APPLICATION NOTE

CELLCYTE X™ | Quantitative live cell imaging assays for immunology

Juliane Strietz, PhD, Yong Chen, MS

CYTENA, Freiburg, Germany

Abstract

The immune system is a natural defense system that protects the human body against pathogens and malignant cells. A substantial part of the immune response is carried out by immune cells that differ in their cellular functions and growth kinetics. Researchers across the globe aim to obtain a deeper understanding of the cellular processes in these cells and live cell imaging is a valuable tool to provide comprehensive data.

Here, we discuss how CYTENA's CELLCYTE X™ live cell imaging system can benefit immunology research by enabling real-time monitoring of immune cell growth and health, documentation of complex immunological processes such as macrophage differentiation, and evaluation of cellular immunotherapies such as CAR T-cell therapy to eradicate cancer cells.

Introduction

The immune system is a natural defense system that protects the human body against pathogens such as viruses and bacteria but also eliminates malignant cells such as cancer cells.

There are two lines of defense: innate immunity and adaptive immunity. Innate immunity refers to the first line of defense and is an antigen-independent, non-specific defense mechanism with a very fast response time. There is no immunological memory. The adaptive immunity is antigen-dependent and highly antigen-specific but has a slower response time. There is immunological memory, which results in a faster and more efficient immune response if the antigen is encountered subsequently[1].

A substantial part of the immune response is carried out by blood cells that develop from hematopoietic stem cells. These immune cells can be divided into two different lineages. Cells of the myeloid lineage include among other monocytes and their more differentiated form called macrophages. Cells of the lymphoid lineage include T lymphocytes that can be helper T cells or cytotoxic T cells, and B lymphocytes that can present antigens or produce antibodies.

The various types of immune cells differ in their cellular functions and growth kinetics, and researchers aim to obtain a deeper understanding of the cellular processes in these cells. Traditional cell-based assays are often endpoint assays and require the pre-definition of the time points of interest, risking the possibility of overlooking important observations. Additionally, some effects may only be seen in subpopulations or on the single-cell level and not while analyzing the whole cell population, as is the case for plate reader-based assays.

Imaging-based methods tackle these issues but can be challenging because of the need to maintain consistent optimal growth conditions during the assays, especially for more sensitive cells such as primary immune cells. Furthermore, morphological characteristics of immune cells and their changes can be difficult for most imaging systems with automated autofocus to capture.

In this application note, we used the live cell imaging system, the CELLCYTE X, to address different topics in immunology. We tracked the morphology and proliferation of different immune cell types, and documented changes in growth behavior during macrophage differentiation. We further discussed how the CELLCYTE X can be used to analyze immune cell health during activation-induced cell death, a feedback mechanism that prevents overreaction of the immune system, and how it can be utilized to evaluate cellular immunotherapies such as CAR T-cell therapy to eradicate cancer cells.

Material and methods

Cell culture

Jurkat, Nalm-6 and THP-1 cells were cultured in RPMI-1640 medium (Thermo Fisher) supplemented with 10% fetal bovine serum (FBS) (Sigma) and 1% penicillin/streptomycin (P/S) (Thermo Fisher) at 37°C and 5% CO_2. They were passaged twice per week and seeded for the different studies as described below.

Immune cell proliferation study

For proliferation studies, Jurkat, Nalm-6 and THP-1 cells were resuspended, counted and seeded with the indicated cell numbers (2.5k, 5k, 10k, 20k, 40k per well) in 200 µL medium per well of a 96-well flat bottom microtiter plate (Corning, #3595). The cells were allowed to settle for 30 minutes at room temperature to ensure a homogeneous distribution of cells across the well. Subsequently, the microtiter plate was placed in the CELLCYTE X, which was housed in an incubator. To minimize condensation, the plate was equilibrated to 37°C for 30 minutes before the scan was started. The cell growth was monitored every 2 hours for 96 hours using the 10X Standard scan module. Cell confluency was analyzed using the cell confluence recipe in CELLCYTE Studio, the system's acquisition and analysis software.

