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TITLE:

Simultaneous Study of the Recruitment of Monocyte Subpopulations Under Flow *In vitro*

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KEYWORDS

Leukocyte trafficking, monocyte recruitment, inflammation, timelapse imaging, confocal microscopy, adhesion, transmigration, monocyte subpopulations, chemokines, integrins, adhesion molecules

SUMMARY

Here, we present an integrated protocol that measures monocyte subpopulation trafficking under flow *in vitro* by use of specific surface markers and confocal fluorescence microscopy. This protocol can be used to explore sequential recruitment steps as well as to profile other leukocyte subtypes using other specific surface markers.

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.

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[1–3]. 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 barrier[5]. 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[7–9]. 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 function[10]. Consistently, endothelial cell stimulation with inflammatory cytokines such as human tumor necrosis factor (TNF)α or interleukin (IL-1)beta (conventional inflammation) is sufficient to trigger the complete recruitment of classical $CD14^{+}CD16^{-}$ monocytes. However, simultaneous actions of vascular endothelial growth factor (VEGF)A and TNFα (angiogenic factors-driven inflammation) are required to provoke the transmigration of the $CD16^{+}$ proangiogenic pool of monocytes[3]. 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*[11–13]. 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 *versus* 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)

1.1. 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.

1.2. 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.

1.3. 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.

1.4. Tighten the cord and the cannula connection firmly with a length of wire.

1.5. 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.

1.6. Perfuse the vein with 12 mL of 1 mg/mL collagenase type I (0.22 µm-filtered).

1.7. Close the stopcock at the cord ends and incubate the cord at 37 °C for 12 min.

1.8. Gently massage the cord to detach endothelial cells from the vein lumen.

1.9. 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.

1.10. Connect an empty 50 mL syringe to the other end of the umbilical cord

1.11. Open the stopcock and perfuse the vein from one end whilst reciprocally collecting from the other end.

Note: The collected suspension contains endothelial cells.

1.12. Centrifuge this cell suspension at 200 x g for 5 min.

1.13. 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).

1.14. Remove the coating solution from the T75 flask and rinse once with PBS.

1.15. Seed the cells collected from step 1.13 into the T75 flask and place it in the incubator at 37 °C with 5% CO_2.

1.16. 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.

1.17. 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.

1.18. 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.

1.19. 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.

1.20. Discard the supernatant from step 1.19, resuspend the cell pellet in freezing solution (FCS containing 10% DMSO) at a density of $5x10^5$ cells/mL in cryotubes, and freeze at -80 °C or in liquid nitrogen until use.

1.21. To check HUVEC purity:

1.21.1. 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.

1.21.2. Incubate at room temperature for 10 min.

1.21.3. Add 100 µL of PBS and centrifuge at 400 x g for 30 s.

1.21.4. 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).

2.1. Coat a T75 flask with 1 mL of the coating solution at 37 °C for 30 min.

2.2. Rapidly defreeze HUVEC at 37 °C for 2 min and resuspend the cells in 10 mL of complete M199.

2.3. Centrifuge the cells at 200 x g at room temperature for 5 min and discard the supernatant.

2.4. Resuspend the cell pellet in 10 mL of complete M199.

2.5. Transfer the cell suspension in the pre-coated flask. Place the flask in the incubator at 37°C with 5% CO_2. Change the cell culture medium every 2 days.

3. HUVEC Culture in 0.4 µ-Slide Chamber

3.1. 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.

3.2. Wash the chambers with 100 µL of PBS.

3.3. Detach the cells from an 80-90% confluent HUVEC of a T75 flask.

3.4. Rinse HUVEC with 5 mL of PBS and detach them with 5 mL of 0.05% trypsin at 37 °C for 5 min.

3.5. 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.

3.6. Resuspend the cell pellet at 10^6 cells/mL and distribute 30 µL (30,000 cells) per chamber.

3.7. Incubate the cells in an incubator at 37 °C with 5% CO_2 for 1 h.

3.8. Add 150 µL of complete M199 to each chamber and culture the cells for 5 days in the incubator at 37 °C and 5% CO_2. Change the medium every 2 days.

4. HUVEC Staining for Monocyte Recruitment Assay Under Flow

4.1. 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.

4.2. Wash HUVEC twice with M199 medium warmed at 37 °C.

4.3. 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% CO_2 for 10 min.

4.4. Wash once with complete M199 and incubate the cells with complete M199 in the incubator at 37 °C and 5% CO_2 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.

4.5. 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% CO_2.

5. Isolation of Human Pan Monocytes and Staining of Subpopulations

5.1. 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.

5.2. 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.

5.3. 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.

5.4. Centrifuge at 200 x g at room temperature for 5 min. Discard the supernatant.

5.5. Resuspend the cell pellet with 10 mL of staining buffer (PBS- 1 mM EDTA containing 0.5% bovine serum albumin BSA).

5.6. Centrifuge at 200 x g at room temperature for 5 min. Discard the supernatant.

5.7. Repeat steps 5.5 and 5.6.

5.8. Resuspend the cell pellet with 10 mL of staining buffer. Take an aliquot of 10 µL for a cell count.

5.9. 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 50-100x10^6 PBMC.

5.10. **For the recruitment of CD14+ *versus* CD14- PBMC under flow**:

5.10.1. Wash the pellet three times with flow buffer (M199 containing 0.5% BSA) and resuspend the mononuclear cells in flow buffer at 6x10^6 cells per mL.

5.10.2. Make aliquots of 200 µL for each assay. Incubate at 37 °C until 20 min before the assay.

5.10.3. 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.

5.10.4. Centrifuge the aliquot at 400 x g for 30 s.

5.10.5. Discard the supernatant and resuspend the pellet with 200 µL of flow buffer.

5.11. **For the recruitment of monocyte subpopulations under flow**:

5.11.1. Isolate monocytes with a pan monocyte isolation kit according to manufacturer instructions.

Note: The following isolation protocol is for 50x10^6 cells. It can be scaled up or down as long as it is within the manufacturer's recommendations.

5.11.2. Centrifuge the PBMC suspension at 200 x g at room temperature for 5 min.

5.11.3. Discard the supernatant and resuspend the pellet with 400 µL of staining buffer.

5.11.4. Add 50 µL of Fc-receptor blocking reagent and 50 µL of Pan Monocyte antibody cocktail.

5.11.5. Incubate at room temperature for 10 min.

5.11.6. Add 400 µL of staining buffer and 100 µL of magnetic beads conjugated anti-biotin antibody. Incubate at room temperature for 15 min.

5.11.7. Add 2 mL of staining buffer and use a MACS LS column coupled with a magnet.

5.11.8. Place the LS column on the magnet and add 1 mL of staining buffer. Discard the flow-through.

5.11.9. Pass the PBMC suspension in the column and collect the clear flow though containing pan monocytes in a new 15 mL tube.

5.11.10. Add the staining buffer to top up to 5 mL.

5.11.11. Take an aliquot and check the quality of the monocyte isolation with a flow cytometer.

5.11.12. Determine the pan monocyte count.

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

5.11.13. Centrifuge the remainder of monocytes from step 5.11.11 at 200 x g for 5 min.

5.11.14. Discard the supernatant.

5.11.15. Resuspend the cell pellet in 5 mL of flow buffer (M199 containing 0.5% BSA).

5.11.16. Repeat 5.11.13 to 5.11.14 twice to eliminate any trace of EDTA.

5.12. Make monocyte suspension in flow buffer (M199 with 0.5% BSA) at 6 x 10^6 cells/mL.

5.13. Make aliquots of 200 µL of monocytes for each recruitment assay.

5.14. Keep the aliquot at 37 °C in the incubator until 20 min before injection.

5.15. Add 5 µL of anti-CD16-PE antibody and Hoechst 33342 (2 µM final) to each aliquot.

5.16. Mix and incubate at 37 °C for 10 min.

5.17. Centrifuge the aliquot at 400 x g for 30 s.

5.18. Discard the supernatant and resuspend the pellet with 250 µL of flow buffer.

5.19. Add 30 µL of the monocyte suspension in one chamber of the slide to serve for setting the acquisition parameters on the confocal microscope.

5.20. 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

6.1. Ensure that the cell incubator for the imaging set at 37 °C.

Note: A diagram of the flow system is shown in **Figure 2**.

6.2. 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.

6.3. 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.

6.4. Connect part I and part II tubing by inserting the Luer connector males to a female Luer lock coupler (**Figure 2A**).

6.5. Put the free end of the silicone tubing in the reservoir containing the flow buffer (M199 + 0.5% BSA) warmed at 37 °C.

