APPLICATION NOTE

Evaluation of GM-CSF and TNFα-induced apoptosis with EarlyTox Cell Viability Assay Kits

Introduction

Tumor necrosis factor α (TNFα) is one of the key inflammatory cytokines which modulates various events in several cellular pathways. It is known to induce apoptosis in hematopoietic cells, such as U937, via activation of intracellular caspase cascades. Granulocyte-macrophage colony-stimulating factor (GM-CSF), on the other hand, is a member of hematopoietic growth factors that promotes proliferation and/or differentiation. However, it has been reported that U937 cells show growth inhibition and apoptosis in response to GM-CSF. While both TNFα and GM-CSF induce apoptosis in U937 cells, the time course appeared to be distinct between these two cytokines. When combined, a highly synergistic effect of the two cytokines was observed.

EarlyTox™ Cell Viability Kits are a family of reagents for assessing a spectrum of cellular conditions, including viability and various apoptotic events. These reagents are designed for homogeneous no-wash assays which are optimized for fluorescence microplate readers. Here, we used three different kits, Live/Dead Assay, Caspase-3/7 R110 Assay, and Caspase-3/7 NucView 488 Assay, to measure the effect of TNFα and GM-CSF treatment on U937 cells (Figure 1).

- The EarlyTox™ Live/Dead Assay Kit contains two markers for live or dead cells that are suitable for use with mammalian cells. Calcein AM is a widely used live-cell marker, and Ethidium homodimer-III (EthD-III) enters cells through compromised membrane and binds to DNA to produce a bright red fluorescence in dead cells.
- The EarlyTox™ Caspase-3/7 R110 Assay Kit provides a single-step, homogenous assay which requires cell lysis for endpoint analysis of the apoptosis process.
- The EarlyTox™ Caspase-3/7 NucView 488 Assay Kit enables detection of apoptosis in intact cell populations through the use of NucView 488 Caspase-3 substrate. This reagent is not toxic to cells and can be used for kinetic studies of apoptosis.

In this cell-based study, the fluorescent signals were measured on a SpectraMax® i3x Multi-Mode Microplate Reader from the bottom with the Well Scan feature, which averages signals from 12 points (user definable) across the bottom of the well to avoid well-to-well variation due to potential non-uniformity of cell growth.

Benefits

- **Homogeneous assay with easy, mix-and-read workflow**
- **Optimized for fluorescence microplate readers**
- **Increased throughput with microplate reader format**
- **Preconfigured protocol in SoftMax® Pro Software**

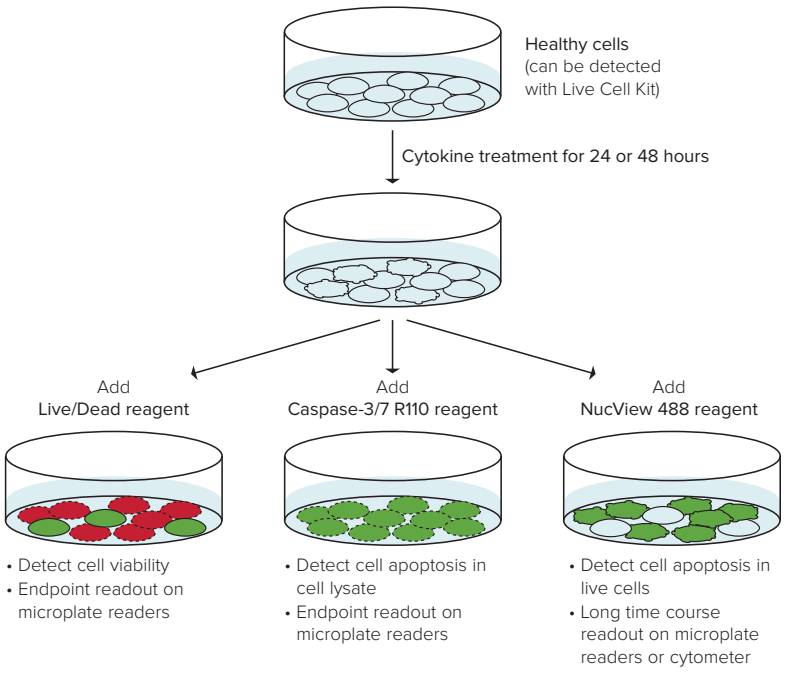

Figure 1. Evaluation of GM-CSF and TNFα-induced apoptosis.

Materials

- U937 cells (ATCC Cat.# CRL-1593.2)
- Cytokines:
  - Human TNFα (Peprotech Cat# 300-01)
  - Human GM-CSF (Peprotech Cat# 300-03)
- EarlyTox Cell Viability Reagents (Molecular Devices, LLC):
  - EarlyTox Live/Dead Assay Kit (Cat.# R8340)
  - EarlyTox Caspase-3/7 R110 Assay Kit (Cat.# R8346)
  - EarlyTox Caspase-3/7 NucView 488 Assay Kit (Cat.# R8348)
- PBS
- 96-well, flat, clear-bottom, black TC-treated microplates (Corning cat. #3904)
- SpectraMax i3x Multi-Mode Microplate Reader and SpectraMax® MiniMax 300 Imaging Cytometer

Methods

Cell culture and treatment

U937 cells were cultured in RPMI 1640 medium supplemented with 10% FBS and Penicillin/Streptomycin.