Macrophage differentiation study

THP-1 monocytic cells were resuspended, counted and seeded at 10k cells per well in a 96-well microtiter plate. Phorbol 12-myristate 13-acetate (PMA) (PeproTech) was dissolved in DMSO at 1 mg/mL and added to the wells to obtain 5 ng/mL, 50 ng/mL or 500 ng/mL final concentration in the well.

Lipopolysaccharide (LPS) (Thermo Fisher) was ordered as ready-to-use solution with a concentration of 2.5 µg/µL and was added to the cells to obtain 10 ng/µL, 100 ng/µL or 1000 ng/µL final concentration in the well. Control cells were left untreated.

Cell growth was imaged every 3 hours for 96 hours using the 10X Standard scan module. The cell confluency was analyzed using the cell confluence recipe in CELLCYTE Studio.

Activation-induced cell death study

For AICD, Jurkat cells were resuspended, counted and seeded at 10k cells per well in a 96-well microtiter plate. C.LIVE Tox Green (1 mM in DMSO) (CYTENA, CY.CL.KIT.002) was added to obtain a final concentration of 250 nM in the well.

Ionomycin (Cayman) was dissolved in DMSO to obtain 1 mg/mL stock concentration. Jurkat cells were grown in the absence or presence of a combination of 10 ng/mL PMA and 750 ng/mL Ionomycin (1X) or dilutions thereof (0.5X, 0.25X, 0.125X, 0.0625X). Cell growth and health were monitored every 3 hours for 96 hours using the 10X Standard scan module. The confluency and green object count were quantified using the cell confluence and object count recipe in CELLCYTE Studio, respectively.

Cellular immunotherapy study

The cellular immunotherapy study was performed in collaboration with the research group of Dr. Susana Minguet of the University of Freiburg's CIBSS and BIOSS research centres.

Nalm-6 cells were resuspended, counted and seeded at 20k cells per well in a 96-well microtiter plate. CYTENA's C.LIVE Caspase-3 Green NucView (5 mM in DMSO) was added to the cells to obtain a final concentration of 2.5 µM in the well.

CAR T cells were generated as described elsewhere[2]. CAR T cells were added to the Nalm-6 cells in a 1:1 or 3:1 effector-to-target ratio and the co-cultures were monitored every hour for 12 hours using the CELLCYTE X with the 10X Standard scan module. The total intensity of C.LIVE Caspase-3 was quantified using the object count recipe in CELLCYTE Studio.

Results and discussion

Immune cell growth and proliferation

Immune cells play an important role in health and disease of the human body and many researchers study the different types of immune cells for their phenotypes and functions. Different cell lines often vary in their morphology and growth behavior, and it can be challenging to monitor and quantify these differences in growth kinetics. Live cell imaging documents the morphology of cells and quantifies their proliferation continuously over time.

