

Video Article

Simultaneous Study of the Recruitment of Monocyte Subpopulations Under Flow In Vitro

Patricia Ropraz¹, Beat A. Imhof², Thomas Matthes¹, Bernhard Wehrle-Haller³, Adama Sidibé³

¹Hematology service, Centre Médical Universitaire (CMU), Medical faculty, University of Geneva

²Department of Pathology and Immunology, Centre Médical Universitaire (CMU), Medical faculty, University of Geneva

³Department of Cell Physiology and Metabolism, Centre Médical Universitaire (CMU), Medical faculty, University of Geneva

Correspondence to: Adama Sidibé at Adama.Sidibe@unige.ch

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Abstract

The recruitment of monocytes from the blood to targeted peripheral tissues is critical to the inflammatory process during tissue injury, tumor development and autoimmune diseases. This is facilitated through a process of capture from free flow onto the luminal surface of activated endothelial cells, followed by their adhesion and transendothelial migration (transmigration) into the underlying affected tissue. However, the mechanisms that support the preferential and context-dependent recruitment of monocyte subpopulations are still not fully understood. Therefore, we have developed a method that allows the recruitment of different monocyte subpopulations to be simultaneously visualized and measured under flow. This method, based on time-lapse confocal imaging, allows for the unambiguous distinction between adherent and transmigrated monocytes. Here, we describe how this method can be used to simultaneously study the recruitment cascade of pro-angiogenic and non-angiogenic monocytes in vitro. Furthermore, this method can be extended to study the different steps of recruitment of up to three monocyte populations.

Video Link

The video component of this article can be found at <https://www.jove.com/video/58509/>

Introduction

Monocytes constitute a phagocytic component of innate immunity that is essential for fighting pathogens, cleaning up damaged tissues, angiogenesis, and the pathophysiology of many diseases including cancers^{1,2,3}. Monocytes are bone marrow-derived cells composed of heterogeneous subpopulations that circulate in the blood but can be recruited to the site of inflammation in peripheral tissue through specific molecular mechanisms. The recruitment cascades of monocytes, as for leukocytes in general, implicates different steps including capture, rolling, crawling, arrest, transendothelial migration (transmigration) and migration through the vessel wall (basement membrane and mural cells)⁴. These steps mainly involve inflammation-induced molecules on the endothelial luminal surface such as selectins, glycoprotein ligands, chemokines, intercellular and junctional adhesion molecules, and their receptors on leukocytes such as selectin ligands and integrins. Trafficking pathways through either the endothelial cell junctions (paracellular) or through the endothelial cell body (transcellular) can be used by leukocytes to cross the endothelial barrier⁵. Whilst monocytes have historically been documented to transmigrate through the transcellular route, potential divergences in their migratory pathway have been proposed as monocytes are no longer considered a homogeneous cell population. It is now becoming clear that monocyte diversity can be defined by each of their differences and commonalities, with respect to their distinctive extravasation cascades^{3,6}. Therefore, in order to unambiguously discriminate between monocyte subpopulations, it is crucial to visualize and phenotype the behavior of each of these different subpopulations during the recruitment process.

Monocytes from human, pig, rat and mouse were subdivided into phenotypic subpopulations with certain ascribed functions and specific migratory behaviors^{7,8,9}. For example, in humans, monocytes can be divided into three subsets based on their surface expression of CD14, a coreceptor for bacterial lipopolysaccharide, and CD16, the Fc-gamma receptor III. Human monocyte subpopulations include classical CD14⁺CD16⁻, intermediate CD14⁺CD16⁺ and non-classical CD14^{dim}CD16⁺ cells^{6,9}. The classical CD14⁺CD16⁻ monocytes were shown to be mainly inflammatory whereas the pool of CD16⁺ monocytes were collectively found to present TIE2 expression and proangiogenic function¹⁰. Consistently, endothelial cell stimulation with inflammatory cytokines such as human tumor necrosis factor (TNF) α or interleukin (IL-1) β (conventional inflammation) is sufficient to trigger the complete recruitment of classical CD14⁺CD16⁻ monocytes. However, simultaneous actions of vascular endothelial growth factor (VEGF) α and TNF α (angiogenic factors-driven inflammation) are required to provoke the transmigration of the CD16⁺ proangiogenic pool of monocytes³. Historically, the traditional Transwell system under static conditions, the parallel plate flow chamber, and the μ -slide flow chambers have been used to quantitatively analyze the recruitment of one leukocyte population at a time *in vitro*^{11,12,13}. Whilst these protocols have been validated, a more robust method that allowed the simultaneous analysis of multiple monocyte subpopulations would be considered more insightful. Such methodologies must account for multiple interactions and the differing frequencies of

each respective population and also provide a mechanistic understanding of the similarities and specificities for the recruitment cascades that define each monocyte subset.

Here, we present a method based on the time-lapse imaging of monocyte recruitment under flow which allows the migratory cascades of different monocyte subpopulations to be studied simultaneously by using confocal microscopy. This method integrates certain critical features that mimic endothelial cell inflammation, as well as the hemodynamics of circulating monocytes in post-capillary venules, the main location of leukocyte recruitment *in vivo*. The proposed method uses human umbilical vein endothelial cells (HUVEC), which are generated through a well-established protocol of isolation from human umbilical cords. This clinical resource has the advantage of being easily available as a biological by-product, whilst also providing a reasonable yield of endothelial cells that can be isolated from the umbilical vein. We also used fluorescent dyes and immunofluorescence to distinguish between the different cellular components, and confocal microscopy to unambiguously define monocyte positioning (luminal vs. abluminal) over time. The protocol presented here has been developed to simultaneously measure the transmigration levels of monocyte subpopulations. Moreover, it should be noted that this methodology can be extended to study other leukocytes subpopulations and recruitment processes by use of different biomarkers and labelling.

Protocol

Human materials were used with the informed consent of volunteer donors and in accordance with the Swiss Ethics Committees on clinical research.

