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Static Adhesion Assay for Human Peripheral Blood Mononuclear Cells

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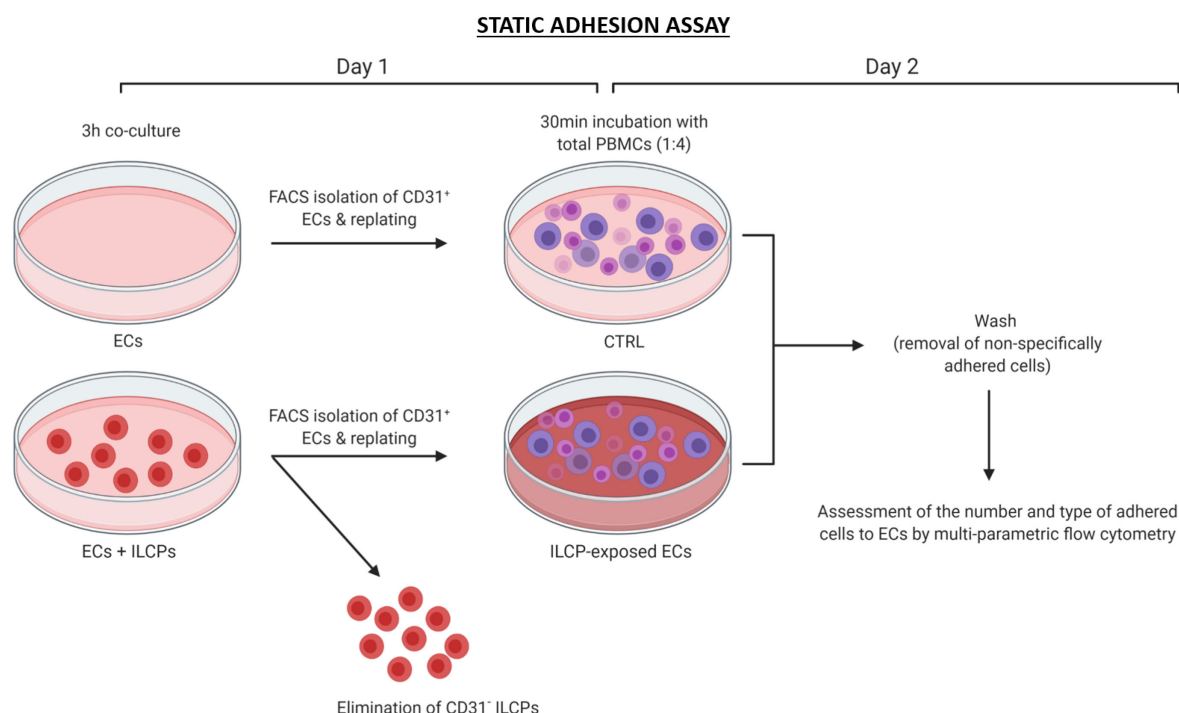
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[Abstract] Blood endothelial cells (ECs) constitute the primary physical barrier to be crossed by circulating leukocytes, once attracted to a site of ongoing inflammation/infection. Upon a pro-inflammatory stimulus, such as tumor necrosis factor (TNF), ECs upregulate adhesion molecule expression to favor the adhesion and, subsequently, the transendothelial migration of the attracted lymphocytes. To address the ability of a cell to transmigrate through a monolayer of ECs, the classical transmigration assay is usually performed (Muller and Luscinskas, 2008). In the present protocol, adapted from Safuan *et al.* (2012), we describe an *in vitro* assay for assessing the functionality of the second step of the transendothelial migration, *i.e.*, the firm adhesion of peripheral blood mononuclear cells (PBMCs) to ECs, under static conditions. By pre-incubating primary human umbilical cord ECs (HUVECs) with either innate lymphoid cell progenitors (ILCPs) or TNF, we were able to upregulate adhesion molecules on the EC surface. Then, by adding total PBMCs, we were able to both quantitatively and qualitatively analyze the cellular subtype and number of PBMCs that adhered to the pre-treated ECs. The important advantage of this technique is the possibility to perform functional studies on ECs biology since, differently from transwell-based strategies, it allows a good recovery of ECs at the end of the assay. Overall, this assay enables to interrogate how/if different stimulations/cell types can influence EC ability to retain PBMCs *in vitro*, under static conditions.