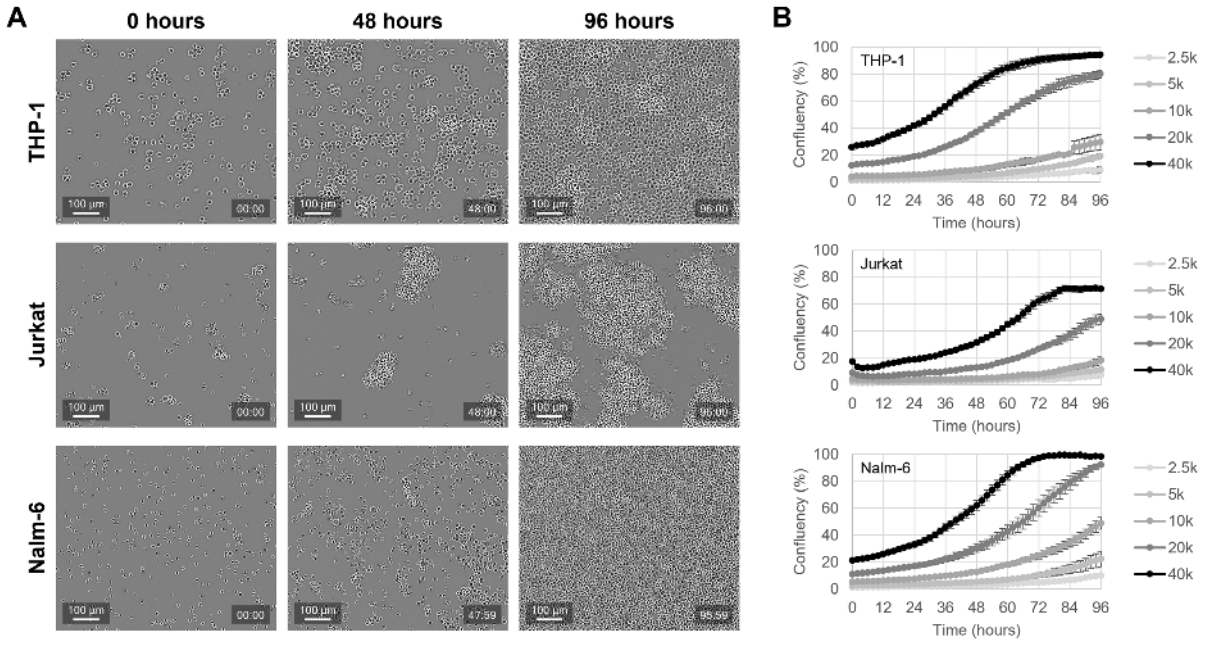

Figure 1: *Different immune cell lines differ in their growth behavior. (A) THP-1, Jurkat and Nalm-6 cells were seeded with the indicated cell numbers per well and their growth was monitored every 2 hours for 96 hours. (B) Cell confluency was analyzed using the cell confluence recipe in CELLCYTE Studio. Depicted are mean ±SEM, n = 3.*

THP-1 is a human monocytic cell line that has been extensively used to study monocyte and macrophage functions[3,4]. Jurkat is a T lymphocytic cell line that is an established model for T cell activation and TCR signaling[5]. Nalm-6 is a B lymphocytic cell line that expresses CD19, a common antigen found on B cell malignancies, and is used as model target for anti-CD19-directed CAR T cells[6].

Enhanced contour images from the CELLCYTE X showed that THP-1 cells have a relatively big cell size compared to other immune cell lines and that they tend to grow as single cells in suspension. Jurkat cells are comparably smaller and grow mainly as suspension cell clusters. Nalm-6 cells have approximately the same cell size as Jurkat

cells but grow as single cells in suspension **(Figure 1A)**. These observations have been confirmed in literature where THP-1 cell diameter is documented with 15-20 µm and Jurkat and Nalm-6 cells with 6-12 µm diameter[7].

Growth of the different cell lines was quantified as confluency over time and showed a steady proliferation for all three cell lines **(Figure 1B)**. As expected, wells with a higher seeding density reached full confluency faster. Additionally, it was noted that Jurkat cells did not reach full confluency in the observed time frame due to their growth as cell clusters.

Taken together, the CELLCYTE X and its reliable autofocus allowed the continuous imaging of various immune cells growing in suspension with differing growth morphologies. Additionally, cell proliferation could be analyzed in real time, providing comprehensive data of the cell growth.

Differentiation of monocytes into macrophages

Monocytes and macrophages mediate the innate immune response and inflammation. Monocytes can be recruited from the blood to differentiate and reside in tissues as macrophages. Compared to their progenitors, macrophages are long-lived and very specialized in their functions; they maintain tissue homeostasis and eliminate pathogens and infected cells via phagocytosis and cytokine secretion.

Since primary tissue macrophages are difficult to expand *ex vivo*, monocytic cell lines such as THP-1 are often used as models for research purposes; they can be stimulated with phorbol esters to differentiate into a macrophage-like phenotype[3].

Inflammatory responses of monocytes and macrophages can be triggered by pathogen components such as LPS that are part of the cell walls of gram-negative bacteria. Therefore, bacterial LPS has been commonly used in research to activate monocytes and macrophages in order to study the inflammation process[8].