1. Isolation and Freezing of Human Umbilical Vein Endothelial Cells (HUVEC)

1. Add 5 mL of coating solution to a T75 flask (0.1 mg/mL collagen G and 0.2% gelatin in phosphate buffered saline PBS at pH 7.4) for 30 min at 37 °C before initiating HUVEC isolation.
2. Clean the cord with PBS, wipe it with sterile compresses, and place it in a sterile 20 cm Petri dish. Cut the ends of the cord with sterile scissors.
3. Identify the single large vein and the two small arteries. Gently insert a cannula with a three-way stopcock attached to it into the vein extremities at the cord ends.
4. Tighten the cord and the cannula connection firmly with a length of wire.
5. Perfuse the cord twice with 20 mL of RPMI medium containing 100 U/mL penicillin, 100 U/mL streptomycin and 250 ng/mL amphotericin B to wash the cord's veins. This process makes the appearance of the cord whiter and clearer. Empty the vein before collagenase addition by collecting the RPMI with a syringe at one end.
6. Perfuse the vein with 12 mL of 1 mg/mL collagenase type I (0.22 µm-filtered).
7. Close the stopcock at the cord ends and incubate the cord at 37 °C for 12 min.
8. Gently massage the cord to detach endothelial cells from the vein lumen.
9. Take 30 mL of RPMI containing 10% fetal calf serum with a 50 mL syringe and connect it to one end of the umbilical cord.
10. Connect an empty 50 mL syringe to the other end of the umbilical cord
11. Open the stopcock and perfuse the vein from one end whilst reciprocally collecting from the other end.
NOTE: The collected suspension contains endothelial cells.
12. Centrifuge this cell suspension at 200 x g for 5 min.
13. Discard the supernatant and resuspend the cell pellet with 10 mL of complete M199 medium (M199 containing 20% FCS, 15 µg/mL endothelial cell growth supplements, 100 µg/mL heparin sodium, 0.5 µM hydrocortisone, 10 µg/mL L-Ascorbic Acid, 100 U/mL penicillin, 100 U/mL streptomycin and 250 ng/mL amphotericin B).
14. Remove the coating solution from the T75 flask and rinse once with PBS.
15. Seed the cells collected from step 1.13 into the T75 flask and place it in the incubator at 37 °C with 5% CO₂.
16. The next day, rinse the flask 3 times with the complete M199 medium to remove residual red blood cells and then change the medium every 2 days until confluence.
17. At 80–90% confluence, rinse the HUVEC monolayer once with 5 mL of PBS and detach the cells with 5 mL of 0.05% trypsin in 1 mM EDTA at 37 °C for 5 min. Add 4 mL of M199 and 1 mL of FCS to stop the trypsin action. Flush the flask to detach all HUVEC.
18. Collect an aliquot of 50 µL to be used for staining of VE-cadherin, PECAM-1 and gp38, and analyze by flow cytometry to check HUVEC purity.
19. Collect the remainder of HUVEC from step 1.18 in a 15 mL tube and centrifuge at 200 x g for 5 min at room temperature.
20. Discard the supernatant from step 1.19, resuspend the cell pellet in freezing solution (FCS containing 10% DMSO) at a density of 5 x 10⁵ cells/mL in cryotubes, and freeze at -80 °C or in liquid nitrogen until use.
21. **To check HUVEC purity:**
 1. Add 1 µL of anti-human VE-cadherin-FITC antibody, 1 µL of anti-human PECAM1-PE antibody, and 1 µL of anti-human Podoplanin-APC antibody to the aliquot of 50 µL of HUVEC collected at step 1.18.
 2. Incubate at room temperature for 10 min.
 3. Add 100 µL of PBS and centrifuge at 400 x g for 30 s.
 4. Discard the supernatant and resuspend in 100 µL of PBS. Data can now be acquired by flow cytometry techniques.
NOTE: HUVEC are positive for VE-cadherin and PECAM-1, and negative for Podoplanin.

2. HUVEC Defrosting

NOTE: Use HUVEC at low passage for experiments (maximum 5 passages).

1. Coat a T75 flask with 1 mL of the coating solution at 37 °C for 30 min.
2. Rapidly defreeze HUVEC at 37 °C for 2 min and resuspend the cells in 10 mL of complete M199.

3. Centrifuge the cells at 200 x g at room temperature for 5 min and discard the supernatant.
4. Resuspend the cell pellet in 10 mL of complete M199.
5. Transfer the cell suspension in the pre-coated flask. Place the flask in the incubator at 37 °C with 5% CO₂. Change the cell culture medium every 2 days.

3. HUVEC Culture in 0.4 µ-Slide Chamber

1. Five days before starting the flow experiment, pre-coat the chambers of a 0.4 µ-slide with 30 µL of PBS containing 0.1 mg/mL collagen G, 0.2% gelatin at 37 °C for 30 min.
2. Wash the chambers with 100 µL of PBS.
3. Detach the cells from an 80–90% confluent HUVEC of a T75 flask.
4. Rinse HUVEC with 5 mL of PBS and detach them with 5 mL of 0.05% trypsin at 37 °C for 5 min.
5. Flush and collect the cell suspension in complete M199 and count the cells by the most convenient method. Centrifuge at 200 x g for 5 min at room temperature.
6. Resuspend the cell pellet at 10⁶ cells/mL and distribute 30 µL (30,000 cells) per chamber.
7. Incubate the cells in an incubator at 37 °C with 5% CO₂ for 1 h.
8. Add 150 µL of complete M199 to each chamber and culture the cells for 5 days in the incubator at 37 °C and 5% CO₂. Change the medium every 2 days.

4. HUVEC Staining for Monocyte Recruitment Assay Under Flow

1. Prepare the labeling medium made of M199 and 1 µM of CMFDA (5-chloromethylfluorescein diacetate) and warm it at 37 °C for 5 min before cell labeling.
2. Wash HUVEC twice with M199 medium warmed at 37 °C.
3. Replace the medium with 30 µL of warmed labeling medium containing 1 µM of CMFDA and place into the incubator at 37 °C and 5% CO₂ for 10 min.
4. Wash once with complete M199 and incubate the cells with complete M199 in the incubator at 37 °C and 5% CO₂ for 30 min.
NOTE: It is important to remove all traces of serum before addition of the labeling solution, otherwise it may alter HUVEC staining.
5. Replace the medium with complete M199 containing either human TNFα (500 U/mL) or a mix of human TNFα (500 U/mL) with human VEGFA (1 µg/mL) for 6 h in an incubator at 37 °C and 5% CO₂.