6.6. Pull on the plunger of the 20 mL syringe to fill the tubing with flow buffer.

6.7. Place the syringe on the pump and secure it.

6.8. Set the pump in withdraw mode (as opposed to infuse) and specify the flow rate.

6.9. Determine the flow rate according to the IBIDI slide used by using the following formula:

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

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 website[14]. The flow buffer viscosity is 0.0072 dyn.s/cm^2. Shear stress at the post-capillary venules is about 0.5 dyn/cm^2.

6.10. Connect the slide (**Figure 2B**):

6.10.1. Clamp the silicone tubing around the female Luer Lock Coupler and disconnect the two Luer connector males from the coupler.

6.10.2. Connect them to the reservoirs of the slide containing stimulated HUVEC and fill with medium. Avoid air bubbles during this step.

6.10.3. Take off the clamps and ensure that the connection is not leaking.

6.11. 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

7.1. Use a 40X objective (see **Table of Materials**) for imaging.

7.2. Activate the 405 nm (blue monocyte nuclei), 488 nm (green endothelial cells) and 561 nm (red CD16+ subset) lasers.

7.3. 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.

7.4. Place the chamber to be acquired under the microscope.

7.5. Choose 3 fields of views within 1 cm radius for multi-position confocal imaging.

7.6. Define the basal and the apical sides of endothelial cells

7.7. Set a z-stack to the 10-12 µm range (0.5 µm step). Run a time-lapse acquisition every 1 min.

7.8. After 3 min of imaging, inject 200 µL of monocyte suspension ($6x10^6$ 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).

7.9. Image for at least 30 min. Once finished, stop imaging and stop the flow. Clamp the tubing to disconnect them from the slide.

7.10. Fix the slide with 4% paraformaldehyde at 4 °C for 10 min.

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

8. Analyze the Data with ImageJ

8.1.1. Count the number of total adherent monocytes in each field. Determine the cell count per mm^2.

8.1.2. 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.

8.1.3. Divide the count of transmigrated leukocytes by the total number of adherent leukocytes. The transmigration rate is presented as a percentage of adherent monocytes.

8.1.4. For illustration, the apical and the basal sides can be shown simultaneously to illustrate the events occurring in each of these endothelial compartments.

Note: Non-transmigrated monocytes are positioned at the apical plane, whereas transmigration occurs with movement from the apical to the basal plane. In addition, a black hole appears in the green channel around the nuclei of transmigrated monocytes. This black hole corresponds to the space occupied by the body of transmigrated monocytes underneath endothelial cells stained in green. This discontinuation in green at the basal plane follows the movement of transmigrated monocytes in the abluminal compartment.

REPRESENTATIVE RESULTS

Determining the state of HUVEC activation induced by TNFα

The bio-activity of the inflammatory cytokine TNFα 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 TNFα 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[15–17]. An easier and simpler way to check the activation status of HUVEC after TNFα 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 TNFα in comparison to unstimulated cells. Similar elongation is observed when HUVECs are stimulated by a mix of TNFα 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 TNFα 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 TNFα only. However, this rate increased when endothelial cells were stimulated simultaneously with TNFα 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 TNFα or TNFα+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 TNFα or TNFα+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 LEGENDS

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.

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.

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 TNFα (500 U/mL) or a mix of TNFα (500 U/mL)+VEGFA (1 µg/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 µm

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 µm. **(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 transmigrated 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 µm. **(D)** Quantification of monocyte adhesion to TNFα-stimulated *versus* unstimulated HUVEC over time. **(E)** Quantification of monocyte transmigration rate over time. N = 3 biological replicates. Data are presented as mean ± S.D.

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 TNFα-activated HUVEC. Scale bar = 40 µm; 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 TNFα-activated HUVEC monolayer. N = 4 biological replicates, data are presented as mean ± 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 TNFα+VEGFA-activated HUVEC. Scale bar = 40 µm; 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 TNFα+VEGFA-activated HUVEC monolayer. N = 4 biological replicates, data are presented as mean ± S.D. *p < 0.05; Mann-Whitney test. **(E)** Localization of $CD16^+$ monocytes before (10 min) and after (15 min) transmigration through TNFα+VEGFA-stimulated HUVEC. The orthogonal views are shown. Scale bar = 40 μm

Figure 6: Simultaneous investigation of the transmigration of CD14+ *versus* CD14- PBMC under flow. (A) Adhesion of $CD14^+$ *versus* $CD14^-$ PBMC to TNFα-activated HUVEC under flow. **(B)** Adhesion of $CD14^+$ *versus* $CD14^-$ PBMC to TNFα+VEGFA-activated HUVEC under flow. **(C)** Transmigration rate (%) of $CD14^+$ *versus* $CD14^-$ PBMC through TNFα-activated HUVEC under flow. **(D)** Transmigration rate (%) of $CD14^+$ *versus* $CD14^-$ PBMC through TNFα+VEGFA-activated HUVEC under flow. Data are mean ± S.D. N = 4 biological replicates. *p < 0.05; Mann-Whitney test.

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 μm

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 μm

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 μm

Supplemental Movie 4: View at the apical plane of the recruitment of monocyte subpopulations to TNFα-activated HUVEC. Expanded view at the apical plane of the simultaneous recruitment of monocyte subpopulations under flow to TNFα-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 μm.

Supplemental Movie 5: View at the basal plane of the recruitment of monocyte subpopulations to TNFα-activated HUVEC. Expanded view, at the basal plane, of the simultaneous recruitment of monocyte subpopulations under flow to TNFα-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 μm.

Supplemental Movie 6: View at the apical plane of the recruitment of monocyte subpopulations to TNFα+VEGFA-activated HUVEC. Expanded view, at the apical plane, of the simultaneous recruitment of monocyte subpopulations under flow to TNFα+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 μm

Supplemental Movie 7: View at the basal plane of the recruitment of monocyte subpopulations to TNFα+VEGFA-activated HUVEC. Expanded view, at the basal plane, of the simultaneous recruitment of monocyte subpopulations under flow to TNFα+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 μm

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 *versus* 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 TNFα[3]. However, the combination of TNFα 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 microscopy[3]. 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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Figure 1