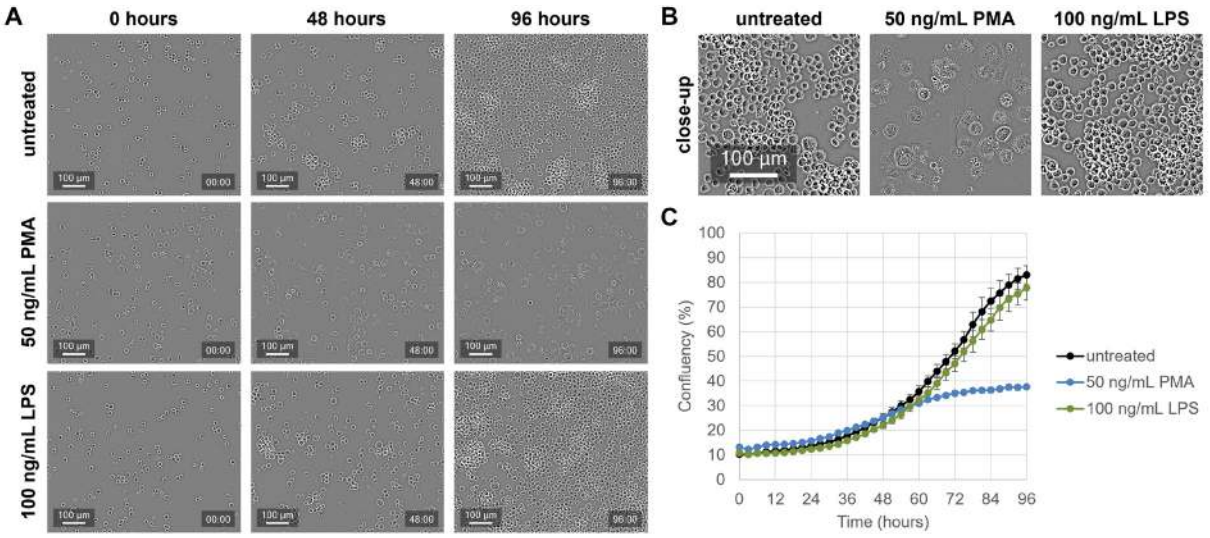

Figure 2: *Proliferation of THP-1 cells is impaired by PMA but not by LPS. (A) THP-1 cells were seeded and treated with either 50 ng/mL PMA or 100 ng/mL LPS. Their growth was monitored every 3 hours for 96 hours. (B) Changes in morphology were documented more in detail after 96 hours. (C) The cell confluency was analyzed using CELLCYTE Studio. Depicted are mean ±SEM, n = 3.*

Here, THP-1 monocytes were seeded and either treated with the phorbol ester PMA to induce macrophage differentiation or activated with *E.coli*-derived LPS to evaluate if an effect on proliferation could be observed. The cells were monitored every 3 hours for 96 hours and the cell confluence was quantified with CELLCYTE Studio **(Figure 2)**.

THP-1 cells treated with PMA changed their morphology over time. The cytoplasmic volume increased significantly, leading to an increase in cell size. The cells also started to adhere to the cell culture plate, resulting in a widespread morphology **(Figure 2A and B)**. The observed changes were in accordance with studies associating macrophage differentiation with a reduction in the nucleocytoplasmic ratio and an increase in cell adherence[9,10].

Additionally, data analysis showed a decreased cell confluence growth over time compared to the untreated control cells suggesting a decrease in cell proliferation **(Figure 2C)**. This effect is potentially even more significant than simple quantification of the confluency might suggest since the reduction of proliferation is partially masked by the described increase in cell size. Here, the CELLCYTE X allowed a comprehensive understanding of the differentiation process since it provided the visual insight in parallel to the quantification of confluency. The system tracked the entire process undisrupted through drastic morphology changes, resulting in accurate measurements of immune cell response to different stimuli.