5. Isolation of Human Pan Monocytes and Staining of Subpopulations

1. Use either a buffy coat of concentrated human blood, or 20 mL of freshly isolated human blood, collected on the day of the experiment in EDTA vacutainer tubes.
2. Dilute the blood in PBS-1 mM EDTA (1:1) and pipette gently 20 mL of the diluted blood on top of the 20 mL of density gradient media. Centrifuge at 400 x g for 30 min at room temperature with slow acceleration and without brake.
3. Collect the peripheral blood mononuclear cell (PBMC)-platelet layer (between density gradient media and plasma layers) into a new 50 mL tube containing 40 mL of PBS- 1 mM EDTA. Top up to 50 mL with PBS- 1 mM EDTA.
4. Centrifuge at 200 x g at room temperature for 5 min. Discard the supernatant.
5. Resuspend the cell pellet with 10 mL of staining buffer (PBS- 1 mM EDTA containing 0.5% bovine serum albumin BSA).
6. Centrifuge at 200 x g at room temperature for 5 min. Discard the supernatant.
7. Repeat steps 5.5 and 5.6.
8. Resuspend the cell pellet with 10 mL of staining buffer. Take an aliquot of 10 µL for a cell count.
9. Check PBMC populations and count cells rapidly with a flow cytometer.
NOTE: The characteristic lymphocyte and monocyte populations can be observed (**Figure 1A**). From 50 mL of fresh human blood expect about 50-100 x 10⁶ PBMC.
10. **For the recruitment of CD14+ versus CD14- PBMC under flow:**
 1. Wash the pellet three times with flow buffer (M199 containing 0.5% BSA) and resuspend the mononuclear cells in flow buffer at 6 x 10⁶ cells per mL.
 2. Make aliquots of 200 µL for each assay. Incubate at 37 °C until 20 min before the assay.
 3. Add 5 µL of anti-CD14-PE and Hoechst 33342 at a final concentration of 2 µM to each aliquot. Mix and incubate at 37 °C for 10 min.
 4. Centrifuge the aliquot at 400 x g for 30 s.
 5. Discard the supernatant and resuspend the pellet with 200 µL of flow buffer.
11. **For the recruitment of monocyte subpopulations under flow:**
 1. Isolate monocytes with a pan monocyte isolation kit according to manufacturer instructions.
NOTE: The following isolation protocol is for 50 x 10⁶ cells. It can be scaled up or down as long as it is within the manufacturer's recommendations.
 2. Centrifuge the PBMC suspension at 200 x g at room temperature for 5 min.
 3. Discard the supernatant and resuspend the pellet with 400 µL of staining buffer.
 4. Add 50 µL of Fc-receptor blocking reagent and 50 µL of Pan Monocyte antibody cocktail.
 5. Incubate at room temperature for 10 min.
 6. Add 400 µL of staining buffer and 100 µL of magnetic beads conjugated anti-biotin antibody. Incubate at room temperature for 15 min.
 7. Add 2 mL of staining buffer and use a MACS LS column coupled with a magnet.
 8. Place the LS column on the magnet and add 1 mL of staining buffer. Discard the flow-through.
 9. Pass the PBMC suspension in the column and collect the clear flow though containing pan monocytes in a new 15 mL tube.
 10. Add the staining buffer to top up to 5 mL.

11. Take an aliquot and check the quality of the monocyte isolation with a flow cytometer.
12. Determine the pan monocyte count.

NOTE: Only monocyte population can be observed (**Figure 1B**).

13. Centrifuge the remainder of monocytes from step 5.11.11 at $200 \times g$ for 5 min.
14. Discard the supernatant.
15. Resuspend the cell pellet in 5 mL of flow buffer (M199 containing 0.5% BSA).
16. Repeat 5.11.13 to 5.11.14 twice to eliminate any trace of EDTA.

12. Make monocyte suspension in flow buffer (M199 with 0.5% BSA) at 6×10^6 cells/mL.
 13. Make aliquots of 200 μ L of monocytes for each recruitment assay.
 14. Keep the aliquot at 37 °C in the incubator until 20 min before injection.
 15. Add 5 μ L of anti-CD16-PE antibody and Hoechst 33342 (2 μ M final) to each aliquot.
 16. Mix and incubate at 37 °C for 10 min.
 17. Centrifuge the aliquot at $400 \times g$ for 30 s.
 18. Discard the supernatant and resuspend the pellet with 250 μ L of flow buffer.
 19. Add 30 μ L of the monocyte suspension in one chamber of the slide to serve for setting the acquisition parameters on the confocal microscope.
 20. Keep the aliquots of monocyte suspension from step 5.18 at 37 °C.
- NOTE:** This suspension is ready to be injected in the flow system.

6. Preparation of the Fluidic System

1. Ensure that the cell incubator for the imaging set at 37 °C.
- NOTE:** A diagram of the flow system is shown in **Figure 2**.
2. Assemble the tubing part I: Insert a Luer connector male to one end of a piece of silicone tubing (8 cm long and 3 mm thick) and connect the other end to an in-line Luer injection set. Connect the latter Luer connector to a piece of silicone tubing (40 cm and 3 mm thick) at one end.
- NOTE:** Optionally, a 3-way tap connected to a 5 mL syringe can be inserted between the in-line Luer injection set and the silicone tubing for eventual air bubble removal.
3. Assemble the tubing part II: Connect a 20 mL syringe to one end of a length of silicone tubing (1 m long and 3 mm thick). Insert a Luer connector male to the other end of the tubing.
 4. Connect part I and part II tubing by inserting the Luer connector males to a female Luer lock coupler (**Figure 2A**).
 5. Put the free end of the silicone tubing in the reservoir containing the flow buffer (M199 + 0.5% BSA) warmed at 37 °C.
 6. Pull on the plunger of the 20 mL syringe to fill the tubing with flow buffer.
 7. Place the syringe on the pump and secure it.
 8. Set the pump in withdraw mode (as opposed to infuse) and specify the flow rate.
 9. Determine the flow rate according to the IBIDI slide used by using the following formula:

$$\text{Flow rate} \left(\frac{\text{ml}}{\text{min}} \right) = \frac{\text{Shear stress} \left(\frac{\text{dyn}}{\text{cm}^2} \right)}{\text{Buffer viscosity} \left(\frac{\text{dyn.s}}{\text{cm}^2} \right) * \text{Slide factor}}$$

Note: The slide factor is dependent on the IBIDI slide used for the experiment. For the μ -slide I^{0.4} Luer lock used in this example, the slide factor is 131.6. For specific slide factors, see the company website¹⁴. The flow buffer viscosity is 0.0072 dyn.s/cm². Shear stress at the post-capillary venules is about 0.5 dyn/cm².

10. **Connect the slide (Figure 2B):**
 1. Clamp the silicone tubing around the female Luer Lock Coupler and disconnect the two Luer connector males from the coupler.
 2. Connect them to the reservoirs of the slide containing stimulated HUVEC and fill with medium. Avoid air bubbles during this step.
 3. Take off the clamps and ensure that the connection is not leaking.
11. Place the slide under the microscope for time-lapse imaging and start the pump.

7. Time-lapse Imaging of Monocyte Recruitment Under Flow by Confocal Microscopy

1. Use a 40x objective (see **Table of Materials**) for imaging.
 2. Activate the 405 nm (blue monocyte nuclei), 488 nm (green endothelial cells) and 561 nm (red CD16+ subset) lasers.
 3. Use the chamber that contains the monocytes to set the acquisition parameters.
- NOTE:** To detect both non-transmigrated and transmigrated monocytes, the pinhole and intensity of the laser 405 nm are set high. Thus, non-transmigrated monocytes are slightly visible in the basal plan. However only transmigrated monocytes present an unstained area around the nucleus corresponding to the new space occupied underneath endothelial cells.
4. Place the chamber to be acquired under the microscope.
 5. Choose 3 fields of views within 1 cm radius for multi-position confocal imaging.
 6. Define the basal and the apical sides of endothelial cells
 7. Set a z-stack to the 10–12 μ m range (0.5 μ m step). Run a time-lapse acquisition every 1 min.
 8. After 3 min of imaging, inject 200 μ L of monocyte suspension (6×10^5 cells/mL) through the in-line Luer injection port.
- NOTE:** Rapidly monocytes appear in the apical focal plane, adhere and start transmigration (transit from the apical to the basal plan).
9. Image for at least 30 min. Once finished, stop imaging and stop the flow. Clamp the tubing to disconnect them from the slide.
 10. Fix the slide with 4% paraformaldehyde at 4 °C for 10 min.