On the day of the experiment, cells were seeded at 100,000 cells/80 µL/well. 20 µL of 5X concentration of the Cytokine(s) was added to each well so the final concentration was 1X and the volume was 100 µL. The treatment was carried out in a 37°C incubator with 5% CO_2 for 24 or 48 hours.

Cell viability and apoptosis assays

After treatment, reagents were prepared and added to each well as described below.

For Live/Dead assay: A 2X working solution of calcein AM/EthD-III was prepared by adding calcein AM and EthD-III stock solutions to PBS for a concentration of 6 µM for each dye. 100 µL of the 2X working solution was added to each assay well, resulting in a final volume of 200 µL and a final concentration of 3 µM for each dye. The plate was incubated at room temperature for one hour, protected from light. Fluorescence was measured on the SpectraMax i3x reader using the settings indicated in Table 1.

	Live/Dead		Caspase-3/7 R110	Caspase-3/7 NucView 488
Parameter	Setting		Setting	Setting
Read mode	Fluorescence			
Read type	Well Scan			
Wavelengths	Live	Excitation = 495 nm Emission = 530 nm	Excitation = 490 nm Emission = 520 nm	Excitation = 490 nm Emission = 520 nm
	Dead	Excitation = 530 nm Emission = 645 nm		
PMT and optics	Flashes per read: 6 Read from bottom			
Well Scan setting	Pattern: Fill Points per well: 12 Density: 4 Point spacing: ~1.25			

Table 1. Plate reader settings.

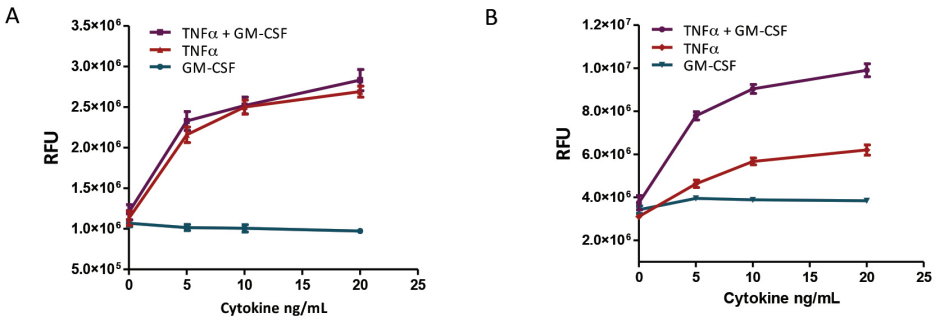

Figure 2. Concentration response of TNFα and GM-CSF induced apoptosis in U937 with Caspase-3/7 R110 assay. U937 cells were treated with the cytokine(s) in a 37°C incubator with 5% CO_2 for 24 hrs **(A)** or 48 hrs **(B)** An apoptosis assay was performed by adding the Caspase-3/7 R110 reagent directly to the wells at the specified time point and incubated at room temperature for one hour.

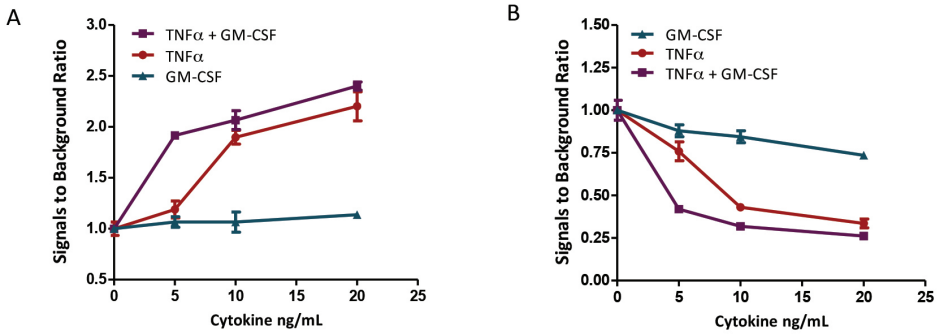

Figure 3. Concentration response of TNFα and GM-CSF induced apoptosis in U937 with Live/Dead assay. U937 cells were treated with the cytokine(s) in a 37°C incubator with 5% CO_2 for 48 hrs. Live/Dead assays were performed by adding the Live/Dead reagent directly to the wells after 48 hours and incubated at room temperature for one hour. Results are normalized to no treatment control. **(A)** Dead cell population. **(B)** Live/Dead ratio.

For Caspase-3/7 R110 assay: Substrate assay buffer was prepared by adding enzyme substrate $(AC\text{-}DEVD)_2$-R110 (2 mM) to cell lysis/assay buffer at a ratio of 50 µL to 1 mL buffer. 100 µL of substrate assay buffer was added to each well, resulting in a final volume of 200 µL per well and a final concentration of 50 µM substrate. The samples were then incubated at room temperature for one hour, protected from light. Fluorescence was measured on the SpectraMax i3x reader using the settings indicated in Table 1.