Graphic abstract:



The workflow of the Static Adhesion Assay.

Keywords: Static adhesion, Endothelial cells, Adhesion molecules, Transendothelial migration, Peripheral blood immune cells, Fluorescence-activated cell sorting

[Background] The leukocyte transendothelial migration (TEM) is the outward passage of leukocytes through intact vessel walls into a tissue, and it generally occurs at the endothelial cell-cell junction level (Schimmel *et al.*, 2017). To address the ability of a cell to transmigrate through a monolayer of ECs *in vitro*, the transwell-based transmigration assays are the most commonly performed (Muller and Luscinskas, 2008). Assays using transwell inserts are very popular due to their simplicity. ECs are grown on top of a polycarbonate filter that separates two fluid compartments. Leukocytes are added to the upper chamber and migrate across the ECs, through the filter, to the lower compartment, where a chemoattractant is generally added. Besides the advantage of allowing to address the ability of a cell to functionally transmigrate through a monolayer of ECs, a few limitations are present: (i) transwell-based assays are extremely expensive; (ii) EC monolayers are difficult to visualize at the microscope, and therefore it is complicated to decide whether to continue or not with the assay. (iii) Transwell-based assays do not allow to investigate cell-cell interactions and mediators of the firm adhesion/arrest step of the TEM process, and so, regulators of the transmigration itself. (iv) Finally, it is really complicated to recover ECs grown on the upper surface of a polycarbonate filter. Conversely, the static adhesion assay is an easy, low cost, and versatile tool, not only to investigate regulators of the transmigration, but also to gain insights into the ability of different cell types to adhere or not to a monolayer of ECs *in vitro*,

under static conditions. Moreover, ECs can be collected at the end of the assay and employed in further downstream experiments.

Materials and Reagents

1. 24-well flat bottom polystyrene plate (Corning, Costar, catalog number: CLS3524)
2. 48-well flat bottom polystyrene plate (Corning, Costar, catalog number: CLS3548)
3. 1.5 mL Eppendorf tubes
4. 50 mL Falcon tube
5. 0.22 μ m filter
6. 75 cm² Tissue Culture Flasks (TPP, catalog number: TPP 90075)
7. 5 mL round bottom tubes with snap cap (Corning, Costar, catalog number: 352058)
8. Human Umbilical Vein Endothelial Cells (HUVEC) (Lonza, catalog number: LZ-CC-2517). Store cryopreserved cells at -80°C
9. EGM Endothelial Growth Medium Bullet Kit (Lonza, catalog number: LZ-CC-3124). Once prepared, use the supplemented EGM within 6 weeks. Store at 4°C and, each time before use, warm up at 37°C only the amount of medium needed.
10. Accutase (Gibco, catalog number: A110501). Store at 4°C and warm up at 37°C before use.
11. 9 mL of blood in Li Heparin tubes from human healthy donors. Process fresh and store the isolated PMBCs at 4°C until usage.
12. Lymphoprep (STEMCELL, catalog number: 07851). Store at 4°C, and let equilibrate at RT in the dark before use.
13. rhTNF (Peprotech, catalog number: 300-01A). Store at -20°C, and avoid multiple thawing/freezing cycles.
14. Distilled water*
**Note: This item can be ordered from any qualified company.*
15. Potassium chloride (KCl) (Merck, catalog number: 104936)
16. Potassium dihydrogen phosphate (KH₂PO₄) (Acros Organics, catalog number: ACRO271080025)
17. Sodium dihydrogen phosphate (NaH₂PO₄) (VWR Chemicals, catalog number: 28013.264)
18. Sodium chloride (NaCl) (Merck, catalog number: S9625-500G)
19. Sodium hydroxide (NaOH) (Merck, catalog number: 43617-1L)
20. RBC lysis buffer (Invitrogen, catalog number: 00-4333-57). Store at RT.
21. BSA – for FACS buffer (Sigma-Aldrich, catalog number: A7906). Store lyophilized stock at 4°C.
22. BSA – for Sorting Buffer (Sigma-Aldrich, catalog number: 126609). Store lyophilized stock at 4°C.
23. Sodium azide pure (NaN₃) (AppliChem, catalog number: A1430). Store lyophilized stock at 4°C.
24. UltraPure 0.5M EDTA, pH 8.0 (Invitrogen, catalog number: 15575-020). Store at 4°C.
25. Flow cytometry antibodies (store all antibodies at 4°C in the dark):

