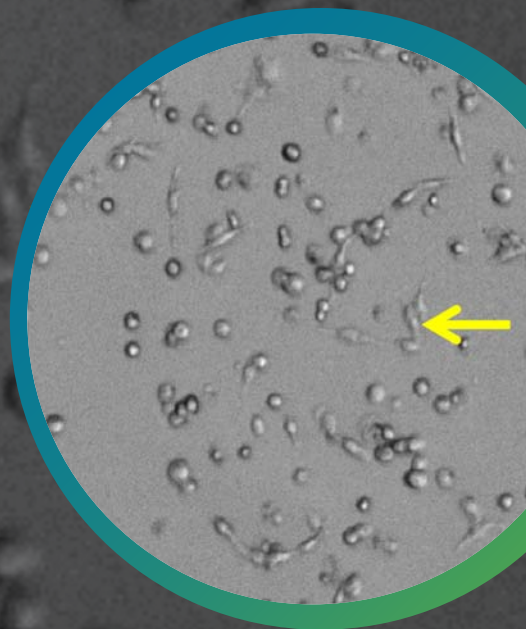


APPLICATION NOTE

Quantify TNF α secretion by THP-1-derived macrophages with an AlphaLISA assay

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Introduction

Macrophages are a type of white blood cell that plays a role in detecting and destroying infectious microorganisms, removing dead cells and debris, and in wound healing. A major cellular component of the tumor microenvironment, tumor-associated macrophages are often associated with poor prognosis and are under investigation as potential targets for anticancer therapy.¹

Derived from the blood of an acute monocytic leukemia patient, the human monocytic cell line THP-1 is widely used to study the function of monocytes and macrophages, as well as to conduct research in cancer and immunology. THP-1 cells normally grow in suspension, but when treated with phorbol 12-myristate 13-acetate (PMA) and lipopolysaccharide (LPS), they can be induced to differentiate into adherent macrophage-like cells that secrete IL-8, TNF α , and other markers, and can thus serve as a model for the biology of human macrophages, including their interaction with cancer cells.^{2,3} By using methods that can sensitively quantify levels of markers like TNF α , one can explore the cell signaling pathways involved and identify compounds that reduce TNF α secretion.⁴

Benefits

- Biomarker detection at picogram levels
- Homogeneous assay for rapid screening
- Automated curve fitting and calculation of concentrations

In this application note, effects of several anti-inflammatory compounds on TNF α secretion by THP-1-derived macrophages were measured using the AlphaLISA High Performance (HP) Human Tumor Necrosis Factor alpha (TNF α) Detection Kit. Using the SpectraMax i3x Multi-Mode Microplate Reader and this homogeneous assay, picogram levels of TNF α were detected in culture medium removed from THP-1 cells activated with PMA and LPS, and further treated with the compounds SB202190, statin, and resveratrol.

Materials

- SpectraMax i3x Multi-Mode Microplate Reader (Molecular Devices P/N i3x)
- AlphaScreen Detection Cartridge (384 HTS) (Molecular Devices P/N 0200-7018POS)
- MiniMax Imaging Cytometer
- AlphaLISA High Performance (HP) Human Tumor Necrosis Factor alpha (TNFα) Detection Kit, 500 assay points (Perkin Elmer cat. #AL3157C)
- THP-1 cells (ATCC cat. #TIB-202)
- Phorbol 12-myristate 13-acetate (PMA, Sigma cat. #524400)
- Lipopolysaccharide (LPS, Sigma cat. #L2630)
- SB202190 (Sigma cat. #S7067)
- Resveratrol (Sigma cat. #554325)
- Stattic (Sigma cat. #S7947)
- 96-well black-wall, clear-bottom cell culture microplate (Corning cat. #3603)
- Solid white, low-volume 384-well microplate (Corning cat. #4513)

Cell differentiation and treatment

Day 1–2: THP-1 cells were plated at 20,000 cells per well in a 96-well cell culture microplate and treated with 20 ng/mL PMA to induce differentiation for 48 hours. Cells were imaged at day 1 and day 2 on the MiniMax Imaging Cytometer to monitor changes in morphology.