Stimulation of THP-1 cells with LPS did not result in morphology changes **(Figure 2A and B)** and did not influence cell proliferation **(Figure 2C)**. This observation is corroborated by literature that describes LPS as a potent activator of monocytes and macrophages. Past studies have shown that exposing THP-1 cells to LPS leads primarily to upregulation of inflammation-related genes and enhanced cytokine secretion but not to changes in morphology or proliferation[8,11].

Activation-induced cell death

T lymphocytes are part of the adaptive immune response. They are characterized by the expression of specific T-cell receptors (TCRs) with which they can recognize specific antigens. T lymphocytes can have helper T cell functions, such as secreting cytokines and activating other immune cells, or they can be cytotoxic T cells that can directly kill virus-infected cells or cancer cells. When T cells encounter their antigen, they get activated and undergo clonal expansion and proliferation to reach sufficient cell numbers to efficiently eliminate their target.

Since overreactive T cells can be harmful for the body and T-cell homeostasis needs to be maintained, activated T cells need to be removed from the immune system after their function is fulfilled. This is achieved by a feedback mechanism called activation-induced cell death (AICD). During T cell activation, stimulation of the TCR leads simultaneously to the upregulation of the death receptor Fas and its ligand FasL in the T cells triggering Fas-mediated apoptosis and T-cell elimination[12].

To study AICD, researchers co-stimulate the immortalized T-cell line Jurkat with PMA and ionomycin, mimicking TCR stimulation and activation leading to AICD[13].

To monitor this process with live cell imaging, Jurkat cells were seeded in a 96-well plate and stimulated with a combination of 10 ng/mL PMA and 750 ng/mL ionomycin

(1X), or dilutions thereof (0.5X, 0.25X, 0.125X, 0.0625X). To detect dead cells, CYTENA's C.LIVE Tox Green was added to the cultures. This dye can only enter dead cells with lost membrane integrity and emits green fluorescence when it binds to DNA, presenting a valuable real-time readout of cell death. The growth and health of the Jurkat cells were monitored every 3 hours for 96 hours and the confluency and green object count were quantified **(Figure 3)**.

