Multi-parametric Phenotypic THP-1 Cell Differentiation and Cytokine Secretion Assay for Evaluation of Anti-Inflammatory Compounds

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INTRODUCTION

Macrophages originate from blood monocytes that leave the circulation to differentiate into various tissues. Macrophages are involved in the detection and phagocytosis of bacteria and damaged cells. In addition, macrophages initiate inflammation by releasing cytokines that activate vascular cells and facilitate adhesion of cytokines to blood vessels and migration into the tissues. Differentiated THP-1 cells have been widely used as an *in vitro* model of macrophages in studies of macrophage involvement in inflammatory responses.[1] The human monocytic cell line THP-1 can be differentiated to macrophages by phorbol 12-myristate 13-acetate (PMA) and activated by LPS. Activated THP-1 cells change morphology and secrete inflammatory cytokines. Monitoring the expression levels of cytokines is an important physiological read-out for cell-based models of inflammation. We present results from a multi-parametric cell-based assay that uses phenotypic imaging for macrophage formation and low volume ELISAs for secreted cytokines to evaluate effects of pharmacological compounds on inflammatory responses. THP-1 cells were stimulated with PMA and LPS for 48 hours. An increase of IL-8, IL-1β and TNF-α was observed upon PMA and LPS activation of THP-1 cells. To evaluate anti-inflammatory compounds, cells were treated with the kinase inhibitors SB202190 and PDTC, and the antibiotic moxifloxacin prior to activation. Then, inhibition of the inflammation responses by those anti-inflammatory compounds was measured by quantifying cytokine secretion. The effects on macrophage formation was also observed using phenotypic imaging. Concentration-dependent decreases in cytokine expression were seen for the compounds SB202190, PDTC, and moxifloxacin consistent with reported mechanisms of actions.

ASSAY BACKGROUND

Differentiation of THP-1 cells into macrophages was quantified by measurement of number of adherent cells using an ImageXpress® Pico Automated Cell Imaging System. The amount of IL-8, IL-1β and TNF-α in cell supernatants was quantified using a low volume, microfluidic-based Pu·MA System ELISA. The PuMA System runs ELISAs small sample volumes (10-20 µl with existing antibody pairs. This enhances the ability to measure multiple cytokines where supernatant volume is limited. The combination of imaging and low volume ELISAs provides an efficient multiparametric assay system that can be used to test the efficacy of anti-inflammatory compounds and provide insight into mechanisms of action.

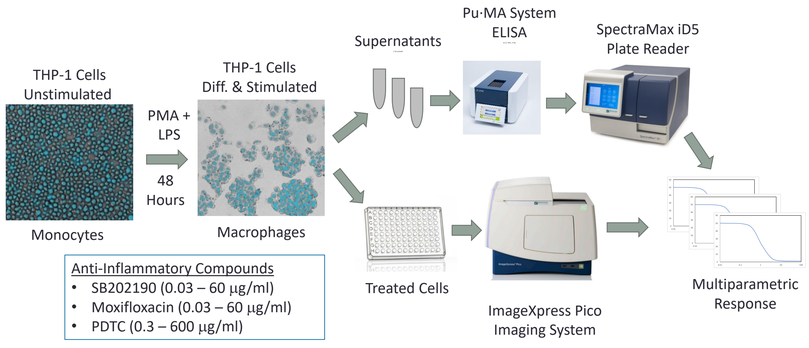


Figure 1. Cartoon of Multiparametric Inflammation assay workflow.

INSTRUMENTATION

The PuMA System is a practical and affordable benchtop instrument that runs ELISA assays using your own antibodies, or precoated flowchips in a streamlined workflow. The PuMA platform has been designed to fit seamlessly into your current laboratory workflow.

- Runs complete ELISA in < 3 hours with "hands-off" processing
- Reduces sample and reagent volumes to 10 - 20 µl
- Works with your existing ELISA kits and antibody pairs

The ImageXpress Pico system includes:

- Four colors + transmitted light
- Environmental control

The system is controlled by CellReporterXpress™ Image Acquisition and Analysis Software

SpectraMax® iD5 Multi-Mode Microplate Reader was used for absorbance measurements, calculation of concentrations and EC_{50} values

References:
[1]Optimized THP-1 differentiation is required for…; Park et al, Inflamm Res. 2007, **56,** 45.
[2]p38α MAP kinase serves cell type-specific inflammatory functions…; Kim et al, Nat Immunol. 2008, **9**, 1019.
[3]PDTC is a potent antioxidant…; Zhu et al, FEBS Letters 2002, **532,** 80.
[4]Anti-Inflammatory Effects of Moxifloxacin…; Weiss et al, Antimicrob Agents Chemother. 2004, **48,** 1974.