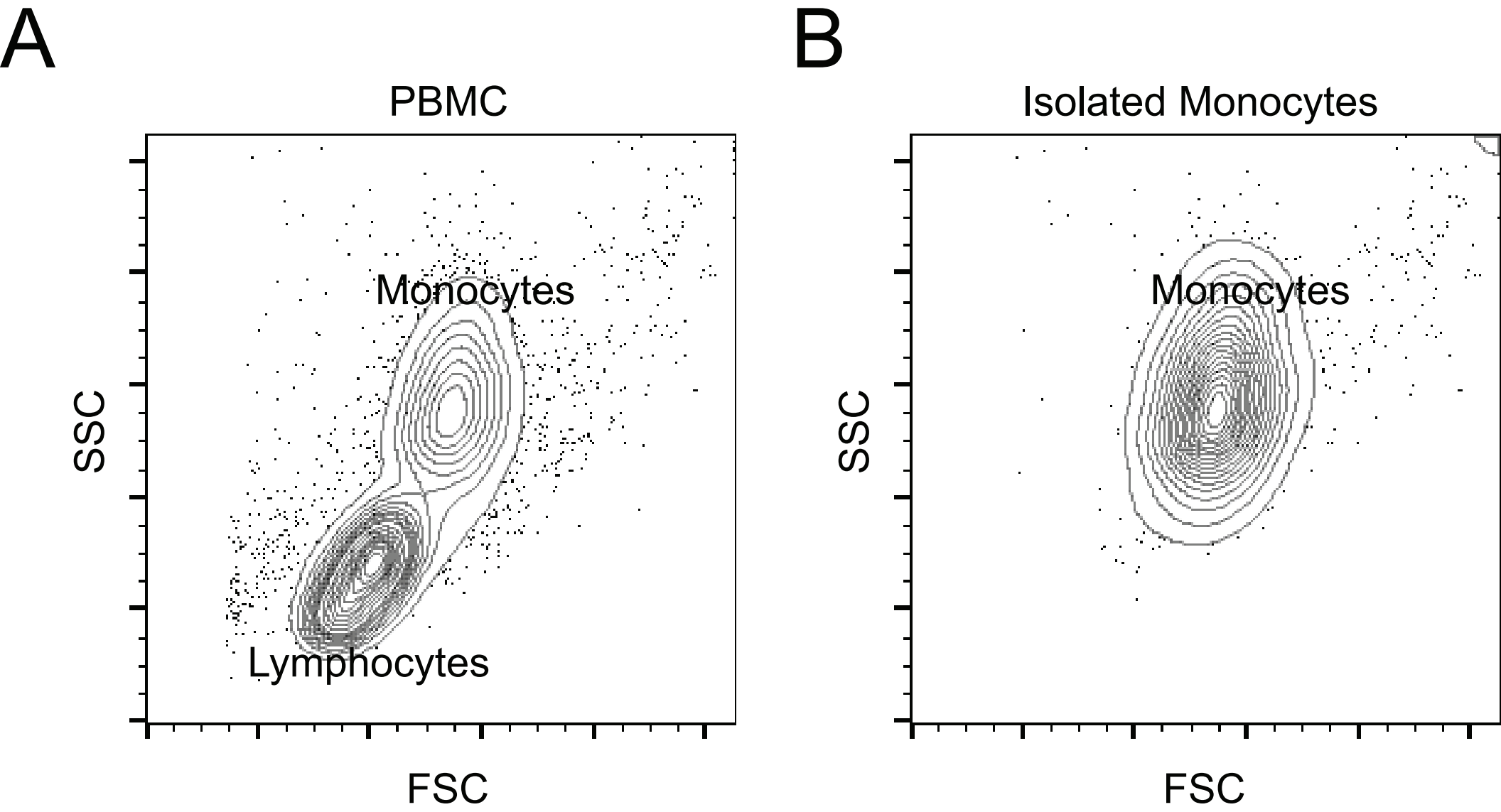


Figure 2

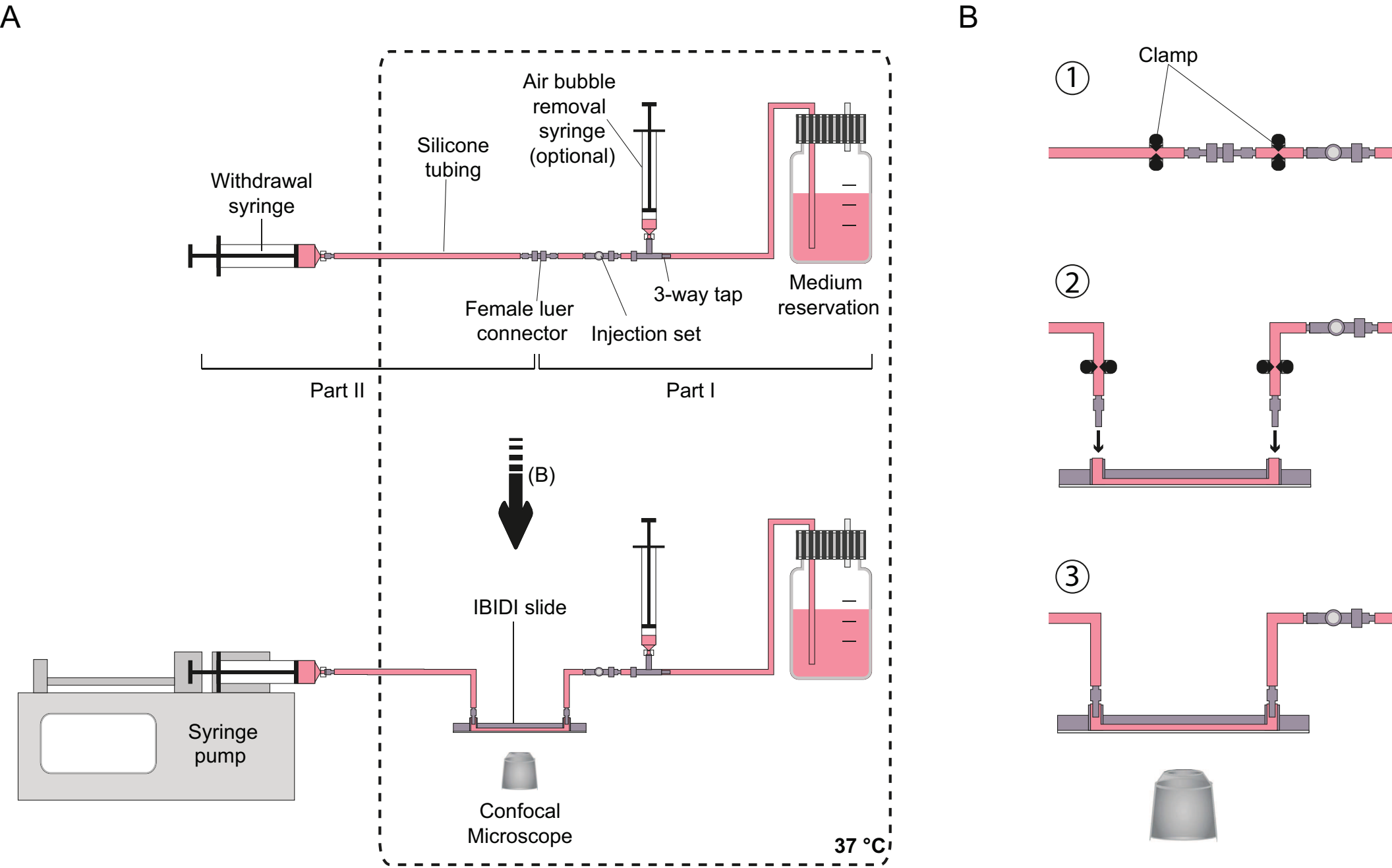


Figure 3

Click here to access/download;Figure;Fig3_EC shape.eps