- a. FITC anti-human CD31 (Biolegend, catalog number: 303104 – 1:100)
 - b. PE anti-human CD56 (Biolegend, catalog number: 362508 – 1:100)
 - c. PE-Cy5 anti-human CD106 (Biolegend, catalog number: 305808 – 1:100)
 - d. Pacific Blue anti-human CD54 (Biolegend, catalog number: 353110 – 1:100)
 - e. APC anti-human CD3 (BC, catalog number: IM2467– 1:100)
 - f. PE-Cy7 anti-human CD4 (BC, catalog number: 737660 – 1:400)
 - g. PE-CF594 anti-human CD14 (BD, catalog number: 562335 – 1:400)
 - h. Alexa Fluor 700 anti-human CD8 (Biolegend, catalog number: 344724 – 1:100)
 - i. APC-H7 anti-human CD19 (BD, catalog number: 641395 – 1:100)
26. CountBright Absolute Counting Beads (Thermo Fisher, catalog number: C36950). Store at 4°C in the dark
27. 1× Phosphate Buffer Solution (PBS) (in-house, see Recipes). Store at RT
28. FACS Buffer (see Recipes). Store at 4°C
29. Sorting buffer (see Recipes). Store at 4°C

Equipment

1. Water bath*
2. Centrifuge*
3. BD FACSAria II (BD Biosciences) – or any other fluorescence-activated cell sorter equipped with a blue laser
4. BD LSRFortessa (BD Biosciences) – or any other flow cytometer equipped with the necessary lasers
5. Cell incubator* (5% CO₂, 37°C)
6. Autoclave*

**Note: These items can be ordered from any qualified company.*

Software

1. BD FACSDiva™ (BD Biosciences, v8.0.2, <https://www.bdbiosciences.com>)
2. FlowJo (Treestar, v10.8.0, <https://www.flowjo.com/>)
3. Prism (GraphPad v9.2.0, <https://www.graphpad.com/>)

Procedure

A. EC culture

1. Thaw cryopreserved ECs at 37°C in a water bath, and transfer the cells to a Falcon tube containing 10 mL of complete EGM. Then, directly transfer the cell suspension to a 75 cm² flask, and place it in the incubator at 37°C.

2. Once the cells have adhered to the flask (~4 h-6 h later), discard the medium to remove traces of DMSO, and add 10 mL of fresh medium.
3. Change the medium every 2-3 days and, once confluent, detach ECs with 1 mL of Accutase at 37°C for 5 min.
4. Add 10 mL of complete EGM, collect the cell suspension, and transfer it to a Falcon tube. Centrifuge at $200 \times g$ for 5 min at RT.
5. Discard the supernatant, resuspend the cell pellet in 1-2 mL of complete EGM. Count and replate ECs.
Note: For "faster" passaging, seed 8,000-10,000 cells/cm².
6. Use Ecs for functional assays between passages 4 and 6 only.
7. Plate Ecs at 80% confluency in a 24-well plate (~100,000 cells/well) in 500 μ L of complete EGM.