INFLAMMATION ASSAY

The protocol for the multiparametric inflammation assay is given below. Upon stimulation, differentiated THP-1 cells will adhere to the plate and secrete upregulate cytokines. Phenotypic changes resulting from THP-1 differentiation are shown in Figure 2. Increases in cytokine secretion from stimulation with PMA and LPS are shown in Figure 3.

- THP-1 cells were plated 20,000 cells per 96well and incubated for 48 hr. Next they were stimulated with a mix of PMA & LPS for 24 hr (0-5 pg/mL of PMA, and 0-100 pg/mL LPS; all from Sigma).
- Anti-inflammatory compounds were added 2 hr prior to cytokine stimulation
- After incubation, 60 µl of supernatant was taken for ELISA analysis from each well. The samples were analyzed fresh or stored at -70C for subsequent analysis.
- Cells were imaged using transmitted light (TL) using ImageXpress Pico system. Before imaging, non-adherent cells were washed 2x with media. Cells were counted in TL.
- Supernatants were diluted 3:1 in assay buffer and analyzed for IL-8, TNFα, and IL-1β using the Pu·MA System flowchips and reagents (all Ab pairs from BioLegend).

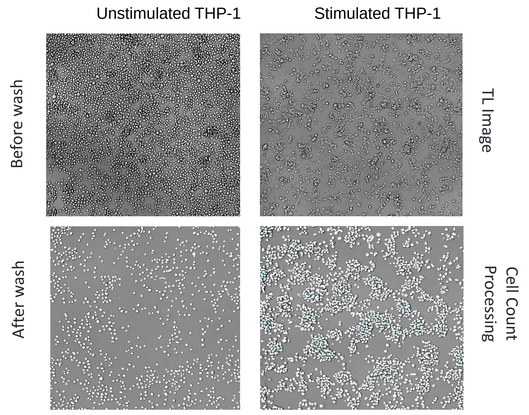
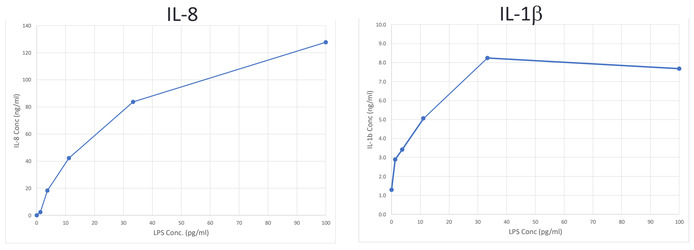


Figure 2. Images of stimulated or un-stimulated THP-1 cells before and after removal of non-adherent cells. Top: Unstimulated (Left) and stimulated (Right) THP-1 cells. Transmitted light images. Bottom: Cell cultures after wash. Analysis masks for cell count shown in white.



IL-8 Pos/Neg (ng/ml)		
	Avg	SD
Stim	127	0.2
Unstim	4.2	2
Fold Ind	30	

IL-1b Pos/Neg (ng/ml)		
	Avg	SD
Stim	7	0.5
Unstim	1.2	0.2
Fold Ind	6	

TNFa Pos/Neg (ng/ml)		
Stim	3	0.1
Unstim	0.2	0.1
Fold Ind	15	

Figure 3. Increase in cytokine secretion as a function of LPS concentration. Amount of IL-8, IL-1β, and TNFα at lowest (Unstim) and highest LPS concentrations (Stim) are provided in tables.

LOW-VOLUME AUTOMATED ELISA

The benefits of microfluidic assays include reduced reagent use and faster time to results. In the Pu·MA System workflow all reagents are pre-loaded into flowchips and then automatically sent through an assay channel where the immunoassay complex is formed. All wash steps are integrated into the protocol. After the protocol is finished quantification is done by reading absorbance on a plate reader.