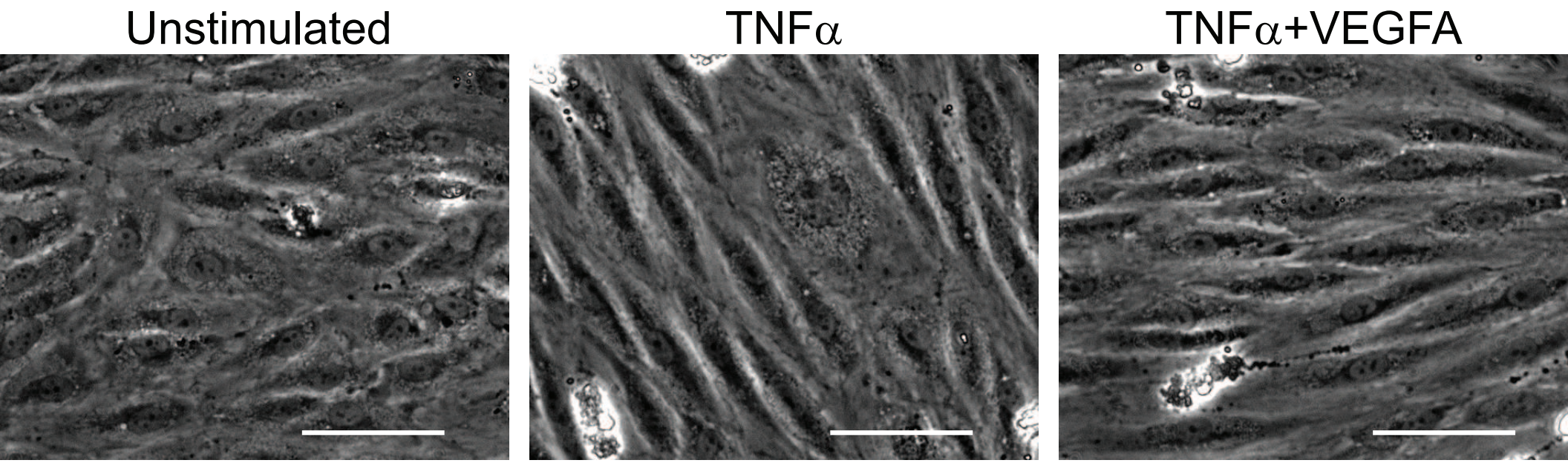


Figure 4

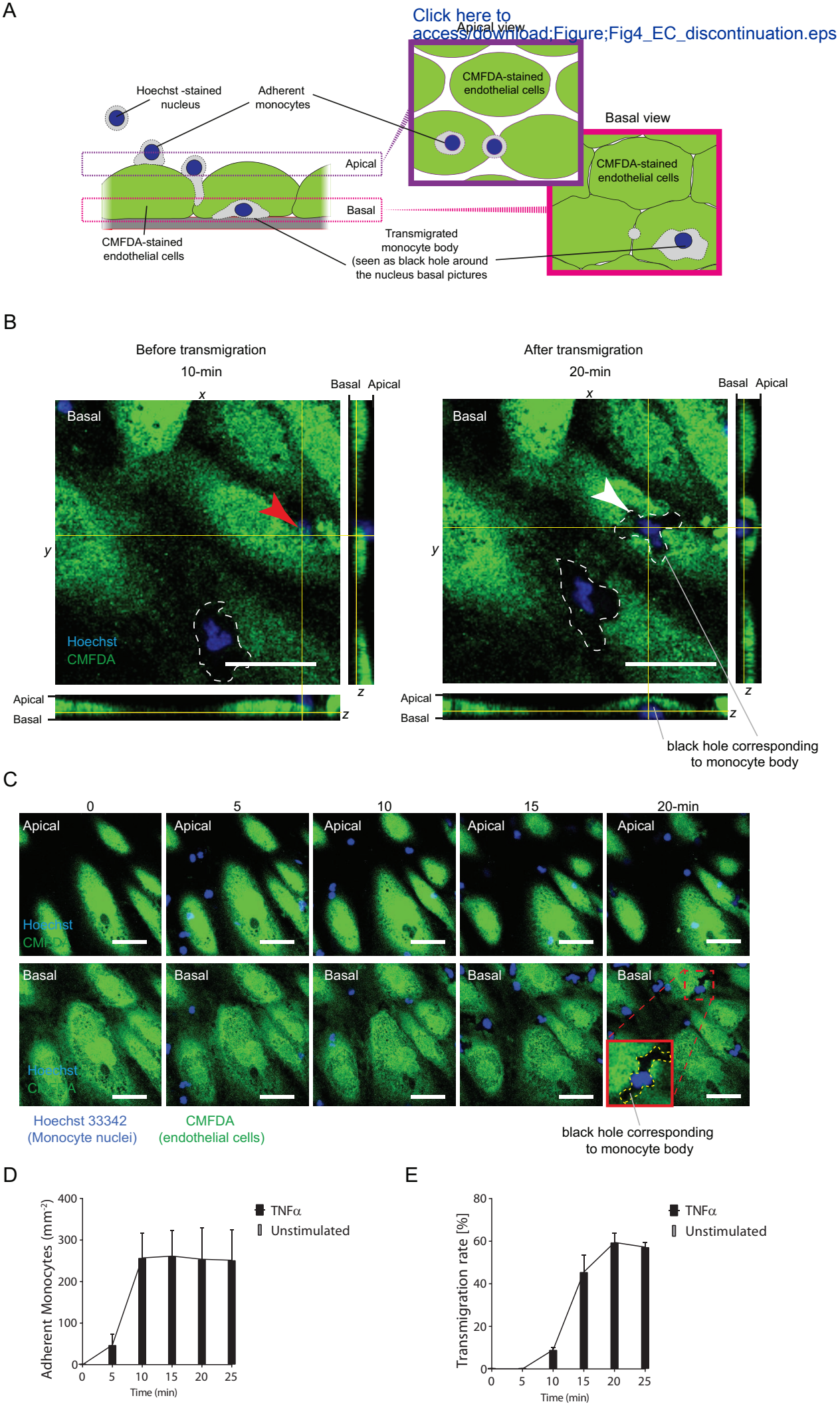
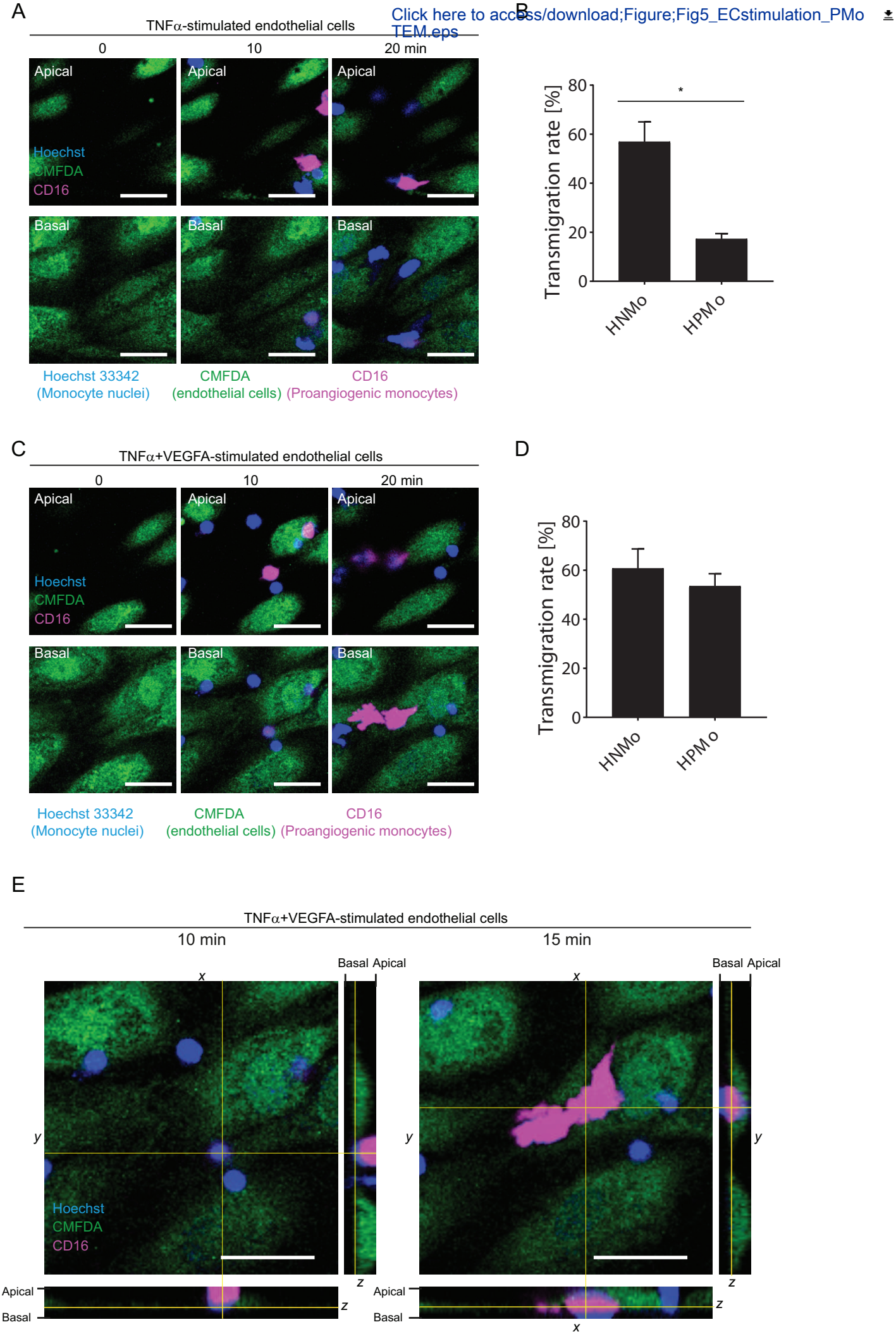