Day 3: Cells were imaged on the MiniMax cytometer prior to washing to visually assess differentiation. Cells were washed with and then incubated in PMA-free medium for 24 hours.

Day 4–6: The compounds SB202190, stattic, and resveratrol were added to cells at concentrations starting at 100 μM, with 1:3 dilutions in medium down to 0.002 μM (11 concentrations total). Two hours after compound addition, LPS was added to cells at a final concentration of 1 μg/mL to induce inflammatory responses for 48 hours. Several wells of PMA-treated control cells received no LPS and no compound treatment.

On day 6, aliquots of the culture medium from treated and control cell wells were removed from the wells and assayed for TNFα with the AlphaLISA High Performance Human TNFα Detection Kit.

TNFα AlphaLISA assay

Please also refer to the assay’s Technical Data Sheet for details on how the reagents are prepared and standard curve and samples for analysis are set up. Here, the High Concentration Protocol (two incubation steps) outlined in the Technical Data Sheet was used in order to obtain higher assay sensitivity compared to the alternative Standard Protocol.

A 12-point standard curve consisting of TNFα standards from 0.3 pg/mL to 100 ng/mL, four replicates per concentration, was set up in a solid white, low-volume 384-well assay microplate. An additional 22 replicate wells containing no standard were included so that the lower limit of detection (LLD) for TNFα could be calculated. From each treated cell well, four aliquots of 5 μL each were transferred to four replicate wells of the 384-well assay microplate.

5 μL of a 10X mix of anti-hTNFα acceptor beads (100 μg/mL) and biotinylated anti-hTNFα antibody (10 nM) were added to each assay well, and the plate was incubated at room temperature for 60 minutes. Then 40 μL of 1.25X SA-donor beads were added to each well, and the plate was incubated at room temperature for 30 minutes. The plate was then read on the SpectraMax i3x reader with an AlphaScreen Detection Cartridge (384 HTS), using the settings shown in Table 1.

Optical Configuration	Alpha-384 HTS Detection Cartridge
Read Mode	Screen
Read Type	Endpoint
Wavelengths	Excitation: 680 nm Emission: 570 nm
PMT and Optics	Excitation Time: 40 ms Integration Time: 80 ms
More Settings	Show Pre-Read Optimization Options: ☒ Normalization: [as needed] Interlaced Reading: [as needed]

Table 1. Instrument settings for SpectraMax i3x reader with AlphaScreen 384 HTS Detection Cartridge. Selecting Pre-Read Optimization Options will enable Microplate Optimization and Read Height Optimization after the plate read is initiated. Each new lot of microplates should be optimized to ensure the center of each well is read. Read Height may be performed for each assay setup. Select the Normalization check box to normalize raw data to counts per second. Select Interlaced Reading to read wells in a checkerboard pattern that minimizes crosstalk.

Results and conclusion

THP-1 cells normally grow in suspension, and they had a spherical appearance when initially seeded in the 96-well plate. However, with incubation in 20 ng/mL PMA, they began to attach to the bottom of the wells and take on a more elongated, flatter appearance. By three days of treatment, many cells had adopted this macrophage-like morphology (Figure 1).

