High-Throughput Multiplexed Inflammation Assay Using Primary Cells

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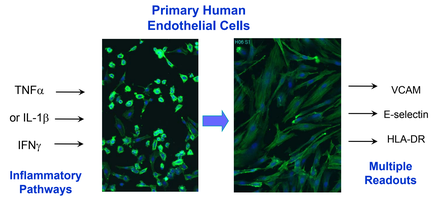
Introduction

An important event in the inflammation response is the expression of cell-surface antigens that facilitate binding of immune cells to blood vessels. The ability to monitor up-regulation of these molecules, such as VCAM, E-selectin, and HLADR on endothelial cells provides an important physiological read-out for cell-based models of inflammation.

We present results from a multiplexed, primary human cell-based assay that uses fluorescence read-outs of inflammation markers to evaluate the effect of different mediators on inflammatory response. Expression of the inflammation markers on primary endothelial cells (HUVEC) stimulated with inflammation cytokines (TNFα or IFNγ) was quantified by measurement of total fluorescence intensity after staining with directly conjugated antibodies.

Comparison is made between IsoCyte scanning cytometer and SpectraMax® M5 microplate reader for fold increase, Z-prime, and throughput. The multiplexed read-outs 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.



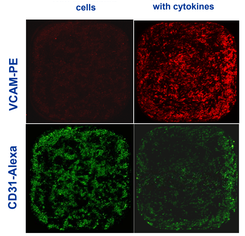
Method

Fluorescence Detection of Inflammation Markers

The **IsoCyte-DL** scanning cytometer platform used for this demonstration was configured with a 20mW 488nm and 40mW 640nm lasers. The 488nm laser was used for Alexa Fluor® 488 (AF488) labeled cell detection using a 510-540nm band pass (bp) filter (Ch1); and Phycoerythrin-labeled detection of the markers using a 560-610nm bp filter (Ch3). APC detection was done at 640 excitation and 660-720nm emission filter. The image acquisition was done at 5 x 5 micron sampling and an entire 96-well or 384-well plate was scanned in < 5 minutes.
The **SpectraMax® M5** plate reader was used with 488 nm excitation and emission at 530nm (cut-off 515nm) for Alexa Fluor® 488 labeled cell detection and 590nm (cut-off 550nm) for Phycoerythrin (PE)-labeled detection

Inflammation Assay

HUVEC cells (Lonza) were stimulated with inflammatory cytokines (TNFα, IFNγ; R&D) for 24h. Cells stained with directly conjugated antibodies (Becton Dickinson) against adhesion molecules as recommended by the BD protocol. Selected antibodies used for optimization:
- PE-conjugated anti-CD106 (VCAM),
- APC-conjugated anti-E-selectin,
- APC-conjugated anti-HLA-DR,
- AF488-conjugated anti-CD31(PECAM)



Results and discussion

Expression of Inflammation Markers Measured by IsoCyte

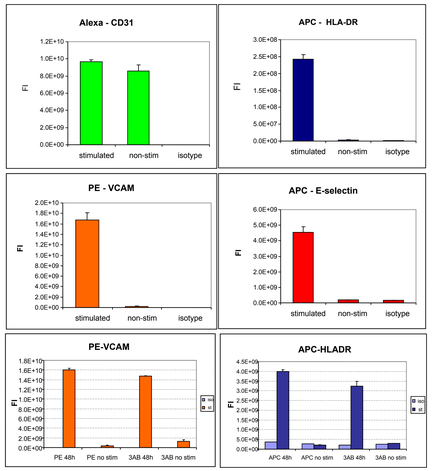
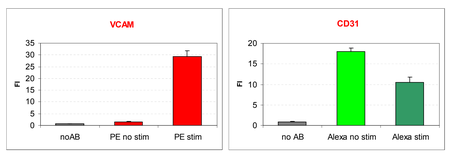


Figure 1. HUVECs were stimulated with cytokines for 24h. Fluorescent intensities were measured by IsoCyte after staining cells with directly conjugated antibodies or appropriate isotype controls. A comparison between single AB (antibodies) and multiplexed AB (3AB) is shown in the bottom charts. The assay allows multiplexing and simultaneous read-outs for different markers and signaling pathways.

Expression of Inflammation Markers Measured by Plate Reader SpectraMax® M5



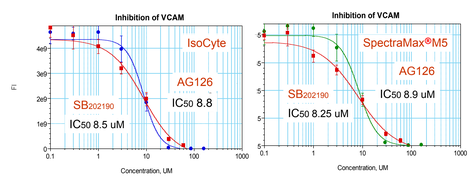


Figure 2. HUVEC cells were stimulated with cytokines for 24h. Fluorescent intensities were measured by SpectraMax® M5. Inhibition constants for different compounds could be determined by either IsoCyte or SpectraMax® M5 instruments.