Figure 5



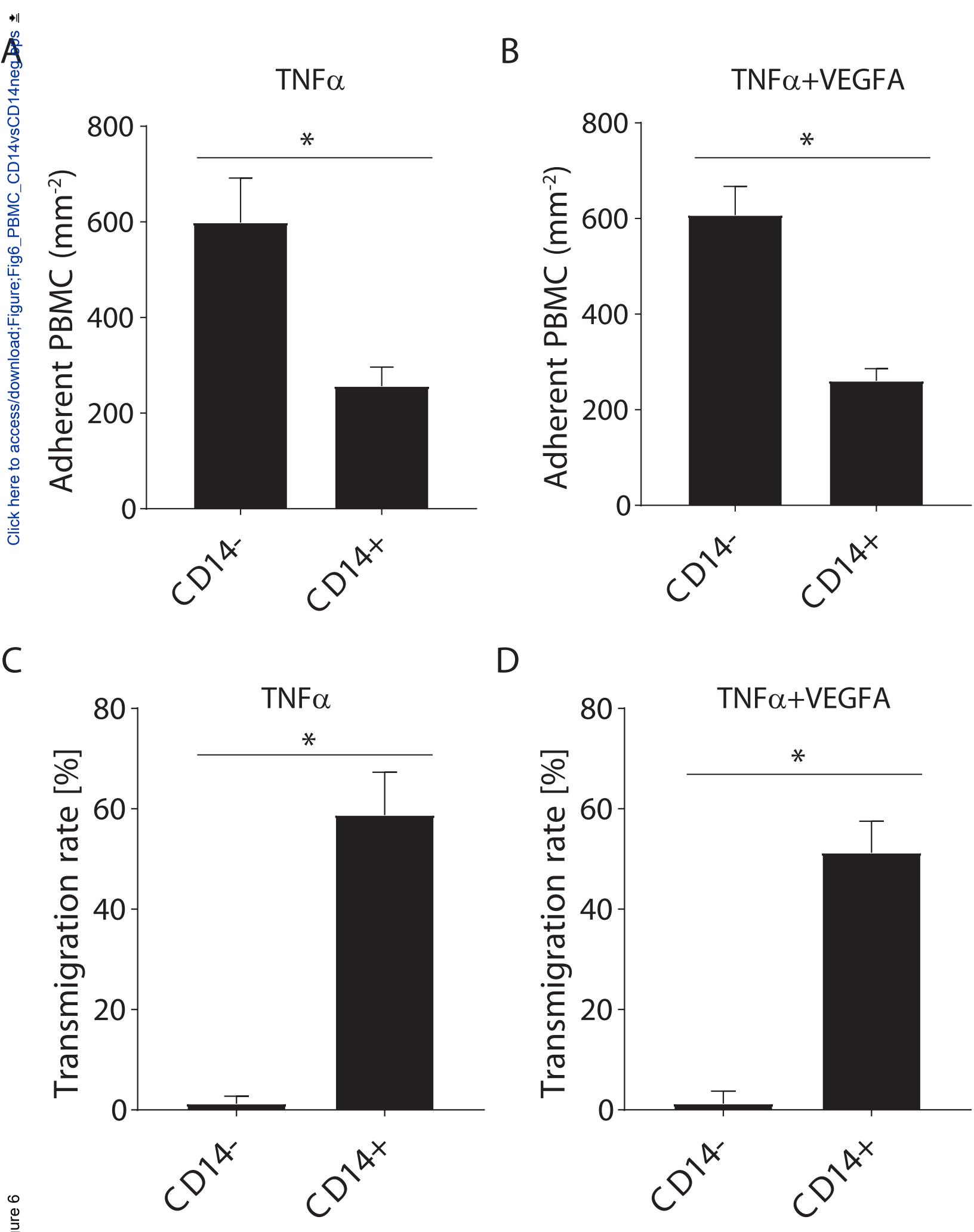


Figure 6

Supplemental Movie 1

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Movie1_Apical_.avi

Supplemental Movie 2

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Movie2_Basal_.avi

Supplemental Movie 3

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Supplemental Movie 4

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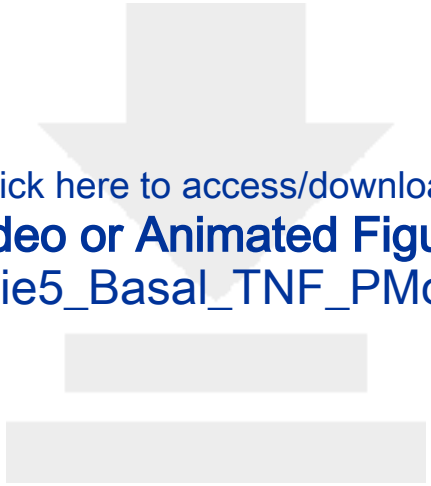
Movie4_Apical_TNF_PMo.avi

Supplemental Movie 5



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Movie5_Basal_TNF_PMo.avi

Supplemental Movie 6

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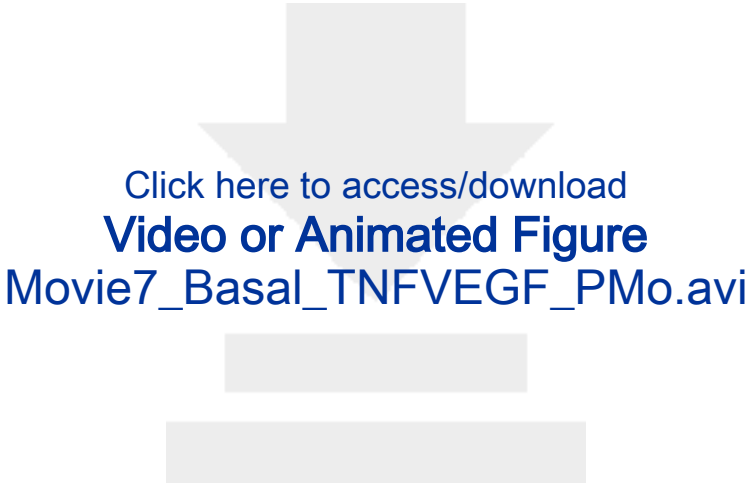
Movie6_Apical_TNFVEGF_PMo.avi

Supplemental Movie 7



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Movie7_Basal_TNFVEGF_PMo.avi

Name of Material/ Equipment	Company
Tissue Culture Flasks 75 cm2	TPP
µ-Slide VI 0.4	IBIDI
Centrifuge Tubes 15 mL	TPP
Centrifuge Tubes 50 mL	TPP
Collagen G	Biochrom
Gelatin	Sigma-Aldrich
Dulbecco's Phosphate Buffered Saline (without MgCl2 and CaCl2)	Sigma-Aldrich
Dulbecco's Phosphate Buffered Saline (with MgCl2 and CaCl2)	Sigma-Aldrich
RPMI-1640 Medium	Sigma-Aldrich
3-Way Stopcocks	BIO-RAD
penicillin 10000 u/ml streptomycine 10000 ug/ml fungizone 25 ug/ml	AMIMED
Collagenase type 1	Worthington
Medium 199 1X avec Earle's salts, L-Glutamine, 25 mM Hepes	GIBCO
Bovine Albumin Fraction V	ThermoFisher
Endothelial Cell Growth Supplement, 150mg	Millipore
Heparin Sodium	Sigma-Aldrich
Hydrocortisone	Sigma-Aldrich
L-Ascorbic acid	Sigma-Aldrich
EDTA disodium salt dihydrate C10H14N2Na2O8 · 2H2O	APPLICHEM
CD144 (VE-Cadherin), human recombinant clone: REA199, FITC	Miltenyi Biotech
CD31-PE antibody, human recombinant clone: REA730, PE	Miltenyi Biotech
Anti-Podoplanin-APC, human recombinantclone: REA446, APC	Miltenyi Biotech
BD Accuri C6 Plus	BD Bioscience
µ-Slide I Luer	IBIDI
CMFDA (5-chloromethylfluorescein diacetate)	ThermoFisher
Recombinant human TNFα	Peprotech
Recombinant human VEGFA	Peprotech
NE-1000 Programmable Syringe Pump	KF Technology
Ficoll Paque Plus	GE Healthcare
Anti-human CD14-PE, human recombinant clone: REA599, PE	Miltenyi Biotech
Pan Monocyte Isolation Kit, human	Miltenyi Biotech
Anti-human CD16-PE, human recombinant clone: REA423, PE	Miltenyi Biotech

LS columns		Miltenyi Biotech
QuadroMACS Separator		Miltenyi Biotech
Hoechst 33342, Trihydrochloride, Trihydrate		ThermoFisher
Silicone tubing		IBIDI
Elbow Luer Connector		IBIDI
Female Luer Lock Coupler		IBIDI
Luer Lock Connector Female		IBIDI
In-line Luer Injection Port		IBIDI
Ar1 confocal microscope		Nikon
40X objective	Nikon	
ImageJ Software		NIH

Catalog Number	RRID of antibodies
90076	
80606	
191015	
191050	
L 7213	
1393	
D8537	
D8662	
R8758	
7328103	
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LS004216	
22340020	
15260037	
02-102	
H3149RT	
H6909	
A 4544	
A2937.0500	
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130-110-807	AB_2657280
130-107-016	AB_2653263
80176	
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130-096-537	
130-106-762	AB_2655403

130-042-401
130-090-976
H1399
10841
10802
10823
10825
10820

40x 0.6 CFI ELWD S Plane Fluor WD:3.6-2.8mm correction 0-2mı

	Comments/Description
Routine culture of isolated HUVEC	
For coating of cell culture flasks	
For coating of cell culture flasks	

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CORRESPONDING AUTHOR:

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Institution:	**Université de Genève**
Article Title:	**Simultaneous study of the recruitment of monocyte subpopulations under flow in vitro**
Signature:	 Date: **May 23th 2018**

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Point-by-point answers to the reviewers' comments

We want to thank the editor and all the Referees for the constructive comments that definitely allowed us to improve our article and contents. We have now addressed the questions pointed out by the referees.

Reply to the editor comments

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Reply: We have been through the manuscript, and fixed outstanding spelling and grammar issues

2. Figure 2: Please include a space between numbers and their units (i.e., 37 °C).

Reply: This has been corrected throughout the text and figures

3. Figure 3: Please define the scale bars in the figure legend.

Reply: The scale bars have now been defined in the figure legends.

4. Figure 6: Please define the asterisk symbols in the figure legend.

Reply: The asterisk symbols used to denote significance are now defined in the figure legend.