B. EC pre-treatment(s)

1. Once the cells are adherent, remove the medium, and wash ECs with 1 mL of PBS 1 \times .
2. Incubate ECs with the following culture conditions:
 - a. Negative control: 500 μ L of complete EGM only.
 - b. Positive control: 500 μ L of complete EGM containing 20 ng/mL of TNF.
Note: TNF is very well known for being a cytokine that can strongly upregulate the expression of E-Selectin, ICAM-1, and VCAM-1 adhesion molecules on ECs in vitro, thus representing a robust positive control.
 - c. Experimental setting: 500 μ L of complete EGM containing ILCPs, at 1:1 ratio.
Note: This protocol can be adapted to any immune cell type of interest and ratios can be adjusted. Please refer to Vanoni et al. (2021) for more details regarding ILCP isolation and culture.
3. Incubate Ecs for 3h at 37°C.
4. After 3 h, discard the supernatant, and perform two washing steps with PBS 1 \times .
5. Detach ECs by adding 150 μ L of Accutase and incubate at 37°C during 5 min.
6. Add 1 mL of complete EGM. Transfer the cell suspension to a 5 mL round bottom tube, and wash with 3 mL of PBS 1 \times .
7. Centrifuge the cells at $200 \times g$ for 5 min at RT.

C. EC sorting

Note: This section is not necessary if you do not incubate ECs with other cell types before performing the static adhesion assay. From Step B7, you can directly go to Step D1.

1. Remove the supernatant, and stain ECs with FITC anti-human CD31 antibody in 50 μ L of Sorting Buffer. Incubate for 20 min at RT in the dark.
2. Wash the cells by adding 1 mL of PBS 1 \times , and centrifuge at $200 \times g$ during 5 min at RT.
3. Resuspend the cells in 500 μ L of sorting buffer and proceed with the sorting, according to the manufacturer's instructions and by following the gating strategy shown below (Figure 1). Collect

the cells in 1.5 mL Eppendorf tubes containing 500 μ L of complete EGM.

4. Centrifuge the cells at $200 \times g$ for 5 min, and replate them in a 48-well flat bottom plate in 250 μ L of complete EGM. Leave overnight at 37°C , or wait until the cells are adherent.

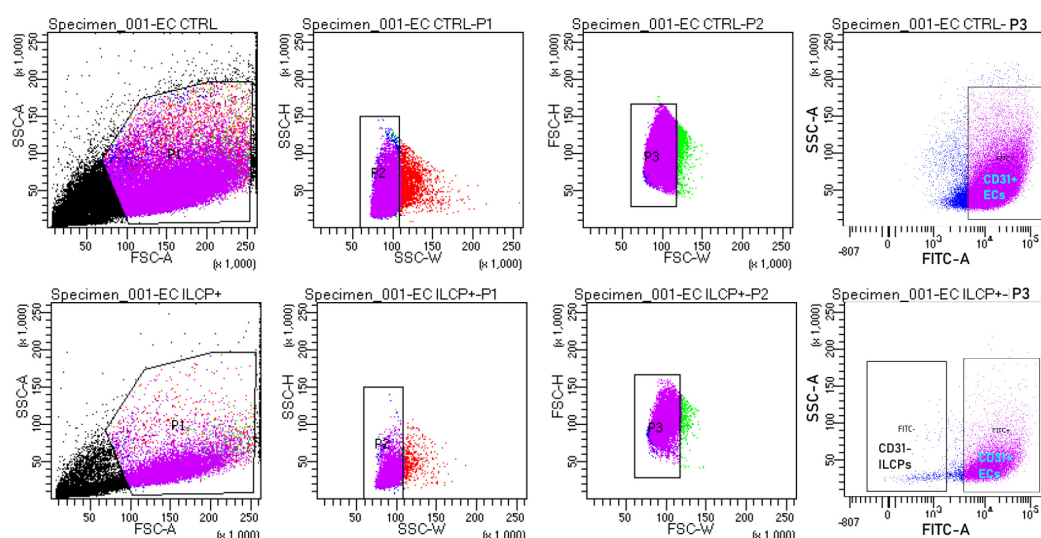


Figure 1. Gating strategy to enrich CD31⁺ ECs.

