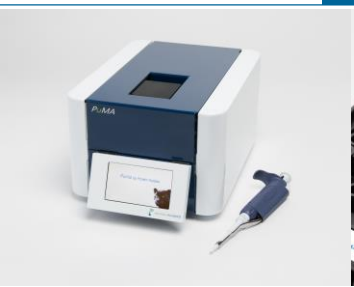

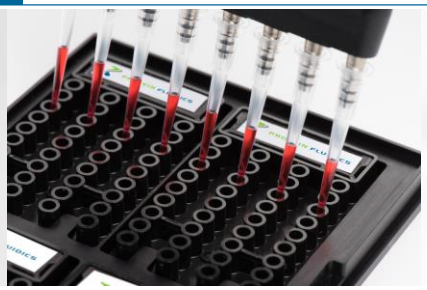

Multiparametric Inflammation Assay

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. In this note 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 PuMA microfluidic-based assay system, PuMA, was used to quantify MCP-1, IL-8, and IL-6 in cell supernatants. The combination of imaging and microfluidic-based assays provides an efficient multiparametric assay

Assay Procedure

The workflow for the multiparametric inflammation assay is shown in Figure 1. Standard cytokine curves for ELISAs run on the PuMA System 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 ml 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 (Molecular Devices, data not shown). Supernatants were diluted 3:1 in assay buffer and analyzed for MCP-1, IL-6 and IL-8 using the PuMA System and reagents (Ab pairs acquired from BioLegend)

PuMA System Complete Workflow – 2-3 hours "hands-off"

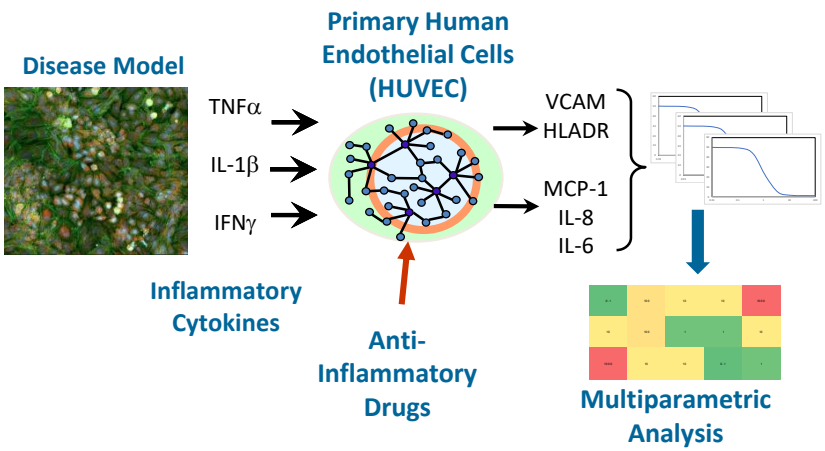

Figure 1. Schematic of Multiparametric Inflammation assay workflow.

Results & Discussion

Endothelial cells play a critical role in inflammation by responding to several endogenous and exogenous proinflammatory stimuli.[1] Three important factors that provide inflammatory signals to endothelial cells are IFN-g, TNF-a, and IL-1b. The purpose of this assay is to assess differences in the signaling pathways, adhesion molecules, and cytokines induced by proinflammatory factors in human umbilical vein endothelial cells (HUVEC) using multi-parametric readouts.[2]

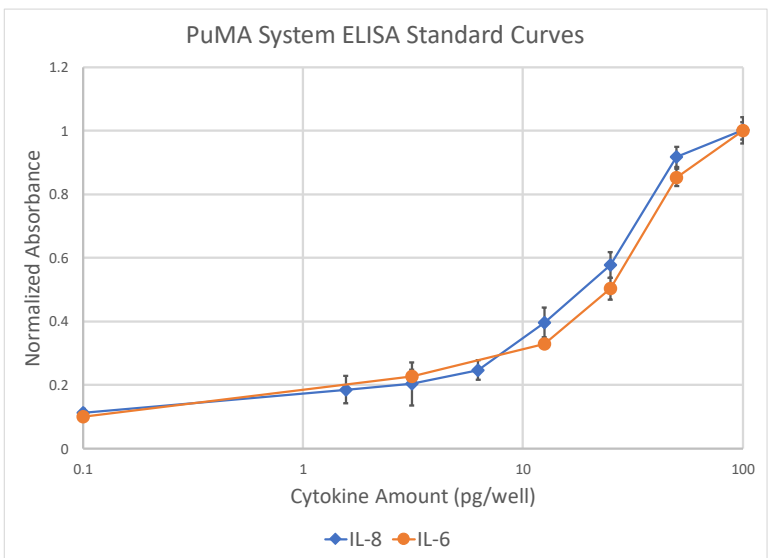

Figure 2. Response of Cytokine Standards for ELISA run on PuMA System. ELISA antibody pairs and standards obtained from BioLegend. Responses scaled to 1; errors bars represent +/- 1 stdev, n=4.