Culture medium removed from wells containing compound-treated THP-1 macrophages, and assayed for TNF α with AlphaLISA, revealed that each of the compounds SB202190, static, and resveratrol inhibited TNF α expression in a concentration-dependent manner. Results were plotted using 4-parameter curve fitting in SoftMax Pro Software, with EC₅₀ values calculated automatically (Figure 2). Curve fit settings include the option to calculate statistics, including an independence parameter that aids in assessing the suitability of a given curve fit for the data set.⁵

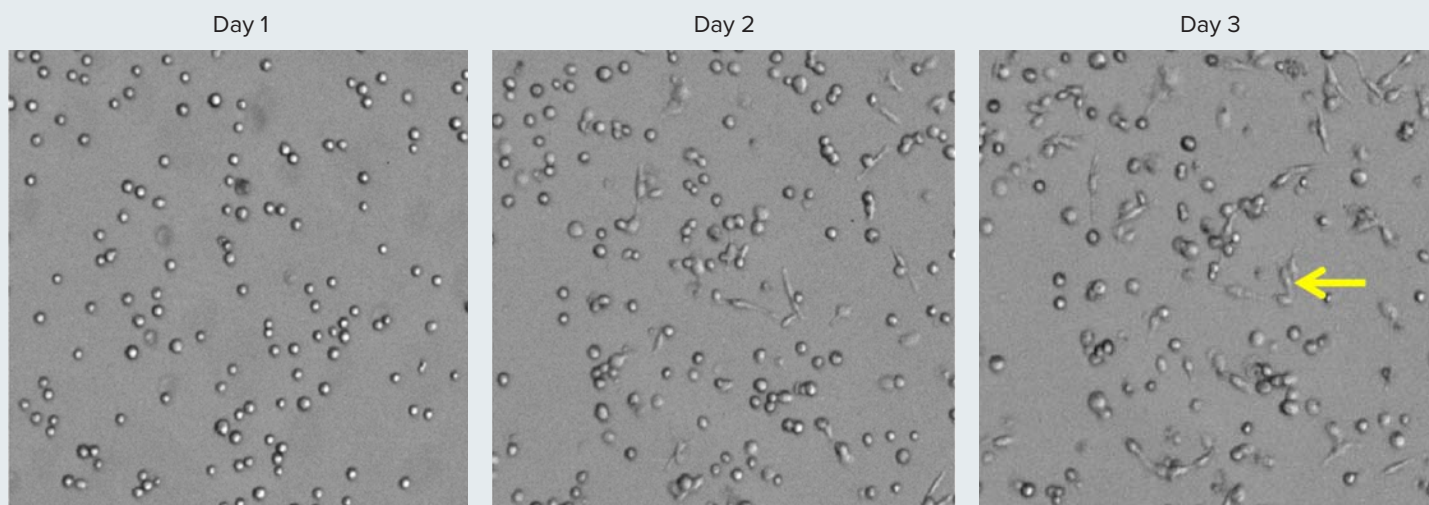


Figure 1. THP-1 cells imaged using MiniMax Imaging Cytometer. On day 1, cells exhibited a rounded morphology. After 24 hours of treatment with PMA (day 2), elongated and flattened cells were observed, with more such cells seen on day 3 (yellow arrow).