5. Supplemental Movies 4-7: The scale bar in the movie is defined as 30 µm while the figure legend says 50 µm. Please revise to be consistent.

Reply: We thank the editor for notifying us of this error. We have now corrected this error and defined the scale bar in figure legends as 30 µm, which is the correct definition.

6. Please provide an email address for each author.

Reply: an email address has now been provided for each author on the submission website.

7. Please add a Summary section before the Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to.

Reply: We have now included in the manuscript a new summary section that describes the protocol and its applications.

8. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

Reply: This is done throughout the manuscript.

9. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

Reply: A space has now been included between all numbers and the corresponding units throughout the manuscript and figures.

10. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

Reply: The centrifuge speeds have now been changed into centrifugal force throughout the manuscript.

11. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

Reply: This has now been corrected in the manuscript.

12. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Falcon, Accuri C6, BD Bioscience, Cell Tracker, Ficoll, Miltenyi Biotec, AutoMACS, etc..

Reply: Generic terms are now used throughout the manuscript. All information that was available has now been included in the table of Materials and reagents.

13. Lines 123-127: Please break up into sub-steps. What happens after centrifugation? Is supernatant discarded or not? Please specify throughout.

Reply: The steps are now more detailed and include this information.

14. Lines 128-130: Does the supernatant come from step k (lines 120-122)? Please specify.

Reply: This has now been clarified, and can be found on lines 128-131.

15. Lines 184-189, 190-196, 266-268: Please break up into sub-steps.

Reply: These steps have now been split into sub-steps that can be found on lines 199 to 288.

16. Lines 247-250, 269-275: Please write the text in the imperative tense. Any text that cannot be written in the imperative tense may be added as a "Note.".

Reply: Every sentence in the protocol has now been written in the imperative tense. All commentaries are now placed in the Note sections.

17. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Reply: The equivalent of about 2.75 pages of text is now highlighted in yellow in the manuscript.

18. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense..

Reply: The relevant text has now been highlighted, and constitutes a cohesive story of the protocol.

19. Please include all relevant details that are required to perform highlighted steps. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Reply: the highlighted text contains all the relevant details needed to perform the experiments. Thus, the sub-steps involved were also highlighted.

20. Line 374: Should B be D instead?.

Reply: This has been corrected within the figure legend.

21. Table of Materials: Please remove trademark (™) and registered (®) symbols. Please provide lot numbers and RRIDs of antibodies, if available. Please use SI abbreviations for all units, include a space between all numbers and their corresponding units. Please use subscripts in chemical formulae to indicate the number of atoms, e.g., CaCl2, MgCl2, etc.

Reply: ALl trademark and registered symbols have been removed. The available details for each product has now been provided in the table of materials file.

22. References: Please do not abbreviate journal titles.

Reply: The full names of all journal title have now been provided in the references.

Reply to the reviewers' comments

Reviewer #1:

Manuscript Summary:

Monocytes play a critical role in the pathophysiological functions including inflammation, tissue damage and autoimmune processes. They are also important markers during some of these processes and may also aid in regeneration mechanisms under ischemic insult. The current manuscript by Ropraz et al. describes a novel method that can identify and visualize monocyte subpopulations using a confocal based imaging method. It should be highlighted that this method can differentiate adherent and transmigrated monocytes, which is key for pathological state of the disease/condition that will be helpful in understanding the molecular signaling cascade that may be triggered.

Reply: We thank the reviewer for this clear summary of the content and the scope of our manuscript.

Minor Concerns:

The review has to be revised for grammatical errors. The terminology used in the manuscript have to be consistent through out. For instance and not limited to, usage of TNFA, alfa has to be denoted by a symbol.

Reply: We have been through the manuscript and corrected all outstanding grammatical errors. Symbols are now correctly used in the manuscript to specify the exact proteins.

Reviewer #2:

Manuscript Summary:

The article entitled "Simultaneous study of the recruitment of monocyte subpopulations under flow in vitro" by Ropraz et al. discusses detailed procedures used for isolation of human venular endothelial cells and monocytes, and quantification of monocyte recruitment in vitro. This is a well written protocol for a fairly complex experimental setup that demonstrates the authors' expertise in the area..

Reply: We thank the reviewer for this clear summary of our manuscript and for the comment. We agree with the referee that the protocol proposed has been written to make the experiments simple to perform.

Major Concerns:

1. Improve the quality of the text within figures.

Reply: We have been through the whole manuscript and rewritten the parts we thought required changes according to this recommendation. We think this renewed version of the manuscript now contains the information in sufficient detail to perform the experiments.

2. Can CD14 and CD16 cell markers be used at the same time to differentiate between the three different populations of monocytes? Perhaps show a figure demonstrating this.

Reply: We thank the reviewer for this interesting suggestion. The double staining with the anti-CD14 and anti-CD16 is theoretically possible to differentiate between the three populations of monocytes. However in our hands, the fast bleaching of fluorescence in far red rendered the double staining of CD14 and CD16 difficult. Thus this limitation would hamper the investigation of the three monocyte subpopulations if used simultaneously.

Minor Concerns:

1. Spell out less known acronyms the first time they are used in the text.

Reply: All the acronyms are now spelled out with the first time of use, as suggested.

2. Write "silicone tubing" instead of "silicon tubing" throughout section 4 and in figure 2.

Reply: We thank the referee for this notification, we have now changed this throughout the manuscript and in the figures.

3. For monoclonal antibodies, state the clone number or indicate if polyclonal antibodies are used.

Reply: The monoclonal antibodies used in the manuscript are associated with their clone information in the material table.

Reviewer #3:

Manuscript Summary:

This is a protocol for studying and comparing human monocyte transmigration under flow. Confocal fluorescence microscopy is used as a read-out and this has advantages: 1. it is possible to simultaneously investigate several different fluorophore-labelled cell types, 2. greater spatial resolution and contrast can be easily achieved especially in the z-direction compared with non confocal methods. I think that the method is in general interesting and useful to the field and as stated in the intro could easily be adapted to other cells.

Reply: We thank the referee for this summary, which we are in total agreement.

However, there isn't too much info on how the scope could be extended. Unfortunately, as it stands, the protocol isn't very well written, several steps are confusing (beyond the many typographical errors that would fall under 'copy-editing') and important information is missing. The protocol will need substantial work before publication.

Reply: We apologize if some steps of the protocol appeared to be confusing to the referee. We have made changes within the protocol to make the message clearer. We feel that there is now all required information to perform the experiment and analyze the results.

Major Concerns:

See above for general comments, specifically:

-HUVEC flow chamber experiments are widely used and there are many sources with protocols. The strength here is the fluorescence / confocal imaging and analysis, however, much of the detail on how this is done is missing. Detailed protocols for the analysis necessary to get the quantitative representative data should be included, particularly as freely-available software has been used.

Reply: As stated above, we think that we have now added enough information to perform the experiment. We have provided the extra details for cell staining, image acquisition under flow and now, as suggested by the referee, we have also explained how to analyze the results. For the analysis, we used ImageJ and the cell count plugin to count the cells. This is now explained. However, we are now developing an easier method of analysis by automating this process.

-Some of the images and videos are unclear, feint cells are visible in the 'apical' images that look similar to transmigrated cells in 'basal' images, this should be explained.

Reply: We agree with the referee that the nuclei of leukocytes in one plane may also be slightly visible in other focal planes. This is due to the size of the pinhole and the laser intensity used in the experiment leading to an overspill of the fluorescence signals. Actually, transmigrated leukocytes present flat nuclei affecting the fluorescence detection. Thus the laser intensity and the pinhole of the microscope are set to be able to detect the leukocyte nuclei through all the layers. What is important to distinguish between crawling and transmigrated cells, is the appearance of an unstained area (in the endothelial cell detection channel) around

the leukocyte nucleus as now shown with the orthogonal projection (figure 4B or figure 5E). This constitutes a critical criteria for identifying transmigrated leukocytes.

Following the suggestion below would show both apical and basal sides of the endothelium simultaneously for instance.

Reply: We agree with the referee that for illustration purposes, it is important to show both the apical and the basal sides of the endothelium simultaneously. This is what we recommend now in the analysis section of the manuscript, as it appears in the figures as well.

-Section on purity of monocytes affecting transmigration efficiency. This section is confusing. Do the authors mean that the percieved rate is influenced by counting non-monocyte cells, or do they mean the other leukocytes are affecting the actual transmigration rate? This should be more carefully written.

Reply: We apologize that the result section on the importance of the purity of monocytes for a reliable analysis of transmigration, appeared confusing to the referee. What we point out in this section is that if monocytes are not properly purified, the contaminant cells would be falsely counted as monocytes leading to factual inaccuracies in the result. We have now added a sentence at the end of this section to precise. This is now clear in the text.