11. Wash the slide with PBS and store the slide at 4 °C for further analysis if needed.

8. Analyze the Data with ImageJ

1. Count the number of total adherent monocytes in each field. Determine the cell count per mm².
2. Count transmigrated monocytes that are present in the basal plan underneath endothelial cells and identified by the presence of a black hole (in the green channel) around the nucleus.
3. Divide the count of transmigrated leukocytes by the total number of adherent leukocytes. The transmigration rate is presented as a percentage of adherent monocytes.
4. For illustration, the apical and the basal sides can be shown simultaneously to illustrate the events occurring in each of these endothelial compartments.

Representative Results

Determining the state of HUVEC activation induced by TNF α

The bio-activity of the inflammatory cytokine TNF α can be vary according to the batch and the repletion of freezing-thawing cycle. It is important to check the activation status of HUVEC with TNF α treatment. This could be performed by staining in parallel some samples of confluent HUVEC for the inflammatory induction of selectins, ICAM-1 and VCAM-1^{15,16,17}. An easier and simpler way to check the activation status of HUVEC after TNF α treatment is the morphological change displayed by endothelial cells under inflammatory stress. As shown in **Figure 3**, HUVEC elongate after 6-h in the presence of TNF α in comparison to unstimulated cells. Similar elongation is observed when HUVECs are stimulated by a mix of TNF α and VEGFA. Recording the activation status of HUVEC is important as the final results of the transmigration of monocytes will depend on the quality of endothelial cell activation.

Monocyte transmigration makes a characteristic discontinuation in endothelial cells

To study monocyte transmigration under flow, we used confocal microscopy with endothelial cells stained in green with CMFDA and the nuclei of isolated monocytes stained in blue with the cell-permeable Hoechst 33342 dye (**Figure 4**). The time-lapse confocal imaging allowed the visualization of monocytes at the apical plane, where their phenotype could be assessed (**Figure 4A-C, Supplemental Movie 1**). Migrating cells undergoing transmigration moved to the intercellular space corresponding to cell-cell junctions before they disappeared from the apical plane and appeared in the basal plane. The transmigrated cells presented a black hole around the nucleus corresponding to the monocyte shapes. This shape constantly changed during monocyte migration underneath endothelial cells (**Figure 4A-C, Supplemental Movies 2-3**). This dynamic black hole made by the monocyte body underneath the endothelial cells, and the monocyte positioning, allowed for the unambiguous identification of transmigrated cells. Quantitation of monocyte recruitment over time showed monocyte adhesion followed by transmigration (**Figure 4D-E**). Although leukocytes can extravasate through both the transcellular and paracellular routes, we could only observe the paracellular transmigration under flow with this method. This is consistent with our previous observations^{3,11,18,19}.

Angiogenic factor driven inflammation promotes the transmigration of CD16⁺ monocytes

By using this method, we analyzed the transmigration of human proangiogenic *versus* non-angiogenic monocytes through an endothelial monolayer stimulated by the inflammatory cytokine TNF α alone or in combination with the angiogenic factor VEGFA. Human proangiogenic monocytes can be identified by the expression of CD16 or TIE2 on their surface. Here, anti-CD16-PE antibody was used to discriminate between pro- and non-angiogenic monocytes. As shown in **Figure 5A-B (Supplemental Movies 4-5)**, the transmigration rate of CD16⁺ monocytes was low when endothelial cells were stimulated with TNF α only. However, this rate increased when endothelial cells were stimulated simultaneously with TNF α and VEGFA (**Figure 5C-E, Supplemental Movies 6-7**). The transmigration rate of non-angiogenic monocytes was similarly high under both inflammatory conditions. For both cell subpopulations, the transmigration occurred exclusively through the paracellular route. This method therefore allows for the transmigration aptitudes of different monocytic populations to be investigated simultaneously.

The purity of monocytes affects the transmigration efficiency

The peripheral blood mononuclear cells are composed of T cells, B cells, NK cells and monocytes. The monocyte isolation method used here requires the depletion of the other leukocyte populations from PBMC. To understand how the lack of monocyte purity affects the results, we used PBMCs and stained for pan-monocytes with an anti-CD14-PE antibody before performing the recruitment assay under flow. As shown in **Figure 6**, HUVEC stimulation with TNF α or TNF α +VEGFA induced the transmigration of only the monocyte population. The other leukocytes composed of T cells, B cells and NK cells did not transmigrate under TNF α or TNF α +VEGFA. Indeed, it has been documented that these leukocytes need other signals for transmigration. Thus, an inefficient isolation of monocytes will lead to an underestimation of monocyte transmigration, as the other leukocytes would be counted as monocytes. This would lead to an erroneous result on monocyte transmigration, due to the contamination of monocyte population with other leukocytes.

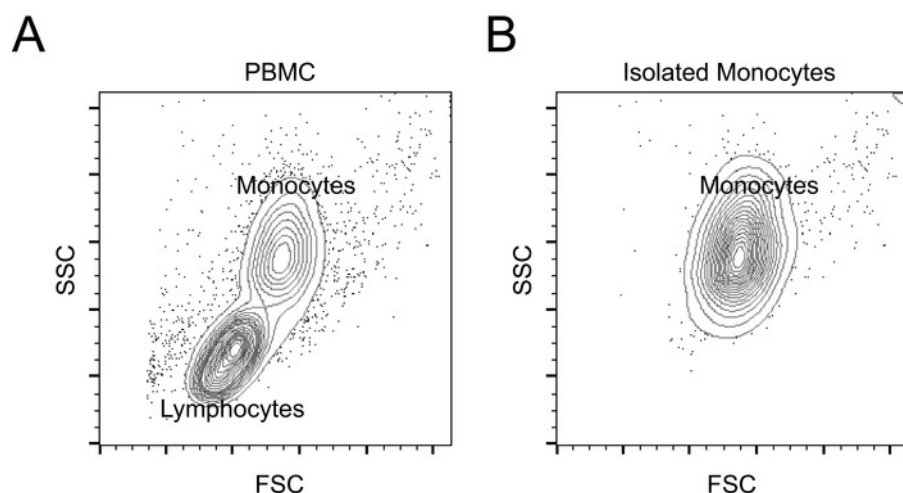


Figure 1: Profiling of isolated monocytes by flow cytometry. (A) Analysis of the morphology of PBMC before lymphocyte depletion. The size (forward scatter: FSC) and granularity (side scatter: SSC) of peripheral blood mononuclear cells were determined by flow cytometry. (B) The size and granularity of isolated monocytes were determined by flow cytometry after lymphocyte depletion. An efficient isolation of monocytes shows a complete depletion of the lymphocyte population. [Please click here to view a larger version of this figure.](#)