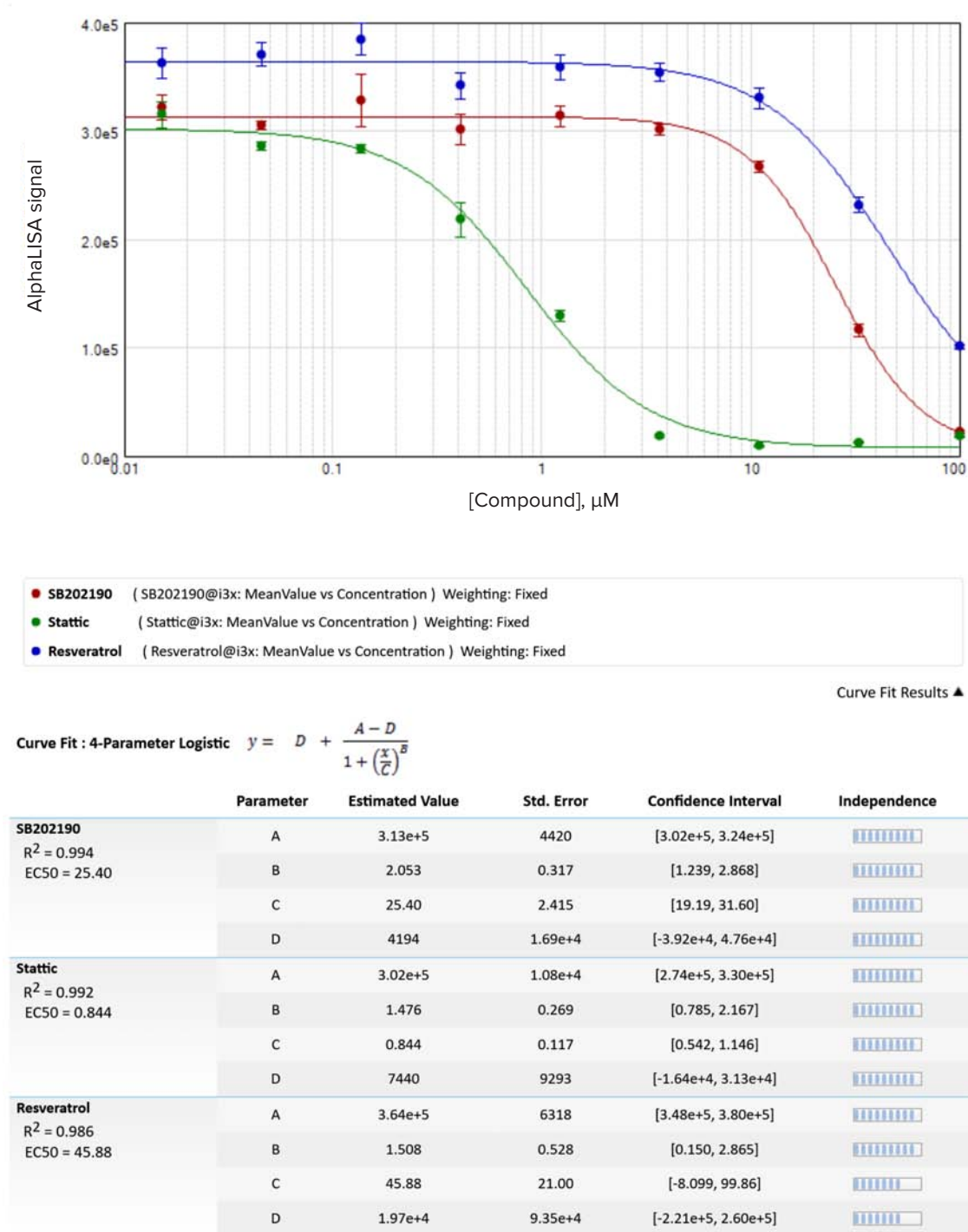
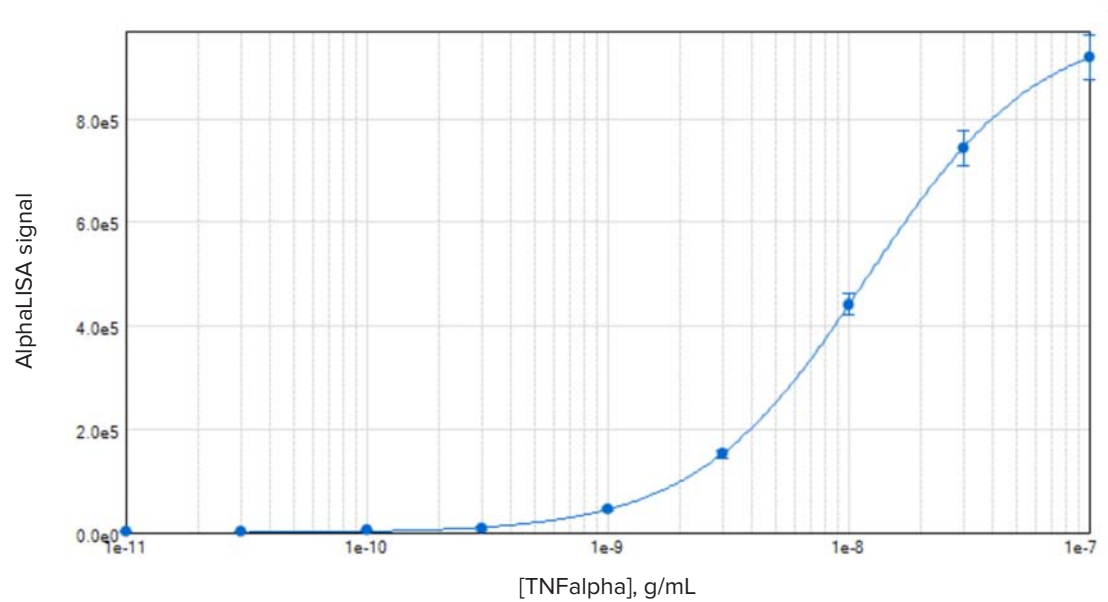