Following 3 h incubation with ILCPs, CD31⁺ ECs have to be sorted, in order to perform the static adhesion assay without any contaminating CD31⁻ ILCPs. As shown in the SSC-A vs. FITC-A dotplot in panel B, ILCPs themselves adhered to ECs during the *in vitro* co-culture. To make ECs go through the same steps, also untreated (CTRL) or TNF-treated ECs (not shown) have to be sorted (examples of unstimulated and TNF-stimulated ECs are shown in Figures 2A and 2B, respectively).

D. Isolation of PBMCs from fresh blood

1. In parallel, in a 50 mL Falcon tube, dispense 12 mL of 1.077 g/mL Ficoll-Hypaque.
2. In another 50 mL Falcon tube, transfer the 9 mL of blood, and add 9 mL of PBS 1 \times .
3. Gently transfer the 18 mL of PBS-diluted fresh blood over the Ficoll-Hypaque solution, being careful to not mix them.
4. Centrifuge at $650 \times g$ during 20 min at RT, without brake (acceleration/deceleration rate of 9/0).
5. Harvest the PBMC layer, and transfer it to a new 50 mL Falcon tube.
6. Wash the PBMCs with PBS 1 \times , by filling up to 50 mL.
7. Centrifuge at $450 \times g$ during 5 min at RT, with brake (9/9).
8. Remove the supernatant, and perform a second wash, by filling up to 40 mL.
9. Centrifuge at $200 \times g$ during 10 min at RT, without brake (9/0).

Note: This step will allow platelet removal.

10. Remove the supernatant and incubate the cell pellet with 500 μ L of RBC lysis buffer during 5 min at 37°C .
11. Wash the PBMCs with PBS 1 \times , by filling up to 50 mL.

12. Centrifuge at $450 \times g$ during 5 min at RT, with brake (9/9).
13. Resuspend the cells in culture medium and perform cell counting.

E. Static adhesion assay

Note: In the original protocol from Safuan et al. (2021), the static adhesion assay lasted 5 min. By performing a kinetic experiment, we observed that 30 min is an optimal incubation period, that allows specific adhesion events to take place.

1. The day after/once ECs have adhered, discard the supernatant and wash the cells with 500 μ L of PBS 1 \times .
2. Add freshly isolated PBMCs at 1:4 ratio (EC:PBMC) to each well, for 30 min at 37°C in a final volume of 200 μ L of complete EGM.
3. After 30 min, discard the supernatant from the wells.
4. Perform two washing steps with 500 μ L of PBS 1 \times , to ensure removal of non-adherent cells from the EC monolayer.
5. Detach ECs, together with adherent PBMCs, by incubating them with 200 μ L of Accutase at 37°C for 5 min.
6. Add 1 mL of complete EGM. Transfer the cell suspension to a 5 mL round bottom tube and wash with 3 mL of PBS 1 \times .
7. Centrifuge the cells at $200 \times g$ during 5 min at RT.
8. Stain the cells using 50 μ L of FACS Buffer, for 20 min at RT in the dark, with the antibodies diluted as listed in the *Materials and Reagents – Flow Cytometry Antibodies* section.
9. In the meantime, let the CountBright Absolute Counting Beads equilibrate at RT.
10. Wash the cells with 2 mL of PBS 1 \times and centrifuge at $450 \times g$ during 5 min at RT, with brake (9/9).
11. Resuspend the cell pellet in 200 μ L of FACS buffer.
12. After having set up the flow cytometer, add 20 μ L of well vortexed (30") CountBright Absolute Counting Beads to the samples, just before acquiring them.
13. Set stopping gate 'beads' at 2000 recorded events.

Data analysis

1. Once all the samples have been acquired, open your ".fcs" files with FlowJo.
2. After having checked the compensation matrix, draw a gate around the ECs and PBMCs, and visualize the different PBMC subsets according to the gating strategy shown in Figure 2.
3. Once the analysis is done, export the results (in particular, the column displaying the absolute number of cells contained in each gated population) to an excel file, and copy-paste the number of adhered CD4⁺ and CD8⁺ T cells, CD14⁺ monocytes, CD19⁺ B cells, and CD56^{dim/bright} pan NK cells to a Prism table, for data visualization.
4. Repeat the experiments at least two times (2 \times positive and negative control – technical

replicates, and with two different PB healthy donors – biological replicates).