HUVECs were incubated with anti-inflammatory compounds then exposed to proinflammatory factors (IFN-γ, TNF-α, and IL-1β). The cytokines MCP-1, IL-8, and IL-6 as well as adhesion molecules VCAM and HLADR were all found to be upregulated in a concentration dependent manner (data not shown) with no anti-inflammatory compounds. The PuMA System was used to quantify the amount of MCP-1, IL-8, and IL-6 in cell supernatants (see Figure 3) showing concentration dependent reduction of the cytokines by the three anti-inflammatory compounds.

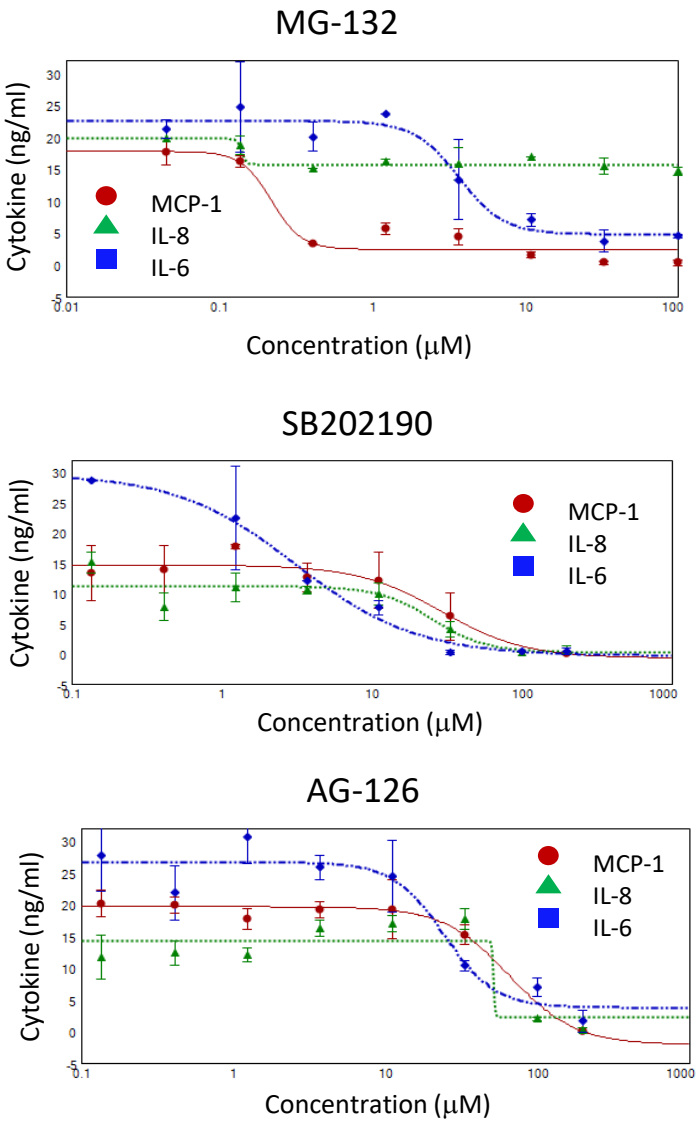

Figure 3. Concentration dependent effect on IL-8, IL-6, and MCP-1 by three anti-inflammatory compounds as measured by PuMA System immunoassay. EC_{50} values derived from a 4-P fit are given in the table.

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

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]

A	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	

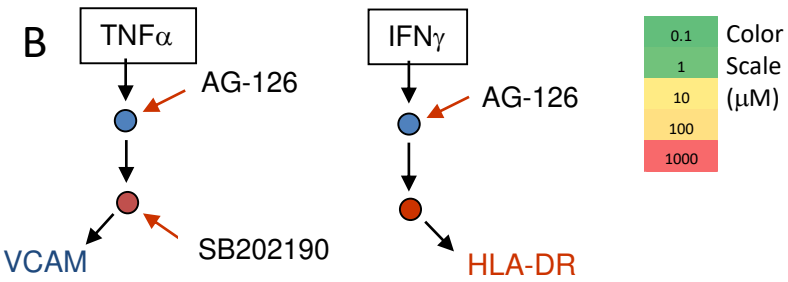

Figure 4. A: Heat map of EC_{50} results from the multiparametric inflammation assay. **B:** Cartoon showing effect of AG-126 and SB202190 on signaling pathways leading to differential expression.

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 while reducing reagent use and improving 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.

[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.

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