Figure 2. TNF α inhibition observed in THP-1-derived macrophages treated with SB202190 (red), stattic (green), and resveratrol (blue). Details for the 4-parameter curve fit are shown, along with the Curve Fit Results table showing the independence parameter, which is translated into bars, where the number of bars indicates the degree of independence. EC_{50} values for each compound are also displayed.

A TNFα standard curve (Figure 3) enabled calculation of actual TNFα levels in assay samples. The curve shown had an assay window from 61.7 pg/mL to 100 ng/mL. This range of AlphaLISA signal fully encompassed the signals observed for samples from treated cells, whose TNFα levels ranged from 0.47 ng/mL to 7.70 ng/mL (Table 2).



[Compound], μM	Average AlphaLISA signal			[TNFα], M		
	SB202190	Stattic	Resveratrol	SB202190	Stattic	Resveratrol
100.000	21875.318	17905.568	101068.068	5.55E-10	4.72E-10	2.04E-09
33.333	116073.32	12663.068	231673.318	2.31E-09	3.59E-10	4.56E-09
11.111	266996.32	9187.568	330201.818	5.33E-09	2.79E-10	6.83E-09
3.704	301719.82	18618.568	353620.318	6.13E-09	4.87E-10	7.45E-09
1.235	313392.57	129115.57	358557.068	6.42E-09	2.55E-09	7.58E-09
0.412	301426.82	218040.32	341897.318	6.13E-09	4.28E-09	7.14E-09
0.137	327780.07	283315.32	384501.818	6.77E-09	5.70E-09	8.31E-09
0.046	304814.57	286359.32	370525.318	6.21E-09	5.77E-09	7.91E-09
0.015	321763.32	314774.82	362926.318	6.62E-09	6.45E-09	7.70E-09

Average AlphaLISA signal			[TNFα], M
PMA-only control			9.07E-10

Table 2. AlphaLISA signal and calculated TNFα concentrations for samples from cells treated with the compounds SB202190, stattic, and resveratrol. PMA-only control cells received no LPS or compound. TNFα concentrations for each sample were calculated by interpolation from the TNFα standard curve.

These results demonstrate the utility of the AlphaLISA assay on the SpectraMax i3x reader. TNF α was quantifiable in media samples taken from macrophages stimulated with LPS and treated with compounds shown to have anti-inflammatory and antioxidant effects, compared to non-stimulated cells. All AlphaLISA signal for the assay fell within the range covered by a TNF α standard curve, thus TNF α concentrations could be calculated accurately and readily compared via analysis in SoftMax Pro Software.

The highest tested concentrations of SB202190, statin, and resveratrol reduced the secretion of TNF α to even lower levels than were observed in non-LPS-stimulated cells. Calculation of curve fit parameter independence in SoftMax Pro Software was used to assess goodness of fit for the 4-parameter curve fit applied to these data sets, and EC₅₀ values were calculated automatically.

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