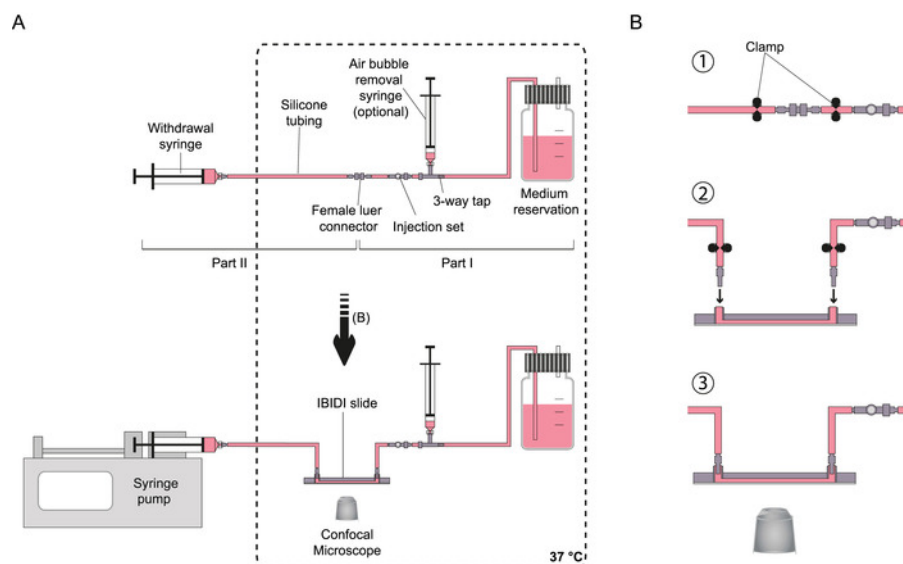


Figure 2: Diagram of the fluidic system. (A) Schematic overview of the perfusion system before and after connection of the slide and mounting on the syringe pump. (B) Diagram of the process of connecting the slide with the tubing using clamps. [Please click here to view a larger version of this figure.](#)

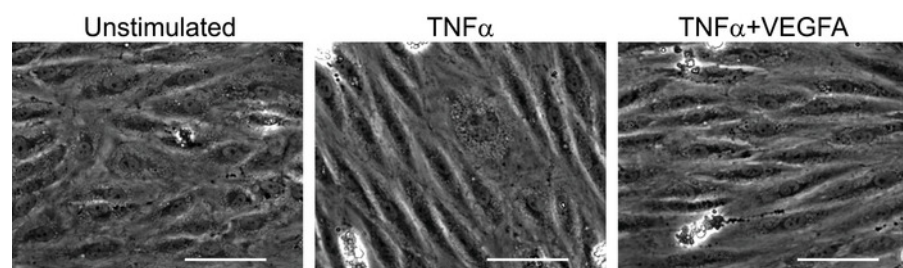


Figure 3: Checking the efficient activation of endothelial cells. The activation of HUVEC by inflammatory stimuli was checked by analyzing the cell shape using phase contrast microscopy. After 6 hours of treatment, HUVEC present an elongated morphology when stimulated with TNF α (500 U/mL) or a mix of TNF α (500 U/mL)+VEGFA (1 μ g/mL) compared to unstimulated cells. This morphological change of HUVEC following the inflammatory stimulation is an easy-to-detect indicator of the cell activation, which should be ensured for the flow assay. Scale bar = 120 μ m [Please click here to view a larger version of this figure.](#)