-The importance of strict criteria for analysis is commented on in the discussion, but this information is missing from the protocol.

Reply: We respectfully don't agree with this comment from the referee. The criteria for identification of transmigrated leukocytes are different between assays based on phase-contrast microscopy and confocal microscopy. As we commented on in the discussion, the similar appearances of transmigrated and strongly adherent monocytes in phase-contrast microscopy render the analysis difficult. Conversely, as shown here in the manuscript for the confocal microscopy, the appearance of the monocyte nucleus underneath endothelial cells and creating a black hole around its nucleus is clearly an unambiguous criteria for defining leukocyte transmigration. We agree that this was not properly stated in the previous version of our manuscript. We have now clearly stated the criteria for defining monocyte transmigration.

-In the discussion using 'cell shadows' as a robust signature of transmigration is discussed. This section seems biased, how would this compare with membrane staining / projecting the confocal stacks 'side-on' perpendicular to the imaging plane to simultaneously see both sides of the endothelium. This comment is also relevant to the one above regarding feint cells in the apical images, or is this what the authors mean? This section should be more clearly re-written.

Reply: We are not sure if we have correctly understood this comment. If it is about the position of the monocyte in the z-axis, and the space occupied by transmigrated monocytes underneath endothelial cells in the abluminal space. To illustrate this, we have now included in both figure 4B and figure 5E orthogonal projections to show that the black holes corresponding to the cell bodies of transmigrated monocytes are unambiguously underneath endothelial cells.

As explained above, the pinhole size, the laser intensity as well as the high z-steps (0.5 µm/step) leads to an overspill of the fluorescence signals rendering the z resolution weak, particularly for monocyte nucleus. This is a technical limitation of the instrumentation used for timelapse imaging. The message of the manuscript is to explain how to perform such experiments with the potential to produce better image quality using different image capture set-ups. However, the proposed protocol in the study will allow for the production of images good enough for analysis.

Minor Concerns:

Representative data, why not include 'xzy' 'side on' projections to illustrate the localisation of the monocytes during transmigration?.

Reply: As stated above, we have now included in figure 4B and Figure 5E the orthogonal projection of the recruitment of monocytes. For example on the z-projection of figure 5E, we can now see that at 10 min, the CD16+ monocyte (pink) is above endothelial cells (in green). However at 15 min, the CD16+ monocytes are positioned underneath the endothelial cells.

Protocol 1:

c) this step is very hard to interpret, no doubt the video will help, but e.g. what 'wire'?

Reply: We have rewritten this section of the text, which we hope has clarified this point.

d) empty the vein how?

Reply: This was performed by simply pulling the syringe plunger, at one end of the cord. This is now stated in the text.

i) how confluent?

Reply: about 80-90% confluence. This is now indicated in the manuscript.

Protocol 2:
several places including e), what temperature?

Reply: We have now corrected this throughout the text. The temperature of all incubations is indicated.

h) What is meant by tilt the slide at 45degC and "...take the same volume from the other."?

Reply: We apologize for stating “45°C” when discussing the tilt of a slide, which we agree is meaningless. We have now corrected this error.

Protocol 3:
b) doesn't make sense, missing word

Reply: We thank the referee for noticing this missing word. This has been corrected now.

Protocol 5
d) incomplete fixing protocol?

Reply: The Protocol describing fixing has been written in a complete form.

Reviewer #4:
Manuscript Summary:
The manuscript written by Ropraz et al. describes a very elegant and robust method to investigate leukocyte trafficking using an in vitro flow chamber assay in combination with confocal microscopy. Although the authors specifically focus on monocyte endothelial transmigration under inflammatory conditions, this method can easily be extended towards the analysis of other recruitment steps as well as to other leukocyte subtypes. The protocol is very well structured and very precise, each step is clearly explained, and critical topics are highlighted. This holds true for the performance of the experiments as well as for the analysis of the videos. Therefore, I am very confident that this method can easily be adapted in other labs and described results will certainly be reproducible. This manuscript clearly demonstrates the expertise of the authors in this field.
Reply: We thank the referee for this summary of our work and comments.

Minor Concerns:
Summary of minor suggestions, corrections and questions (point by point):
Chapter protocol:
1.
A) Coating at which temperature?
Reply: The coating was performed at 37 °C. This is now indicated in the manuscript where necessary.

L) Why is anti-PECAM-PE not used in the assay? This would define endothelial cell borders and would enable - in combination with the CMFDA staining - the differentiation between trans- and para-cellular transmigration. The authors should discuss this.

Reply: We agree that the use of a junctional marker has the advantage of indicating a cell-cell junction. As we showed (Bradfield et al Blood 2007, Sidibé et al Nat Comm 2018), in previous transmigration studies, monocytes exclusively migrate into the abluminal compartment via a paracellular route. The use of VE-cadherin has confirmed this observation. Our confocal microscopy studies are consistent with these observations. The transition of migrating monocytes from the luminal to abluminal compartment always occurred between endothelial cells. For these studies, we did not use anti-PECAM1-PE because the antibody is known to affect the integrity of the endothelial cell monolayer. However, we have already used JAM-C labeling in another protocol, and confirmed using confocal microscopy that monocyte transmigration occurred exclusively at cell-cell junctions. We thank the referee for this suggestion, and we have now included in the discussion the possibility to use junctional staining to visualize the endothelial cell junctions during paracellular transmigration.

2.

A) Which Ibidi flow chambers are used? 0.1 or 0.4? Do the authors degas the chambers 24h prior to the start of the experiment (as recommend by the manufacturer)?

Reply: We use 0.4 µ slides. This has now been indicated in the protocol. To clarify, we do not degas the chambers prior to the start of an experiment.

H) Typo? "Tilt the slide at 45°C…". Please correct to 45°.

Reply: This has now been corrected in the manuscript.

3.

I) Typo? Using anti CD14-FITC to label PBMC makes no sense because of the fluorochrome spectra overlap with CFMDA green tracker labelled HUVECS. On page 9, line 323 the authors use anti-CD14-PE.

Reply: We agree with the referee and apologize for this error. We meant to state CD14-PE, and not CD14-FITC. This has now been corrected throughout the manuscript.

5.

B) Multi position imaging using distant FOVs (field of views) could be critical because stage movement can induce additional flow/ turbulence. What is here the threshold? How far apart should the FOVs be at max to avoid this?

Reply: We agree that the use of very distant fields of views could affect the stability of the slide and the focus over time. Nowadays, confocal microscopes are equipped with the perfect focusing system that could help to maintain focus. In our experiments, we usually defined the fields of views within 1 cm radius without experiencing any focusing or experimental problems. As we did not purposefully investigate the effect of long distance between fields of view, we did not generate any threshold or maximum values. However, in order to not generate any ambiguity, we have now indicated in the protocol that chosen fields of views should be within a 1 cm radius.

E) (including Movie 6/7) Reverse Transmigration occurs rarely but also under physiological/ inflammatory conditions in vivo (see papers e.g. by Sussan Nourshargh). In the movies, not only transmigration from apical to basal plane is occurring but also reverse transmigration. Please refer to the cell in the upper right corner of the movies time point 5:30 to 17:00 min. This cell even re-enters the abluminal site of the HUVECS at last. How often does this happen under these in vitro conditions? Have the authors quantified it?

Reply: We agree with the referee that leukocyte reverse transmigration can also be observed in vitro under flow, and even quantified by this method. By using phase-contrast microscopy, monocyte reverse transmigration has been studied by Bradfield et al (Blood 2007). By using phase-contrast microscopy, we found that about 5 % of monocytes reverse transmigrate after 25 min and this can go up to 10 % after 60 min of transmigration assay under flow. However, we did not analyze monocyte reverse transmigration by the method in this manuscript as we wanted to focus on the transmigration. However as this has been pointed out by the referee, we have now indicated the possibility of analyzing monocyte reverse transmigration using this method.

Chapter representative results:

-HUVEC activation (page 8)

Line 282: Please include reference for ICAM-1/VCAM-1 induction upon TNFα stimulation of HUVECS. Since primary human cells are reacting highly variable, what is the threshold regarding HUVEC activation below which the authors define activation between individual experiments cannot be compared to each other anymore because of too big differences?

Reply: We have now included references for the induction of ICAM/VCAM1 upon TNF stimulation.

