Multi-parametric Human Cell-Based Inflammation Assay for Cytokines and Cell Surface Antigens

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INTRODUCTION

Inflammation is a complex event in which cells respond to various endogenous and exogenous stimuli. Factors such as tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), and interferon gamma (IFN-γ) activate signaling pathways leading to the expression of cell-surface antigens that facilitate binding of immune cells to blood vessels. The ability to monitor up-regulation of molecules, such as MCP-1, IL-8, IL-6, VCAM, and HLA-DR with endothelial cells provides an important physiological read-out for cell-based models of inflammation. We present results from a multiparametric primary human cell-based assay that uses immunoassays for secreted cytokines and fluorescence read-outs of cell surface markers to evaluate the effect of different mediators on inflammatory response. Expression of the inflammation markers on primary human umbilical vein endothelial cells (HUVEC) stimulated with inflammation cytokines (TNF-α, IFN-γ, and IL-1β) was quantified by measurement of total fluorescence intensity after staining with directly conjugated antibodies and microfluidic-based ELISAs. The combination of imaging and microfluidic-based assays provides an efficient multiparametric assay system that can be used to test the efficacy of anti-inflammatory compounds versus toxicity and also provide significant insight into the mechanism of action by selective inhibition of markers triggered by different signaling pathways.

ASSAY BACKGROUND

Endothelial cells play a critical role in inflammation by responding to several endogenous and exogenous proinflammatory stimuli.[1] Here we assessed differences in signaling pathways, adhesion molecules, and cytokines induced by proinflammatory factors (IFN-γ, TNF-α, and IL-1β) in human umbilical vein endothelial cells (HUVEC) using multi-parametric readouts.[2] The PuMA System was used to quantify MCP-1, IL-8, and IL-6 in cell supernatants. The ImageXpress® Nano Automated Imaging System was used to monitor cells surface expression of VCAM and HLADR. The PuMA System runs ELISAs using existing antibody pairs without requiring antibody conjugation using small sample volumes (10-20 µl). This enhances the ability to measure multiple cytokines from a single well for inflammation assays where supernatant volume is limited.

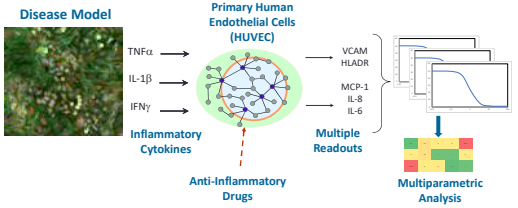


Figure 1. Cartoon of Multiparametric Inflammation assay workflow.

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® Nano Automated Imaging System includes:

- Four colors + transmitted light
- Environmental control

The system is controlled by CellReporterXpress™ Automated Imaging Acquisition and Analysis Software

References:
[1]Proinflammatory Activation Pattern of (HUVEC) cells...; Mako et al, Cytometry Part A, 2010, **77A,** 962.
[2]Method for analyzing signaling networks in complex cellular systems; Plavec I, Sirenko O, et al, PNAS 2004;101(5):1223-8.
[3]Proteasome inhibition leads to NF- k B-independent IL-8 transactivation...; Hipp et al, Eur J Immunol. 2002, **32,** 2208.
[4]p38α MAP kinase serves cell type-specific inflammatory functions...; Kim et al, Nat Immunol. 2008, **9**, 1019.
[5]PDTC is a potent antioxidant...; Zhu et al, FEBS Letters 2002, **532,** 80.
[6]Cytokine responses of human intestinal epithelial-like Caco-2 cells...; Hosoi et al, Int J Food Microbiol, 2003, **82**, 255.

INFLAMMATION ASSAY

The protocol for the multiparametric inflammation assay is given below. Marker expression results from stimulation with cytokine (CK) mix are shown in Figure 2.

- HUVEC were plated 5,000 cells per 96well and incubated for 48 hr. Next they were stimulated with a mix of inflammatory cytokines for 24 hr (5 ng/mL of TNFα, 1 ng/mL IL-1β, 50 ng/mL of IFNγ; all from R&D Systems).
- Anti-inflammatory compounds were added 1 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 -80C for subsequent analysis.
- Cells were then fixed with 4% formaldehyde and stained with antibodies (Ab) for 1 hr and imaged using an ImageXpress Nano Automated Imaging System.
  - Ab's: FITC-mouse anti-human VCAM and PE-mouse anti-human HLA-DR
- Supernatants were diluted 3:1 in assay buffer and analyzed for MCP-1, IL-6 and IL-8 using PuMA Flowchip system and reagents (all Ab pairs from BioLegend)

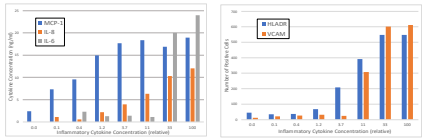


Figure 2. Increase in cytokine secretion (Left) and marker positive cells (Right) in response to stimulation with inflammatory cytokine cocktail. 100 = Maximum CK mix concentration.