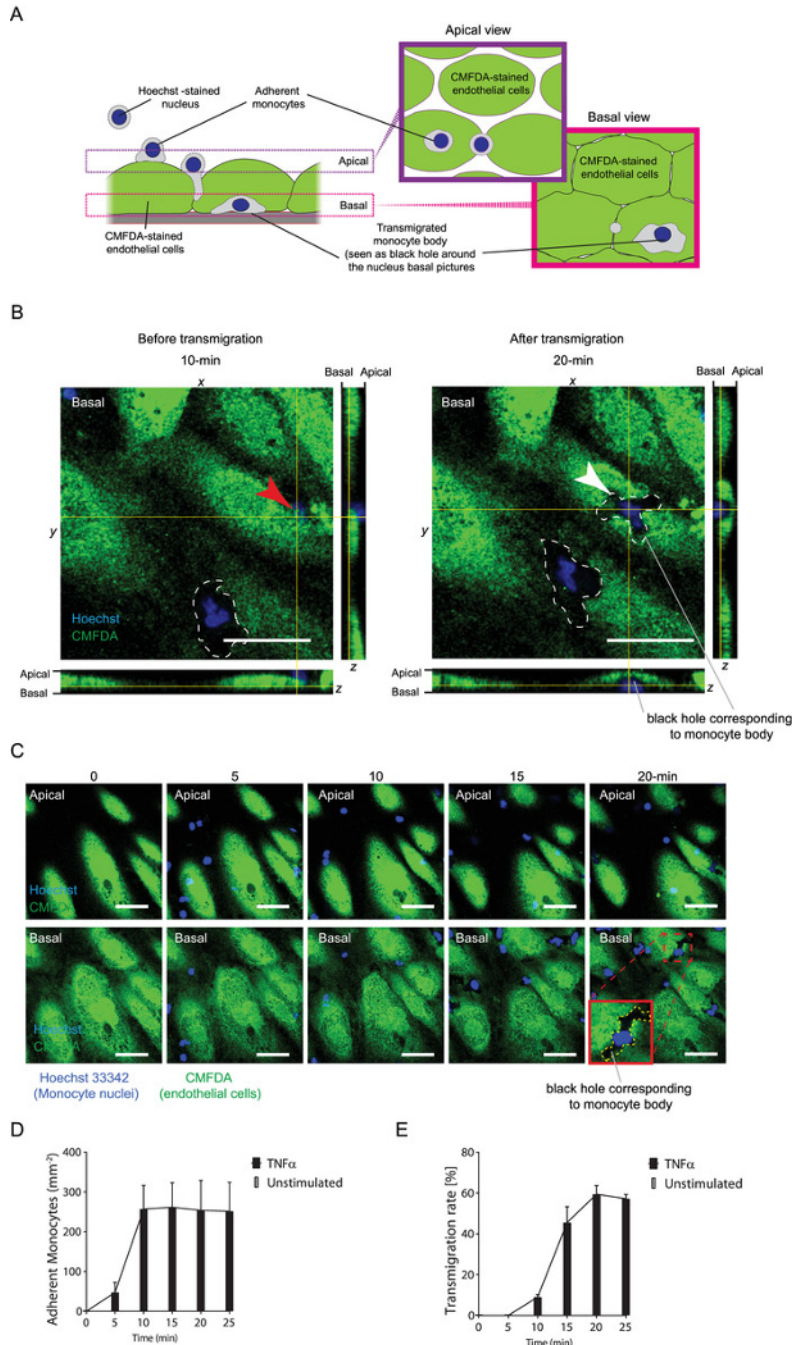


Figure 4: Identification of the transmigrated monocytes by confocal microscopy. (A) Diagram of monocyte transmigration with the expected views at apical and basal planes. The nuclei of monocytes stained with Hoechst 33342 are depicted in blue, and the theoretical shapes of monocytes are depicted with dashed lines around the nuclei. In the basal view, the transmigrated flat monocytes are shown to occupy a space underneath endothelial cell. This space appears as a black hole surrounding the monocyte nucleus on confocal images. (B) Localization of a monocyte before and after transmigration. The orthogonal views are shown, and the appearance of a black hole (delineated with the white dashed line) can be observed after monocyte migration to the endothelial abluminal compartment. A red arrowhead indicates the position of a monocyte before transmigration and the white arrowhead indicates the same cell after transmigration. The orthogonal views show that the transmigrated monocyte is underneath the endothelial cell. Scale bar = 40 μ m. (C) Time-lapse image sequences (from 0 to 20 min) of monocyte recruitment overtime. The apical and basal views are shown. The full sequences can be seen in **Supplemental Movies 1, 2 and 3**. Red squares highlight a transigrated monocyte with a blue nucleus. The black hole corresponding to the flat body of the monocyte underneath the endothelial cell is delineated by a dashed yellow line. Scale bar = 40 μ m. (D) Quantification of monocyte adhesion to TNF α -stimulated *versus* unstimulated HUVEC over time. (E) Quantification of monocyte transmigration rate over time. N = 3 biological replicates. Data are presented as mean \pm S.D. [Please click here to view a larger version of this figure.](#)

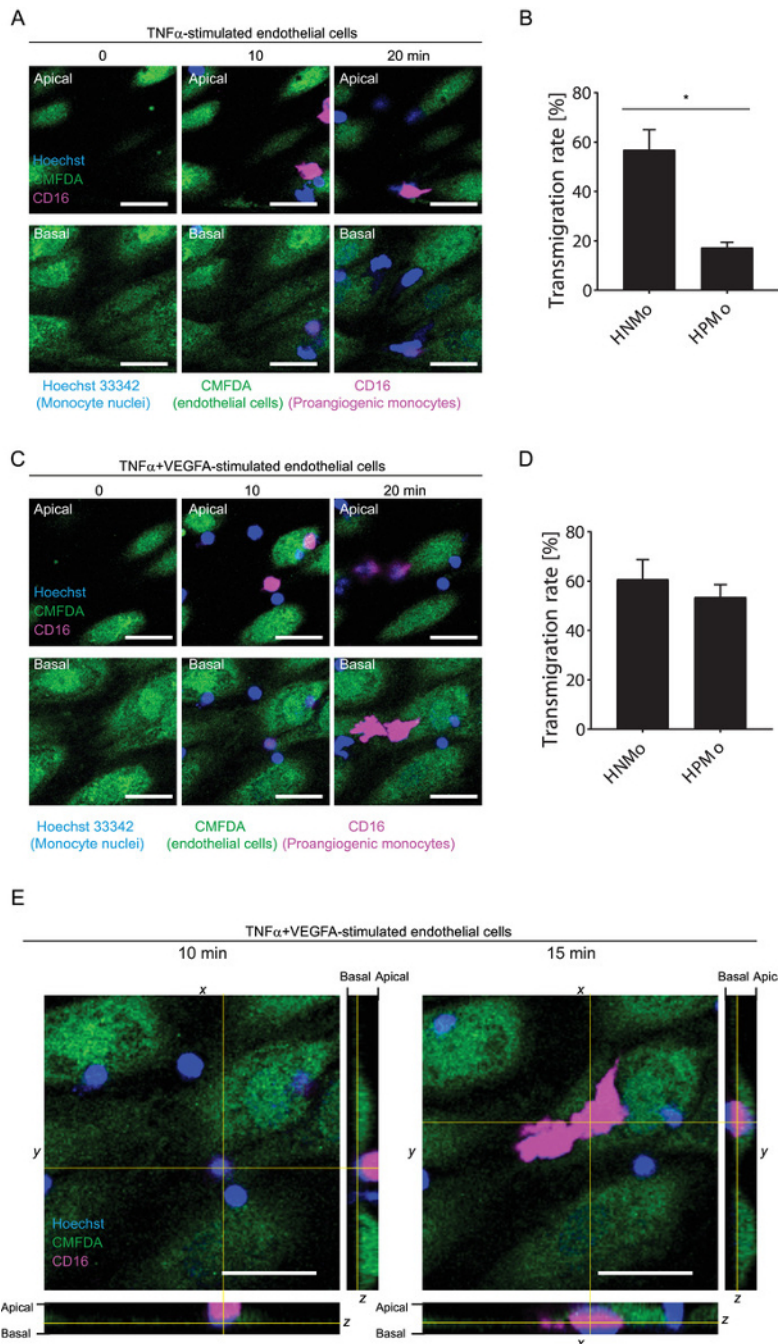


Figure 5: Simultaneous investigation of the transmigration of monocyte subpopulations under flow. (A) Time-lapse image sequences (from 0 to 20 min) of the recruitment of proangiogenic monocytes (CD16⁺) and non-angiogenic monocytes over time through TNF α -activated HUVEC. Scale bar = 40 μ m; the apical and basal views are shown. The full sequences can be seen in **Supplemental Movie 4** for apical and **Supplemental Movie 5** for basal views. (B) Quantitation of the transmigration of human proangiogenic monocytes (HPMo: CD16⁺) and human non-angiogenic monocytes (HNMo) through a TNF α -activated HUVEC monolayer. N = 4 biological replicates, data are presented as mean \pm S.D. *p < 0.05; Mann-Whitney test. (C) Time-lapse image sequences (from 0 to 20 min) of the recruitment of proangiogenic and non-angiogenic monocytes overtime through TNF α +VEGFA-activated HUVEC. Scale bar = 40 μ m; the apical and basal views are shown. The full sequences can be seen in **Supplemental Movie 6** for apical and **Supplemental Movie 7** for basal views. (D) Quantitation of the transmigration of human proangiogenic monocytes (HPMo: CD16⁺) and human non-angiogenic monocytes (HNMo: CD16⁻) through TNF α +VEGFA-activated HUVEC monolayer. N = 4 biological replicates, data are presented as mean \pm S.D. *p < 0.05; Mann-Whitney test. (E) Localization of CD16⁺ monocytes before (10 min) and after (15 min) transmigration through TNF α +VEGFA-stimulated HUVEC. The orthogonal views are shown. Scale bar = 40 μ m [Please click here to view a larger version of this figure.](#)