We agree that the diversity of primary cells can potentially lead to a high variability in the expression level of adhesion molecules as well. We think that this is a very good control of HUVEC activation when compared to unstimulated cells and that it could serve for qualifying individual experiments with the same batch of HUVEC. The expression level of adhesion molecules depends on many parameters including the sensitivity of the detection, thus it is extremely difficult to define a threshold. And if one had to define such threshold, it would also depend on which aspect of leukocyte recruitment one is investigating. It is almost impossible to globally define a threshold of difference for each individual experiment. However, the expression level must, by definition, be increased compared to unstimulated cells. In addition, such expression data could accompany the extent of leukocyte recruitment to have a better understanding of the leukocyte recruitment conditions. . All these aspects are now discussed in the manuscript.

Do the authors always perform control experiments with anti-ICAM1 blocking antibodies? The authors should comment on this and include this information.

Reply: As now discussed in the manuscript, for mechanistic investigations, it is important to use a negative control in which an essential molecule involved in leukocyte recruitment is targeted. We agree that an anti-ICAM1 blocking antibody could serve for this purpose. This is now discussed in the manuscript. However, during a typical recruitment assay experiment, we don't systematically use an anti-ICAM1 blocking antibody although we can monitor for HUVEC activation by simple observation. For mechanistic studies, we use different negative controls, including anti-LFA1, anti-beta1 integrin or the pertussis toxin according to the study context. The context of using such blocking antibodies is now discussed in the manuscript.

-Monocyte transmigration (page 9)
It is not exactly clear how precisely transmigration is defined/ quantified? Black hole as reference is intuitive but what is the criterion to be used to distinguish between a cell being just in the process of transmigration and a cell which has almost completely passed through. What is the exact cut-off? Furthermore, I would suggest including (besides the scheme in figure 4A) representative images of orthogonal views to visualize distinct state to make it clear.

Reply: Monocytes are classified as fully transmigrated when they are underneath the endothelial cells, and the black hole appears around the monocyte nucleus. Only the monocytes that display both these criteria qualify as being transmigrated in this study. In addition, this transition only occurs with migration between endothelial cell-cell junctions. As suggested by the referee we have included representative images of orthogonal views presenting non-transmigrated and transmigrated version of the same monocyte (figure 4B and figure 5E).

-Angiogenic factor driven inflammation
Do the authors check for potential effects of antibody labeling on monocyte transmigration? In other words, did the authors perform control experiments with unlabeled cells and analyzed transmigration rate at the end of the experiment? The authors should discuss this.

Reply: In our recent study (Sidibé et al Nat Comm 2018), we have shown that monocyte labeling with anti-CD16 for example did not affect the recruitment capacity of the monocytes. However, for new leukocyte subtypes or new antibody it is important to assess any effect of the labeling. This is now discussed in the manuscript, as suggested by the referee.

-Figure 4:
To demonstrate that the assay is robust, and results obtained are reproducible, the authors need to include biological replicates (n=3) and perform statistics. (Figure 4C/D) please include results obtained at timepoint 25 min. Error bars equal what, SD or SEM?

Reply: Leukocyte recruitment under flow is a robust assay that is clearly established and has been shown to generate reproducible results published in several papers, from our lab and others. In this manuscript, we present an extension of the classical leukocyte recruitment assay under flow by replacing the phase-contrast microscopy with confocal microscopy with timelapse imaging. Our results are absolutely consistent with what has been observed in phase contrast microscopy. Thus the method is robust, and constitutes an essential improvement of the classical methods based on the phase-contrast microscopy. However as suggested by the

referee, we have now included biological replicates of recruitment under flow by the confocal microscopy. The error bars are SD and are indicated on the revised figures.

-Figure 5C (10 min):
A monocyte nucleus of the same cell is visible in the apical and basal layer at the same time. Is this a cell which is right in the process of transmigration or is it just an "overspill" of the fluorescence signal due to the limitation in z-stack resolution? Deconvolution of the confocal videos should increase spatial resolution in z and therefore help to separate different cells in distinct steps within the process of transmigration more precisely. The authors should comment on this.

Reply: We agree with the referee. This was due to the laser intensity and the pinhole size which led to an overspill of the fluorescence signals. We agree that a deconvolution of the pictures might be helpful to improve the quality of the images. This is now discussed in the manuscript.

-Movies:
Movies are running too fast. Please reduce the frame rate.

Reply: As suggested by the referee, we have now reduced the frame rate to allow easier visualization of the recruitment events over each timeframe.

Reviewer #5:
Manuscript Summary:
The manuscript 'Simultaneous study of the recruitment of monocyte subpopulations under flow in vitro', by Ropraz et.al, presents a method for imaging the dynamics of leukocyte transmigration across the endothelium. This is no doubt an important topic for understanding the inflammatory process and diverse inflammatory pathologies. Here the authors describe preparation of a primary human endothelial cell monolayer, isolation of primary human monocytes and methods for combining the two under physiologic shear flow and imaging the subsequent monocyte adhesion and transmigration. The method is focus on simultaneously imaging of monocytes subsets by labeling them either with fluorescent anti-CD14 or anti-CD16 together with a nuclear stain while the endothelial cells are labeled with a cytoplasmic dye (Cell Tracker CMFDA). The presence of displaced Cell Tracker CMFDA on endothelium co-localized with monocyte nuclei imaged by confocal z-stacks allows for determination of adherent, transmigration and transmigrated monocytes, where as the presence or absence of either CD14 or CD16 fluorescent signal around each nucleus allows for the simultaneous imaging of either the CD14+ and CD14- or the CD16+ and CD16- sub-populations. In this way the investigators examine the differential responses of monocyte subsets while share an identical experimental micro-environments, with emphasis on effects of inflammatory cytokines versus VEGF. The method is generally well described and has some interesting and useful features.

Reply: We thank the referee for this fair description of our work and the comments.

Major Concerns:
None.
Minor Concerns:
General Comments:
1) By focusing on advantages of the current fluorescence imaging methods compared to phase-contrast methods, the discussion gives the false impression that this method is the first to apply fluorescence and confocal imaging to study transmigration dynamics. In fact, many investigator have done so using a range a fluorescent markers and methods over the past ~10 years. A statement of this nature and indicating some of the advantages of the current approach to previous fluorescence methods would be more balanced.

Reply: We agree with the referee that fluorescence, as well as confocal microscopy, were used previously in studies of leukocyte recruitment. However, the modality of use is novel, as our technique allows the investigation of different subpopulations of leukocytes and their migratory behavior. As suggested by the

referee, we have now added a statement to balance and discuss the novelty we have brought to the manuscript.

2) The discussion notes that one of the advantages of the method is that allows for automated analysis. Was automated analysis in fact described in this method?

Reply: We have pointed out the possibility to automate the analysis of leukocyte transmigration using our method. This statement is based on the fact that the black hole surrounding the nucleus of a transmigrated leukocyte can be easily recognized by image analysis software. However, we did not use the automated analysis here though we are developing one in our laboratory for such analysis. We manually counted adherent cells as well as transmigrated ones to calculate the transmigration rate. This is now described in the methods.

3) Shape change as an indicator of endothelial activation, should be presented with the caveat that while our experience has shown this generally to be true for HUVEC (macrovascular endothelial cells), microvascular endothelial cells (that more appropriately model the settings of leukocyte trafficking) do not typically show major shape change following activation.

Reply: We thank the referee for this comment. We included this precise detail in the discussion now.

Specific Comments:
1) The Method begins with description of HUVEC isolations. It seems to jump into instructions without appropriate background or orientation. It would seem worth mentioning that this was the original method for isolating primary endothelial cells, in part because umbilical vein is a readily available source of discarded human tissue. Moreover a large vessel umbilical vein offer potential to collect with many endothelial cells through a convenient methods that basically involves filling the lumen of this vessel with collagenase to elute the innermost layer of cells (i.e., the endothelial cells). Also, if you wish to describe this method here, a schematic should be included. Though in principle an easy method, to the uninitiated a visual aid would be very useful.

Reply: Previously, we wanted to focus on the leukocyte recruitment modalities. However, following the referee's suggestion, we have now included an introduction on HUVEC isolation. However as this isolation will not be part of the video, we did not provide a schematic. We thank the referee for the suggestion.

2) There are some easily fixed ambiguities. For example Section 1, step m. 'Discard the supernatant…' What supernatant? Since the supernatant in question was not from the proceeding step, it is much more clear to simple state: 'Discard the supernatant derived from step k, above'.
There is a similar issue is 2i. What pellet are we washing?

Reply: This has been rewritten to avoid ambiguity. Thus if the instruction is not for an element coming from the preceding step, we have been more precise, as suggested by the referee.

3) 4f. Plonge or plunge?

Reply: It is "plunge" instead. We thank the referee for all this careful reading and suggestions.