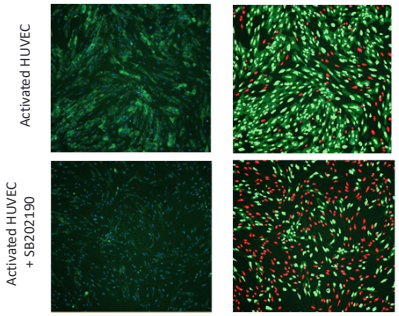


Figure 3. Images of activated HUVEC cells stimulated with CK mix with or without treatment of 10µM SB202190. Left: Cells stained with anti-VCAM antibodies (green) and Hoechst (blue). Right: Analysis masks for nuclei of VCAM-positive cells (light green), cytoplasm of VCAM positive cells (dark green), nuclei of VCAM negative cells (red).

MICROFLUIDIC ELISA

The benefits of microfluidic assays have been well documented including reduced reagent use and faster time to results. However, typical microfluidic-based systems require complex and costly instrumentation and do not work with off-the-shelf ELISA antibody pairs. The PuMA System provides the advantages of microfluidics in a cost-effect platform that works with any existing ELISA antibody pair.

PuMA Flowchip Workflow – 2-3 hours "hands-off"

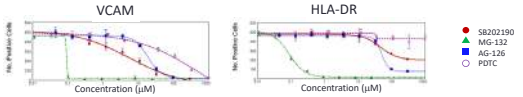
Standard ELISA Workflow – 2 Days

Figure 4. Comparison of assay protocols between PuMA Flowchip and 96-well plate formats.

ANTI-INFLAMMATORY DRUGS

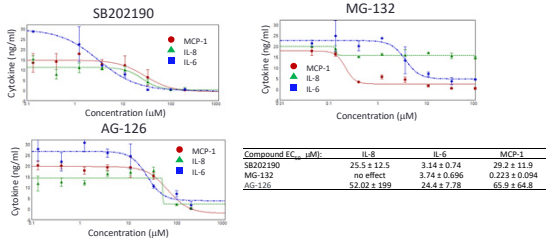
Inflammation is triggered by activation of receptors with cytokines leading to a cascade of signaling events. Kinases activate transcription factors that up-regulate adhesion molecules and cytokines (markers). Different markers are under control of different pathways and transcription factors. We investigated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

- MG-132 a proteasome inhibitor, suppresses MCP-1 & IL-6 but stimulates IL-8[3]
- SB202190 a p38 MAPK inhibitor, acts on JAK/STAT and NFκB pathways[4]
- PDTC an anti-oxidant, suppresses activation of NFκB[5]
- AG-126 a tyrosine kinase inhibitor, suppresses cytokine secretion and adhesion molecule up-regulation[6]



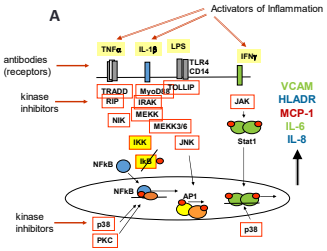
Compounds EC50s (µM):	VCAM pos cells	VCAM ave intensity	HLADR pos cells	HLADR ave intensity
SB202190	9.88 ± 2.71	5.45 ± 1.44	47.5 ± 10.0	35.3 ± 5.62
MG-132	0.09 ± 0.10	0.089 ± 0.036	0.091 ± 0.009	0.10 ± 0.20
AG-126	18.9 ± 2.28	16.21 ± 2.83	51.3 ± 3.55	>100
PDTC	344 ± 137	117 ± 71.9	no effect	no effect

Figure 5. Concentration dependent effect on up-regulation of adhesion molecules by four anti-inflammatory compounds. EC_{50} values derived from a 4-Parameter fit are given in the table.



Compound EC_{50} µM):	IL-8	IL-6	MCP-1
SB202190	25.5 ± 12.5	3.14 ± 0.74	29.2 ± 11.9
MG-132	no effect	3.74 ± 0.696	0.223 ± 0.094
AG-126	52.02 ± 199	24.4 ± 7.78	65.9 ± 64.8

Figure 6. Concentration dependent effect on IL-8, IL-6, and MCP-1 by three anti-inflammatory compounds as measured by PuMA Flowchip immunoassay. EC_{50} values derived from a 4-P fit are given in the table.



C	SB202190	MG-132	AG-126	PDTC
VCAM	9.9	0.09	19	344
HLA-DR	48	0.09	51	>1000
MCP-1	29	0.22	66	
IL-6	3.1	3.7	24	
IL-8	26	>1000	52	

Color Scale (µM)

Figure 7. A: Schematic of signaling pathways involved in inflammation response including NFκB, JNK-AP1, and JAK-STAT. **B:** Cartoon showing effect of AG-126 and SB202190 on signaling pathways leading to differential expression. **C:** Heat map of the multiparametric immunoassay EC_{50} results.

CONCLUSIONS

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

MOLECULAR DEVICES

PROTEIN FLUIDICS