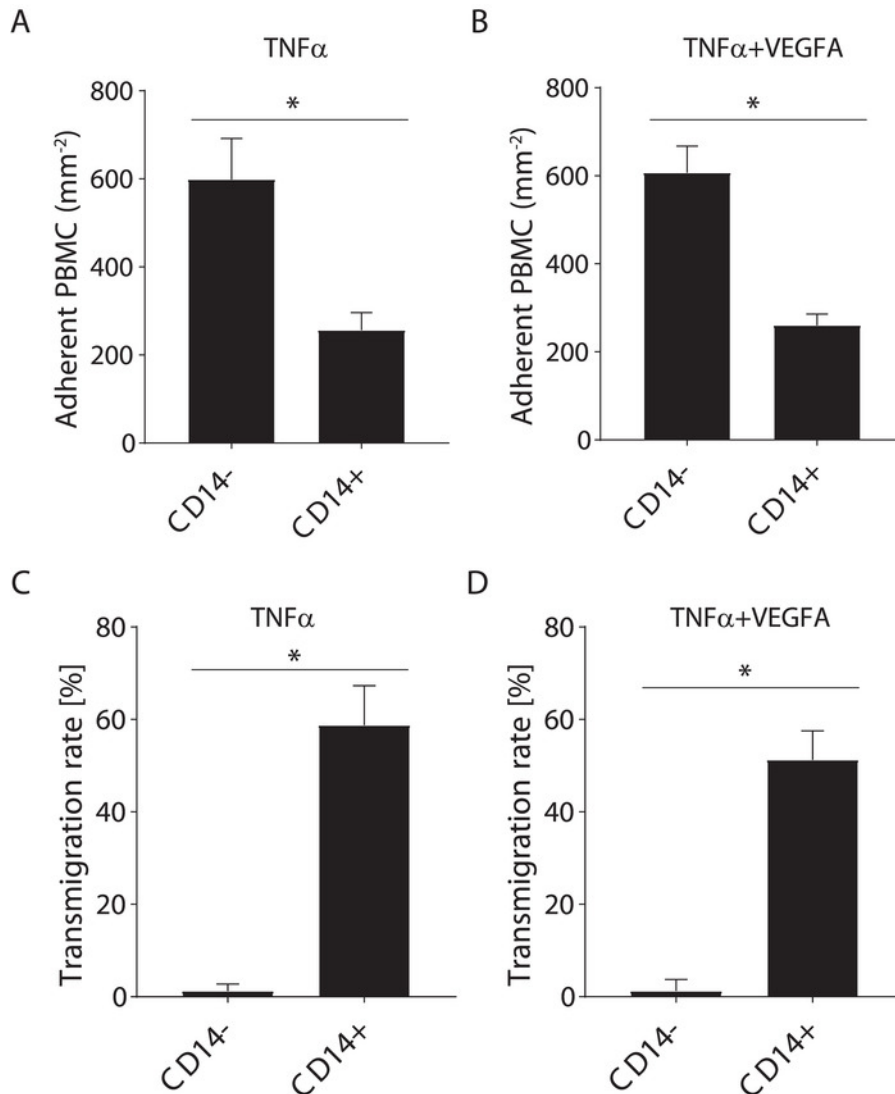


Figure 6: Simultaneous investigation of the transmigration of CD14⁺ versus CD14⁻ PBMC under flow. (A) Adhesion of CD14⁺ versus CD14⁻ PBMC to TNF α -activated HUVEC under flow. (B) Adhesion of CD14⁺ versus CD14⁻ PBMC to TNF α +VEGFA-activated HUVEC under flow. (C) Transmigration rate (%) of CD14⁺ versus CD14⁻ PBMC through TNF α -activated HUVEC under flow. (D) Transmigration rate (%) of CD14⁺ versus CD14⁻ PBMC through TNF α +VEGFA-activated HUVEC under flow. Data are mean \pm S.D. N = 4 biological replicates. *p < 0.05; Mann-Whitney test. [Please click here to view a larger version of this figure.](#)

Supplemental Movie 1: View at the apical plane of pan-monocyte recruitment under flow. Expanded view of the recruitment of pan monocyte under flow at the apical plane. HUVEC were stained with CMFDA and the nuclei of monocytes were live-stained with Hoechst 33342. Scale bar = 50 μ m [Please click here to download this file.](#)

Supplemental Movie 2: View at the basal plane of pan-monocyte recruitment under flow. Expanded view of the recruitment of pan monocyte under flow at the basal plane. HUVEC were stained with CMFDA and the nuclei of monocytes were live-stained with Hoechst 33342. Scale bar = 50 μ m [Please click here to download this file.](#)

Supplemental Movie 3: Maximal projection of z-stacks of pan-monocyte recruitment under flow. Expanded view of the recruitment of pan monocyte under flow as shown in **Supplemental Movies 1 and 2**. HUVEC were stained with CMFDA and the nuclei of monocytes were live-stained with Hoechst 33342. Scale bar = 50 μ m [Please click here to download this file.](#)

Supplemental Movie 4: View at the apical plane of the recruitment of monocyte subpopulations to TNF α -activated HUVEC. Expanded view at the apical plane of the simultaneous recruitment of monocyte subpopulations under flow to TNF α -activated HUVEC under flow. HUVEC were stained with CMFDA and the nuclei of monocytes were live-stained with Hoechst 33342. Human proangiogenic monocyte subpopulations were identified by the surface expression of CD16. Scale bar = 30 μ m. [Please click here to download this file.](#)

Supplemental Movie 5: View at the basal plane of the recruitment of monocyte subpopulations to TNF α -activated HUVEC. Expanded view, at the basal plane, of the simultaneous recruitment of monocyte subpopulations under flow to TNF α -activated HUVEC under flow. HUVEC were stained with CMFDA and the nuclei of monocytes were live-stained with Hoechst 33342. The human proangiogenic monocyte (HPMo) subpopulation was identified by the surface expression of CD16. Scale bar = 30 μ m. [Please click here to download this file.](#)