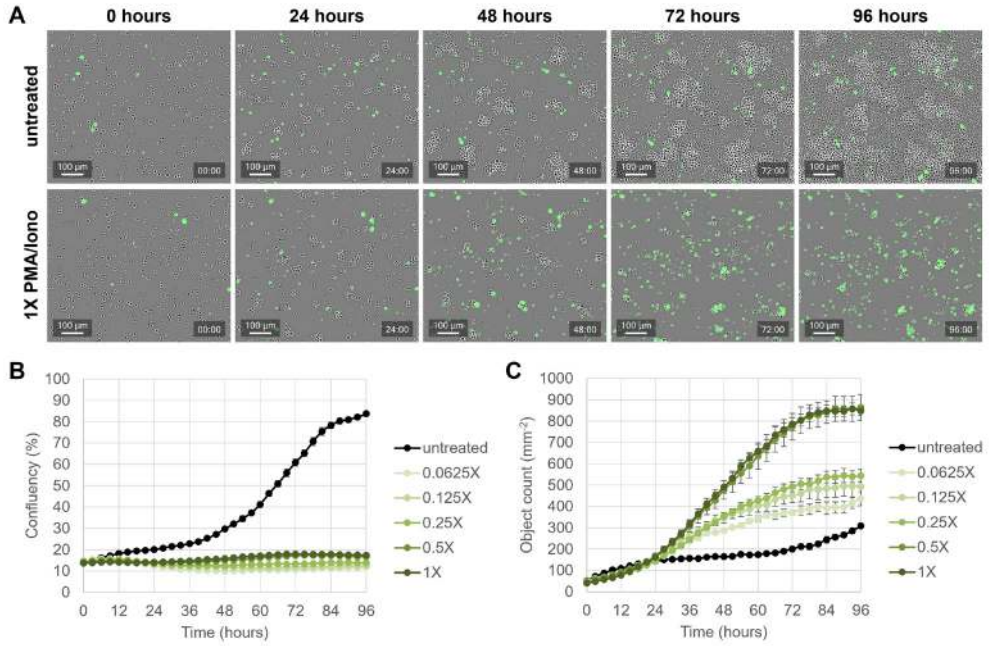

Figure 3: *Jurkat T lymphocytes undergo activation-induced cell death after stimulation with PMA/ionomycin. (A) Jurkat cells were seeded and stimulated with a combination of 10 ng/mL PMA and 750 ng/mL ionomycin (1X) or dilutions thereof (0.5X, 0.25X, 0.125X, 0.0625X). C.LIVE Tox Green was added to detect dead cells. Cell growth and health were monitored every 3 hours for 96 hours and (B) the confluency and (C) the green object count were quantified with CELLCYTE Studio. Depicted are mean ±SEM, n = 5.*

Stimulation with a combination of PMA and ionomycin over the time course of 96 hours reduced cell proliferation significantly as indicated by the steady confluence curves of the treated cells. Untreated control cells showed the expected increase in confluence over time **(Figure 3A and B)**.

Additionally, PMA/ionomycin-treated cells showed an increase in dead cells as indicated by the increase in green object count over time. This effect could be observed in a dose-dependent manner. Control cells showed only minimal cell death, especially at later time points with high cell confluence, which is expected for over-confluent cell cultures **(Figure 3C)**.

These observations suggest that treatment with PMA and ionomycin do indeed trigger AICD, as it has been suggested by other studies before which argue that PMA can activate protein kinase C and that the calcium ionophore ionomycin can raise intracellular Ca^{2+} levels both leading to activation of several intracellular signaling pathways mimicking TCR activation and leading to AICD[14].

In this study, the CELLCYTE X facilitated kinetic readouts where treatment effects could be detected in real time. CYTENA's C.LIVE Tox reagents allowed direct detection of dead cells during the process of AICD. Additional to the automated image analysis, the user can monitor cell morphology in an image-based approach to gain detailed insights in the cell death process.

Cellular immunotherapy

Cytotoxic T cells are not only responsible for the removal of pathogen-infected cells in the human body but can also recognize and eliminate malignant cells such as cancer cells[15]. This process is used in cellular immunotherapy where immune cells of the patient are isolated, modified and expanded *ex vivo*, and then transferred back to the patient as a "cellular drug". Different approaches are being investigated, but one approach that shows great promise is chimeric antigen receptor (CAR) T-cell therapy. CAR T cells are modified to express an artificial receptor that consists of an extracellular antigen-identifying domain, a transmembrane domain, and intracellular co-stimulatory and signaling domains[6]. Immunotherapies that are already approved by the U.S. Food and Drug Administration include Kymriah™ and Yescarta™, which use T cells that express a CAR against CD19, an antigen present on B-cell malignancies such as B-cell leukemia and B-cell lymphoma[16].