Pu·MA System Workflow

Assay Steps

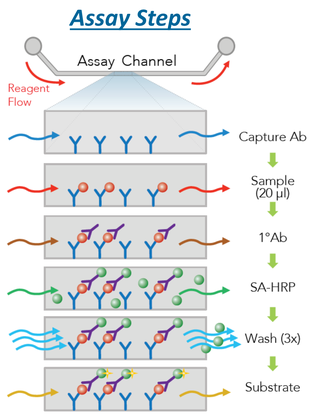


Figure 4. ELISA workflow for automated Pu·MA System and cartoon of automated assay steps in an ELISA protocol. The microfluidic assay channel provides solid support for the capture antibody.

ANTI-INFLAMMATORY DRUGS

Inflammation is triggered by activation of receptors with cytokines leading to a cascade of signaling events. Kinases activate transcription factors that up-regulate adhesion molecules and cytokines (markers). Different markers are under control of different pathways and transcription factors. We investigated three known compounds that effect different parts of the inflammation pathways and measured the response of five markers.

- SB202190 a p38 MAPK inhibitor, acts on JAK/STAT and NFκB pathways[2]
- PDTC an anti-oxidant, suppresses activation of NFκB[3]
- Moxifloxacin inhibits the enzyme bacterial DNA gyrase and prevents replication of bacterial DNA during bacterial growth and reproduction[4]

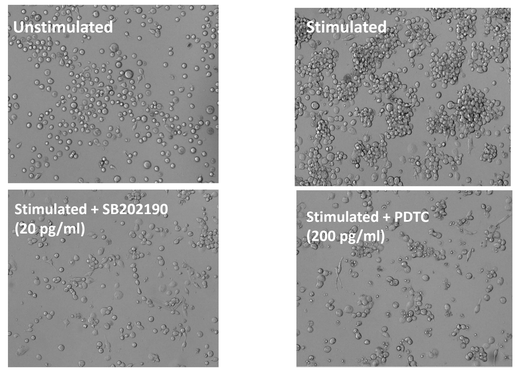


Figure 5. Affect of anti-inflammatory compounds on phenotypic response of THP-1 cells. Top: Transmitted Light images of THP-1 cells with or without PMA + LPS stimulation. Bottom: Stimulated cells incubated with compounds for 4 hr prior to PMA + LPS addition.

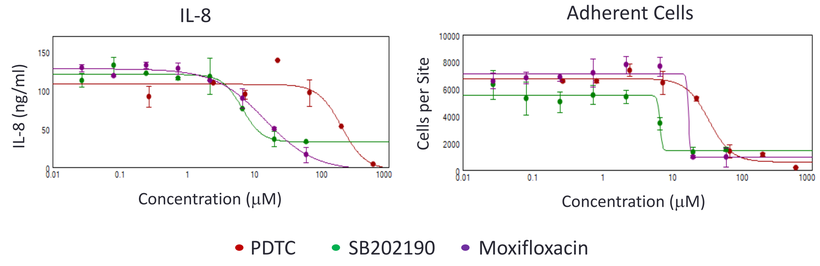


Figure 6. Concentration dependent effects on IL-8 and number of adherent cells by three anti-inflammatory compounds. EC_{50} values derived from a 4-P fit are given in the table below.

EC50, µM	IL-8	IL-1β	TNFα	Adhesion
PDTC	201	42	63	33
SB202190	6.6	9.8	6.4	6.6
Moxifloxacin	17.4	9.9	17.9	17.1

- THP-1 cells were treated with anti-inflammatory compounds for 4 hr then THP-1 cells were activated with 5 pg/ml of PMA and stimulated with 100pg/ml of LPS
- Levels of cytokines in supernatants were measured by Pu·MA System ELISA and SpectraMax iD5 reader
- Numbers of adhered cells were counted by imaging after washing of non-adherent cells
- EC_{50} values were calculated using 4-parametric curve fit with SoftMax® Pro Software

CONCLUSIONS

- We have demonstrated a multiparametric inflammation assay using a THP-1 cell model with automated cellular imaging and a novel microfluidic ELISA system.
- The Pu·MA System performs immunoassays with existing ELISA antibody pairs using microfluidic flowchips that reduce reagent use and improve time-to-results
- The responses of three different inflammation markers to three anti-inflammatory compounds were characterized. Observed differences in behavior were consistent with published mechanisms of action.

MOLECULAR DEVICES

PROTEIN FLUIDICS