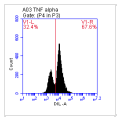

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Flow cytometry based monocyte adhesion assay for quantification of endothelial activation in vitro

Vinnyfred Vincent[1], Himani Thakkar[1], Anjali Verma[2], Atanu Sen[3], Nikhil Chandran[1], Archna Singh[1]

[1]Department of Biochemistry, All India Institute of Medical Sciences, New Delhi, India, [2]Department of Zoology, University of Delhi, New Delhi, India, [3]Department of Cardiac Biochemistry, All India Institute of Medical Sciences, New Delhi, India

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Vinnyfred Vincent

ABSTRACT

Endothelial pro inflammatory activation is a key event in the development of atherosclerosis. In order to study modulation of endothelial activation, quantification of the same in vitro is necessary. At functional level endothelial activation is quantified using monocyte adhesion assay. This involves addition of fluorescently labelled monocytes on top of cultured endothelial cells and quantifying the number of monocytes adhered. Currently this is done using microscopy. He we present a novel flow cytometry based monocyte adhesion assay with clear advantages over previously described methods.

GUIDELINES

1. When doing fluorescent labeling of monocytes, any cell impermeable dye can be used. Make sure to use a dye with high stain index so that there is good separation of negative and positive cells.

MATERIALS

NAME	CATALOG #	VENDOR
TrypLE™ Express Enzyme (1X), phenol red	12605010	Thermo Fisher
DiI Stain (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate ('DiI'; DiIC18(3)))	D282	Thermo Fisher
Human Umbilical Vein Endothelial Cells (HUVECs)		
THP1 monocytes		
Endothelial Cell Growth Media	CCM027	R&D Systems
RPMI 1640	AS162A	Himedia
0.2 % gelatin in PBS		
Cell culture plates (6 well)		
Phosphate buffered saline (PBS) pH 7.4		

SAFETY WARNINGS

Validate that the cell lines are not contaminated with infectious agents, especially if primary human umbilical vein endothelial cells (HUVECs) are isolated in the lab from donors.

BEFORE STARTING

Bring all the reagents to 37^{O}C before starting the assay.

Preparation of human umbilical vein endothelial cell (HUVEC) monolayer

1 Add 1 ml of 0.2% gelatin in PBS to each well of a 6 well plate and keep inside a CO_2 incubator at 37^{O}C for 1 hour. 1h

2 After 1 hour, discard the gelatin and wash with 3 ml PBS. Discard PBS 5m

3 Seed 10^4 HUVECs/cm^2 in each well including a well for negative gating during flow cytometry, in ECGM supplemented with growth factors, 10% fetal bovine serum (FBS) and antibiotics. Keep inside CO_2 incubator at 37^oC and 5% CO_2 till confluent. HUVECs grow as adherent monolayer. 5m

Overnight

Fluorescent labeling of THP1 monocytes

4 THP1 cells are cultured in RPMI 1640 medium with 10% FBS and antibiotics. Count THP1 cells using a hemocytometer. 5x10^5 THP1 cells per well are need for the assay. Count and aliqote the total number of cell needed for the assay into a centrifuge tube including 10^5 cells need for positive gating control during flow cytometry. 15m

5 Stock solution of DIL is made in DMSO at 1 mg/ml. This is added to the THP1 cells in complete media (RPMI with 10% FBS) at 1:1000 dilution with the final concentration in the media of 1 ug/ml. After adding DIL keep the THP1 cells inside CO_2 incubator at 37^oC and 5% CO_2 for 30 minutes. 30m

6 After 30 minutes, centrifuge at 300 g for 5 minutes at room temperature (RT). Discard the supernatant without disturbing the pellet and resuspend in complete media. Repeat this step once more to remain any unbound DIL. 15m

Monocyte adhesion under static conditions

7 Add 5x10^5 THP1 cells per well on top of the HUVEC monolayer and keep inside CO_2 incubator at 37^oC and 5% CO_2 for 30 minutes. Keep 10^5 THP1 cells apart as positive gating control for flow cytometry analysis later. 30m

8 After 30 minutes remove unbound monocytes by washing with PBS thrice. 15m

Quantification of adhesion using flow cytometry

9 Dislodge HUVECs and bound THP1 cells using TrypLE Express dissociation reagent. Add 500 ul of TrypLE to each well and incubate at 37^oC till cells dislodge. Once the cells have dislodged, add 500 ul of complete ECGM to inactivate TrypLE. 5m

10 Transfer to microcentrifuge tubes and centrifuge at 300 g for 5 minutes at room temperature (RT). Resuspend the pellet in PBS and repeat the PBS wash once. Resuspend in 500 ul of PBS. 15m

11 Run pure populations of HUVECs and THP1 cells for setting the vertical gate for analysis. Set a common gate for both HUVECs and THP1 should be used in FSC vs SSC graph. acquire 10^4 events in all tubes. Calculate the percentage of cells on either side of the vertical gate to calculate the number of DIL negative and DIL positive cells. DIL negative cells represent HUVECs and DIL positive cells represent THP1 cells. 15m

12 Calculate the average number of HUVECs adhered to single HUVEC by dividing the number of DIL positive cells out of the 10^4 cells with DIL negative cells. 5m