highly active GFP-NK92 cells that made multiple contacts with different targets in the clusters, leading to cell death of the contacted targets during the course of the assay.

The novel real-time co-culture assay described in this application note demonstrates the utility of eSight for the simultaneous evaluation of invasion and cytotoxicity functions of lymphocytes that are critical for immunotherapy in solid tumors.

References

1. Pickup, M. W.; Mouw, J. K.; Weaver, V. M. The Extracellular Matrix Modulates the Hallmarks of Cancer. *EMBO Rep.* **2014**, *15*, 1243–53.
2. Falasca, M., Raimondi, C.; Maffucci, T. Boyden Chamber. *Methods in Molecular Biology* **2011**, 769, 87–95.
3. Schomer, N.; Boissel, L.; Klingemann, H. Combined Invasion and Cytotoxicity Assay Using Chemokine Secreting Target Cells. **2020**.
4. Morvan, M. G.; Lanier, L. L. NK Cells and Cancer: You Can Teach Innate Cells New Tricks. *Nat. Rev. Cancer* **2016**, *16*, 7–19.
5. Laskowski, T. J.; Biederstädt, A.; Rezvani, K. Natural Killer Cells in Antitumour Adoptive Cell Immunotherapy. *Nat. Rev. Cancer* **2022**. doi:10.1038/s41568-022-00491-0.
6. Edsparr, K.; *et al.* Human NK Cell Lines Migrate Differentially In Vitro Related to Matrix Interaction and MMP Expression. *Immunol. Cell Biol.* **2009**, 87, 489–495.
7. Bunting, M. D.; *et al.* Extracellular Matrix Proteins Regulate NK Cell Function in Peripheral Tissues. *Sci. Adv.* **2022**, *8*, eabk3327.
8. Olofsson, P. E.; *et al.* A Collagen-Based Microwell Migration Assay to Study NK-Target Cell Interactions. *Sci. Rep.* **2019**, *9*.

agilent.com/chem/eSight

For Research Use Only. Not for use in diagnostic procedures.

RA45229.3523148148

This information is subject to change without notice.

© Agilent Technologies, Inc. 2023

Printed in the USA, November 29, 2023
5994-6888EN

Agilent

Trusted Answers