For Caspase-3/7 NucView 488 assay: A 10 µM 2X working solution of NucView 488 substrate was prepared in PBS. 100 µL of working substrate solution was added directly to wells containing 100 µL of cells and medium for a final concentration of 5 µM. Cells were incubated at room temperature for one hour, protected from light. Imaging was performed on the SpectraMax MiniMax cytometer using the 541 nm green fluorescence channel, or detected on the SpectraMax i3x reader. See Table 1 for plate reader settings.

Results

The results showed that U937 cells underwent apoptosis in a dose- and time-dependent manner when treated with TNFα. Treatment of GM-CSF had no effect on U937 for up to 48 hours. However, there was clearly a synergistic effect observed at 48 hours when the cells were incubated with a combination of TNFα and GM-CSF. It has been reported that GM-CSF alone induced apoptosis in U937 cells through a caspase 3-like pathway in a delayed time course (after 72 hours) compared to TNFα, whereas the synergistic effect of these two cytokines had been observed at 24 hours. In our study, the synergy was not apparent until 48 hours. See Figures 2-5.

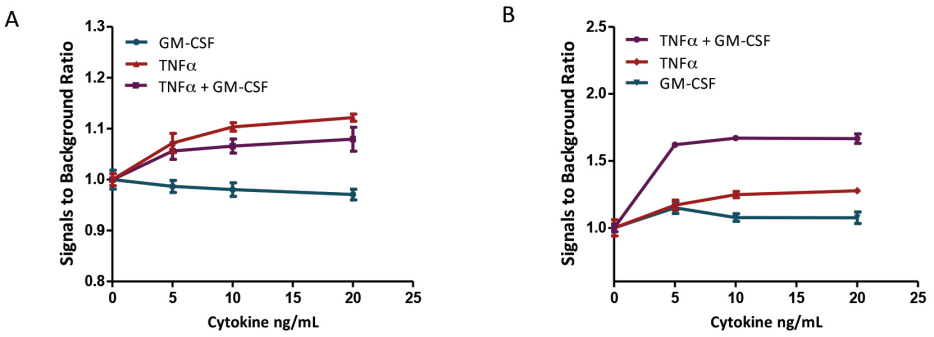

Figure 4. Concentration response of TNFα and GM-CSF induced apoptosis in U937 with Caspase-3/7 NucView 488 assay. U937 cells were treated with the cytokine(s) in a 37°C incubator with 5% CO_2 for 24 hrs **(A)** or 48 hrs **(B)**. An apoptosis assay was performed by adding the NucView Caspase-3/7 Kit reagent directly to the wells at the specified time point and incubating at room temperature for one hour.

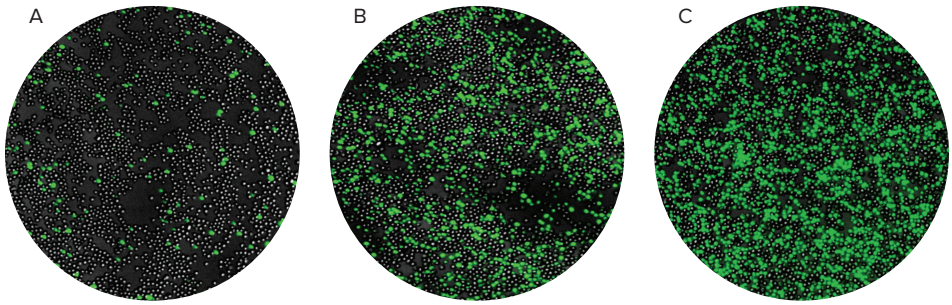

Figure 5. Synergistic effect of TNFα and GM-CSF on inducing apoptosis in U937 with Caspase-3/7 NucView 488 assay using SpectraMax MiniMax cytometer. U937 cells were treated with the cytokine(s) in a 37°C incubator with 5% CO_2 for 48 hrs. An apoptosis assay was performed by adding the NucView Caspase-3/7 Kit reagent directly to the wells at the specified time point and incubating at room temperature for one hour. The imaging data were obtained on the cytometer. Cells treated with **(A)** GM-CSF, **(B)** TNFα, or **(C)** TNFα + GM-CSF are shown.

Conclusion

By using three different reagents that measure different readouts for apoptosis, we demonstrated the mix-and-read protocols that simplified the process of assaying suspension cells. The Well Scan feature of the SpectraMax i3x reader reduced well-to-well variability, correcting for the uneven growth or distribution of cells across the wells. The family of cell viability reagents offers researchers various tools to study cellular events associated with apoptosis and/or cell death.

Reference

Okuma E., Saeki K., Shimura M., Ishizaka Y., Yasugi E., Yuo A. Induction of apoptosis in human hematopoietic U937 cells by granulocyte-macrophage colony-stimulating factor: possible existence of caspase 3-like pathway. *Leukemia* (2000) 14, 612-619.

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