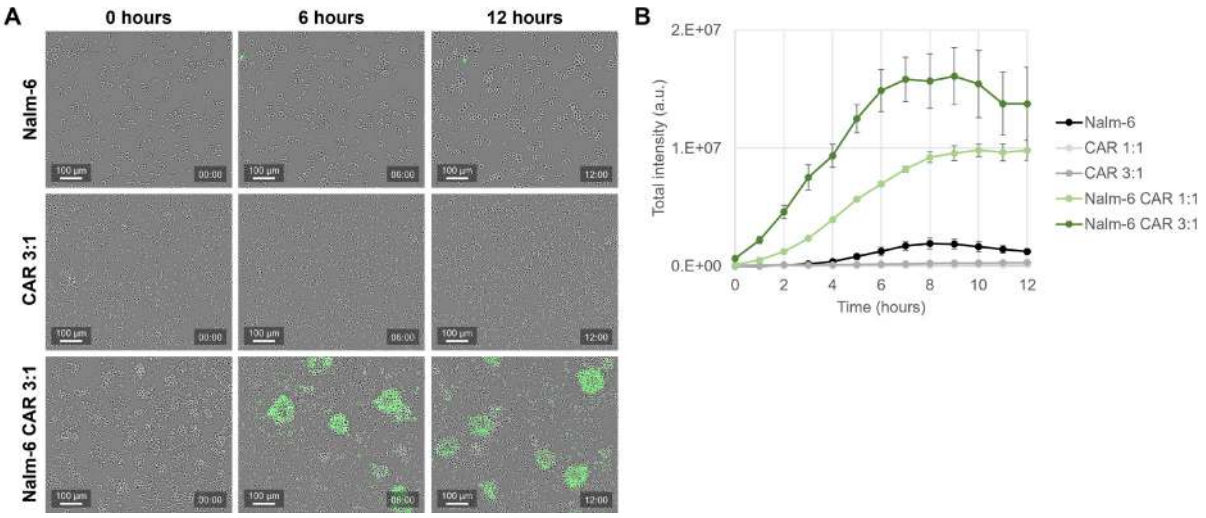

Figure 4: *Anti-CD19-directed CAR T cells eliminate CD19-positive Nalm-6 leukemia cells. (A) Nalm-6 cells were seeded in a 96-well plate and C.LIVE Caspase-3 NucView was added to the cells to detect apoptotic cells. CAR T cells were added to the Nalm-6 cells in a 1:1 or 3:1 effector-to-target ratio and the co-cultures were monitored every hour for 12 hours using the CELLCYTE X. (B) The total intensity of C.LIVE Caspase-3 fluorescence was quantified using CELLCYTE Studio. Depicted are mean ±SEM, n = 3.*

Here, we used Nalm-6 leukemia cells, which are a well-established target for anti-CD19-directed CAR T cells, to document the process of immune cell killing. Nalm-6 target cells were seeded in the absence or presence of anti-CD19-directed CAR T effector cells with an effector-to-target ratio of either 1:1 or 3:1. Apoptotic cells were detected using CYTENA's C.LIVE Caspase-3 Green NucView, a dye that can enter both healthy and dying cells but that is only activated in caspase-positive apoptotic cells where it emits a bright green fluorescence. In addition to the co-cultures, the CAR T cells were also analyzed individually. Because recognition and elimination of malignant cells by T cells can have fast kinetics, the cultures were scanned every hour for 12 hours using the CELLCYTE X **(Figure 4A)** and the total intensity of C.LIVE Caspase-3 Green NucView was analyzed with CELLCYTE Studio **(Figure 4B)**.

Nalm-6 target cells and CAR T effector cells cultured individually showed growth as single cells and only a minimal amount of apoptotic cells as indicated by low total fluorescence intensity values of the green C.LIVE Caspase-3 dye. In contrast, co-cultures of Nalm-6 cells and CAR T cells resulted in cell clusters and showed a significant increase

in green total fluorescence intensity over the time course of 12 hours, suggesting that the CAR T cells can recognize and kill the Nalm-6 cells.

Live cell imaging using the CELLCYTE X also enabled kinetic monitoring of the killing process in detail. It showed that the observed effect was dependent on the effector-to-target cell ratio as samples with higher numbers of CAR T cells showed higher total fluorescence intensities, indicating a more efficient and earlier killing of the target cells. Additionally, hourly scanning enabled a comprehensive real-time documentation of these fast immune cell killing kinetics. The observations made with the CELLCYTE X are in accordance with studies that describe CD19-directed CAR T cells as a remarkably effective treatment for B cell malignancies[16].

Conclusion

The CELLCYTE X live cell imager offers real-time monitoring and analysis of immunology assays. The studies in this application note demonstrated that:

- Robust autofocus algorithm enabled continuous tracking of various immune cells growing in suspension with differing growth morphologies
- Fast kinetics of immune responses were captured using the automatic live-cell imaging platform
- The CELLCYTE X's onboarded analytic software provided versatile and highly specific readouts to document the responses in immune cell growth and health, macrophage differentiation and T cell killing assays
- Comprehensive assessment of immune responses to different stimuli was achieved through both qualitative morphological investigations and quantitative measurement

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