5. Run a paired *t*-test for statistical significance (Prism).

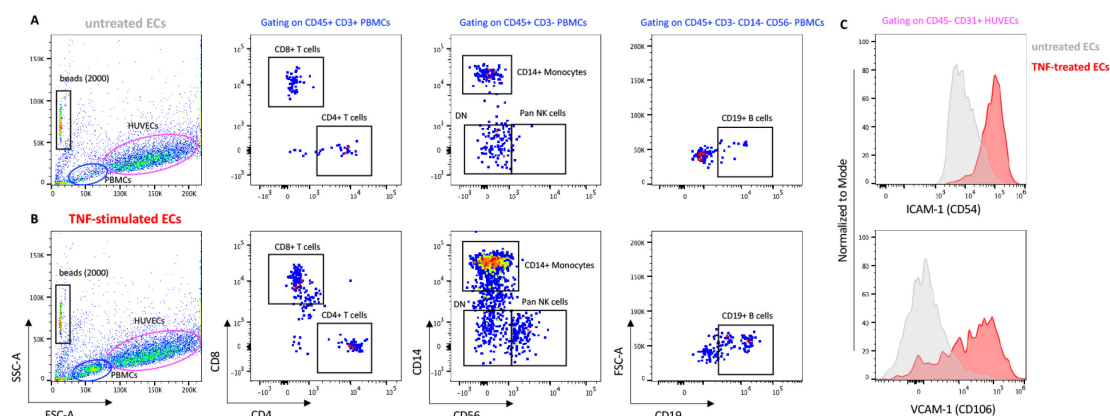


Figure 2. Analysis of the results of the static adhesion assay.

With the use of FlowJo, and according to the used antibody, it is possible to visualize and extract the absolute number of T cells, B cells, Monocytes and NK cells that adhered to ECs *in vitro* under static conditions. As expected, untreated ECs (A) led to the adhesion of much less PBMCs, if compared to TNF-treated ECs (B), thus defining good positive and negative controls, respectively. Since the assay allows the recovery of ECs, it is possible to investigate, for example, adhesion molecule expression upon different stimulations (C).

Notes

This protocol can be adapted to other cell types rather than ILCPs, in order to evaluate the ability of different immune cell populations/soluble molecules/drugs to influence adhesive properties of ECs *in vitro*.

Recipes

- 1× PBS (to be filtered over a 0.22 µm filter)
 - Distilled water
 - NaCl
 - KCl
 - Na₂HPO₄
 - KH₂PO₄
 - NaOH
 Dissolve the following quantities of salts in 800 mL of distilled water:
 - 8 g of NaCl
 - 0.2 g of KCl
 - 1.44 g of Na₂HPO₄

0.24 g of KH_2PO_4

Adjust pH to 7.4 with NaOH, filter and autoclave at 121°C for 20 min.

Adjust volume to 1 L with additional distilled water.

2. FACS Buffer – to stain only

PBS 1×

EDTA 5mM

BSA 0.2%

Sodium azide 0.2%

To prepare the buffer for flow cytometry acquisitions, add to 1 L of PBS 1×:

10 mL of EDTA 0.5 M

2 g of BSA

2 g of sodium azide

3. Sorting Buffer (to be filtered over a 0.22 μm filter)

PBS 1×

EDTA 5 mM

BSA 0.2%

To prepare the buffer for sorting, add to 1 L of PBS 1×:

10 mL of EDTA 0.5 M

2 g of BSA

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Competing interests

On behalf of all the authors of the original work, the authors declare no competing financial or non-financial interests.

Ethics

Venous blood was drawn from healthy donors at the local Blood Transfusion Center, Lausanne, Switzerland, under the approval of the Lausanne University Hospital's Institute Review Board. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

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