Supplemental Movie 6: View at the apical plane of the recruitment of monocyte subpopulations to TNF α +VEGFA-activated HUVEC. Expanded view, at the apical plane, of the simultaneous recruitment of monocyte subpopulations under flow to TNF α +VEGFA-activated HUVEC under flow. HUVEC were stained with CMFDA and the nuclei of monocytes were live-stained with Hoechst 33342. The human proangiogenic monocyte subpopulation was identified by the surface expression of CD16. Scale bar = 30 μ m. [Please click here to download this file.](#)

Supplemental Movie 7: View at the basal plane of the recruitment of monocyte subpopulations to TNF α +VEGFA-activated HUVEC. Expanded view, at the basal plane, of the simultaneous recruitment of monocyte subpopulations under flow to TNF α +VEGFA-activated HUVEC under flow. HUVEC were stained with CMFDA and the nuclei of monocytes were live-stained with Hoechst 33342. The human proangiogenic monocyte (HPMo) subpopulation was identified by the surface expression of CD16. Scale bar = 30 μ m. [Please click here to download this file.](#)

Discussion

Here, we report a method detailing a study of how monocyte subpopulations transmigrate through the inflamed endothelial monolayer. The discussed method used confocal microscopy instead of phase-contrast microscopy, which is also used to study monocyte recruitment under flow^{3,11,19}. One major advantage of using confocal microscopy for time-lapse imaging is the ability to unambiguously discriminate between transmigration and strong adhesion of monocytes. Though the phase-contrast microscopy-based method is also robust, it requires expertise in order to avoid mixing up transmigrated cells and strongly adherent cells. In this case, one needs to establish strict criteria for analysis in order to make a clear difference between these two states of the monocyte recruitment cascade. In addition, it is also important to perform an endpoint analysis by confocal microscopy in order to confirm the global trends observed by the phase-contrast microscopy. Thus, the direct use of confocal microscopy to investigate monocyte recruitment under flow provides clear results on the actual transmigration status of captured monocytes.

One of the major bottlenecks in executing leukocyte recruitment assays under flow and using a phase-contrast microscope is the time spent to perform the analysis and track individual cells from capture to transmigration through the cell-cell junction. Automation of such analysis is possible but difficult to perform due to phase-contrast similarities between crawling and transmigrated monocytes. Here we show by using confocal microscopy that monocyte transmigration was accompanied by a discontinuation of endothelial cell staining in the basal plane corresponding to the shape of the transmigrated monocytes underneath HUVEC. This positioning was confirmed by the orthogonal projection. The transition of monocyte localization occurred exclusively between cell-cell junctions indicative of a paracellular transmigration. This is consistent with our previous data, which showed that under flow in vitro, monocytes transmigrate exclusively through paracellular route with HUVEC^{3,18}. To complement the method proposed here, it is possible to use non-blocking antibodies against junctional proteins such as VE-cadherin, JAMs, or PECAM1 in order to picture the potential sites of monocyte transmigration (paracellular vs. transcellular). We have confirmed that the black shapes surrounding the monocyte nuclei are a robust characteristic of transmigrated cells and a simple event that may be detectable by software. Even though a manual cell counting system is demonstrated here, the black shape formation around the leukocyte nucleus is a criterion that could be used to define leukocyte transmigration in automated analysis, thus saving a lot of time. We are currently working on developing an automated application for such analysis.

Fluorescence and confocal microscopy were previously used in the study of leukocyte recruitment. However, they were not used to investigate the recruitment of different subpopulations simultaneously. Here we propose a modality of using confocal microscopy to study the recruitment of leukocyte subtypes simultaneously in the same microenvironment. We show that confocal microscopy can be used to investigate simultaneously the migratory behaviors of different monocyte subpopulations. As an example, we have used CD16 expression to discriminate between proangiogenic and non-angiogenic monocytes in order to study the transmigration capacity of the two subpopulations in different inflammatory contexts. Consistent with our recent publication, by using the confocal microscopy modality, we have shown that the transmigration rate of CD16⁺ monocytes was lower when the endothelial cell monolayer was stimulated only by TNF α ³. However, the combination of TNF α and VEGFA led to an increase in transmigration of proangiogenic monocytes. The transmigration rate was similarly high for non-angiogenic CD16⁺ monocytes under both inflammatory conditions. We have previously shown that monocyte staining with the anti-CD16 antibody did not present any significant effect on transmigration, confirming this by the analysis of unlabeled monocytes after the transmigration assay, using confocal microscopy³. However, for new leukocyte subtypes or antibodies used to mark them, the labeling effect needs to be assessed. Using this method, up to three different populations of leukocytes can be simultaneously studied. This could be subpopulations that are functionally distinct or similar immune cell types. Although the focus here is on monocyte transmigration, other steps of their recruitment can also be analyzed by this method, including cell behavior before transmigration, such as capture, and migrational directionality. Post-transmigration events such as abluminal retention and reverse transmigration can also be investigated for different leukocyte populations, as an extension of this method. One limitation is the poor detection of the staining in the far-red channel in time-lapse imaging, as well as some overspill of the fluorescence signals that reduce the z-stack resolution. This was mainly related to the instrument used for confocal imaging. The use of image deconvolution could eventually help to improve the image quality and allow further analysis of the different steps of the leukocyte recruitment.

To study leukocyte recruitment under optimal conditions, it is important to check the activation status of the endothelial cell monolayer. Indeed, a deficient activation of endothelial cells leads to a global reduction in monocyte adhesion and transmigration. Endothelial cell activation can be checked by analyzing the expression level of adhesion molecules on endothelial cell surface such as ICAM1 and VCAM1. The level of these adhesion molecules must be increased compared to unstimulated endothelial cells. If no change is detectable in these endothelial adhesion molecules, the cultured HUVEC can be considered as not activated. Assessing the expression level of adhesion molecules can constitute a good quantitative control between different experiments using the same batch of HUVEC. However, the expression level of these adhesion molecules can also vary between different primary culture of endothelial cells limiting the consideration of a global threshold of ICAM1 or VCAM1. The change in shape of macrovascular endothelial cells such as HUVEC is also a good indicator of their activation. This latter change in phenotype

allows a quick and qualitative assessment of HUVEC activation. However, the analysis of adhesion molecules might be a better choice for microvascular cells that don't show major shape-change upon activation with inflammatory cytokines.

For mechanistic studies, relevant negative controls of monocyte transmigration can be performed by using antibodies against endothelial adhesion molecules such as ICAM1, VCAM1 or on leukocyte surface such as Leukocyte Function-associated Antigen (LFA)-1. The use of a relevant negative control is essential for such mechanistic study in monocytes as they express Fc-receptors on their cell surfaces. The purity of monocytes after isolation is also important, in order to avoid contamination by other leukocyte populations and an underestimation of the rate of monocyte transmigration. Another critical parameter is the temperature, which needs to be set at 37 °C for all the assays in order to ensure all experimental observations are relevant and translate accordingly to human *in vivo* cell trafficking.

Disclosures

The authors have no competing financial interests.

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