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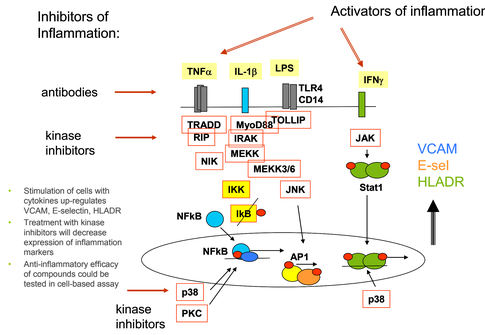
Inhibition of Adhesion Molecules by Anti-Inflammatory Compounds



Comparison of Anti-Inflammatory Potency of Compounds

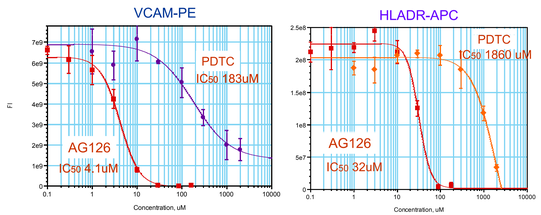


Figure 3. Tyrosine kinase inhibitor AG126 and anti-oxidant PDTC both decrease up-regulation of VCAM and HLA-DR. IC50s for different compounds could be determined by the assay.

Multiplexing of Inflammation Assay

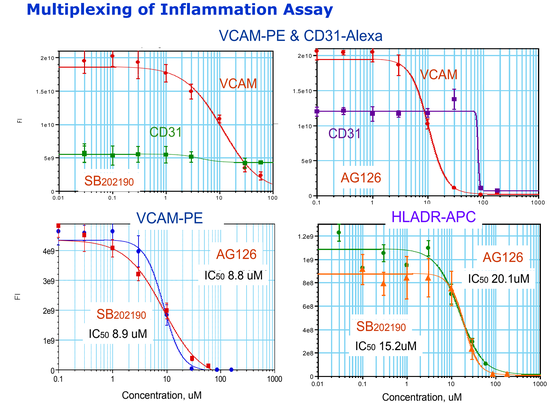
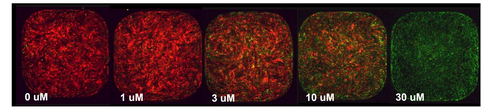


Figure 4. Top. Multiplexing : Inflammation plus toxicity assay
Tyrosine kinase inhibitor AG126 and p38kinase inhibitor SB202190 both decrease up-regulation of VCAM. Dose-dependent inhibition of the expression of VCAM is measured by fluorescence on IsoCyte. Decrease of CD31 marker suggests toxicity effect of AG126 at high doses.

Bottom. Multiplexing: Different inflammation pathways
VCAM and HLA-DR are under control of different signaling pathways: NFkB and Jak-Stat respectively. Both inflammation markers were measured by IsoCyte in the same assay. p38Kinase inhibitor SB202190 and tyrosine kinase inhibitor AG126 decrease up-regulation of VCAM and HLA-DR.

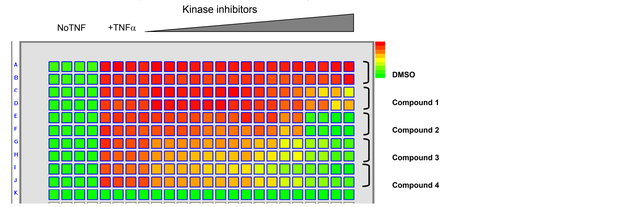
High-Throughput Assay for Anti-Inflammation Efficacy of Compounds

Dose-dependent inhibition of VCAM expression by SB202190:



VCAM-red; CD31-green

VCAM, intensity of fluorescence (heat-map):



IsoCyte	96 well format		384 well format	
	S/C	Z'	S/C	Z'
VCAM	68	0.75	580	0.7
HLA-DR	23	0.72	12	0.8
E-selectin	66	0.81	156	0.7
CD31 (PECAM)	374	0.93	126	0.7

SpectraMax® M5	96 well format		384 well format	
	S/C	Z'	S/C	Z'
VCAM	20	0.73	5	0.75
CD31 (PECAM)	12	0.93	4	0.70

S/C- signal to control, stimulated vs non-stimulated cells; for CD31 – signal to isotype control

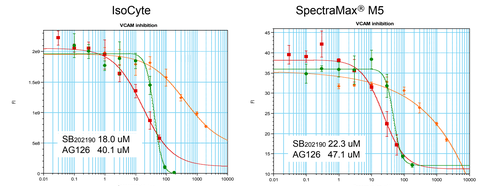


Figure 5. The assay is suitable for either 96-well or 384-well format. Experimental windows were smaller for 384 format in comparison to 96 format. This could be due to instrumental differences or changes in the biological levels of expression of the markers
Z'-values were comparable between 96 and 384 formats.
Despite different sensitivities of IsoCyte and SpectraMax® M5, inhibition constants for each compound could be accurately determined.

Summary

- We demonstrated that measurement of adhesion molecules by directly labeled antibodies provides a powerful assay for testing efficacy of anti-inflammatory compounds
- We have tested expression of several inflammation markers: VCAM, E-selectin, HLA-DR, as well as CD31 on HUVEC stimulated with inflammation cytokines, with or without anti-inflammatory drugs
- We show that up-regulation and inhibition of inflammation markers can be efficiently determined by IsoCyte or SpectraMax® M5 in a multiplexed assay format
  - Data generated for three known anti-inflammatory compounds: AG126, SB202190 and PDTC confirms utility of the assay for testing anti-inflammatory molecules
- Multiplexed inflammation assay can be used for testing efficacy of anti-inflammatory compounds in either 96 or 384 multi-well formats in a high-throughput compatible format

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