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TITLE:
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SHORT ABSTRACT:
The ability of inflamed endothelium to recruit leukocytes from flow is regulated by mesenchymal stromal cells. We describe two in vitro models incorporating primary human cells that can be used to assess neutrophil recruitment from flow and examine the role that mesenchymal stromal cells play in regulating this process.

LONG ABSTRACT:
Stromal cells regulate the recruitment of circulating leukocytes during inflammation through cross-talk with neighboring endothelial cells. Here we describe two *in vitro* "vascular" models for studying the recruitment of circulating neutrophils from flow by inflamed endothelial cells.

A major advantage of these models is the ability to analyze each step in the leukocyte adhesion cascade in order, as would occur *in vivo*. We also describe how both models can be adapted to study the role of stromal cells, in this case mesenchymal stem cells (MSC), in regulating leukocyte recruitment.

Primary endothelial cells were cultured alone or together with human MSC in direct contact on Ibidi microslides or on opposite sides of a Transwell filter for 24hr. Cultures were stimulated with tumor necrosis factor alpha (TNFα) for 4hr and incorporated into a flow-based adhesion assay. A bolus of neutrophils was perfused over the endothelium for 4min. The capture of flowing neutrophils and their interactions with the endothelium was visualized by phase-contrast microscopy.

In both models, cytokine-stimulation increased endothelial recruitment of flowing neutrophils in a dose-dependent manner. Analysis of the behavior of recruited neutrophils showed a dose-dependent decrease in rolling and a dose-dependent increase in transmigration through the endothelium. In co-culture, MSC suppressed neutrophil adhesion to TNFα-stimulated endothelium.

Our flow based-adhesion models mimic the initial phases of leukocyte recruitment from the circulation. In addition to leukocytes, they can be used to examine the recruitment of other cell types, such as therapeutically administered MSC or circulating tumor cells. Our multi-layered co-culture models have shown that MSC communicate with endothelium to modify their response to pro-inflammatory cytokines, altering the recruitment of neutrophils. Further research using such models is required to fully understand how stromal cells from different tissues and conditions (inflammatory disorders or cancer) influence the recruitment of leukocytes during inflammation.

INTRODUCTION:

Inflammation is a protective response to microbial infection or tissue injury that requires tight regulation of leukocyte entry into and exit from the inflamed tissue to allow resolution[1,2]. Cross-talk between endothelial cells (EC) that line blood vessels, circulating leukocytes and tissue-resident stromal cells is essential for coordinating this process[3]. However, uncontrolled recruitment of leukocytes and their ineffective clearance underpin the development of chronic inflammatory diseases[4]. Our current understanding of leukocyte recruitment in health and disease is incomplete and more robust models are needed to analyze this process.

The mechanisms supporting the recruitment of leukocytes from blood through vascular EC in post-capillary venules have been well described[1,2,5]. Circulating leukocytes are captured by specialized receptors (e.g., VCAM-1, E-selectin, P-selectin) which are up-regulated on inflamed endothelium. These transient interactions allow leukocytes to interact with surface bound chemokines and lipid-derived mediators (either endothelial or stromal in origin) that activate integrins expressed by leukocytes[6–11]. This in turn stabilizes adhesion and drives migration across the endothelium and into the tissue[12–15]. Within tissue, recruited leukocytes are subjected to stromal-derived agents that influence their motility, function and survival[16,17].

Growing evidence strongly suggests that signals received at each stage of the recruitment process conditions leukocytes for the next. However, our understanding of leukocyte recruitment remains incomplete and very little is known about the components shaping leukocyte movement within tissue.

In Birmingham we have developed several *in vitro* "vascular" models to study the recruitment of leukocytes from flow[9,18,19]. We now understand that vascular EC act as immediate regulators of leukocyte recruitment responding to changes in their local microenvironment. Specifically, tissue-resident stromal cells can actively regulate the inflammatory response, in part by conversing with neighboring vascular EC to influence their role in recruitment[3]. We have previously shown that various stromal cells modulate the ability of EC to support adhesion and migration of leukocytes in a tissue-specific manner, and that these effects become altered in chronic diseases[13,16,20,21]. Thus, stromal cells establish tissue 'address-codes' that define the context of each inflammatory response[22]. More recently, we have demonstrated that bone-marrow derived MSC (BMMSC) potently down-regulate the response of EC to cytokines, leading to a reduction in the recruitment of both neutrophils and lymphocytes[23].

The mechanisms governing recruitment elucidated *in vitro* have largely used assays incorporating a single cell type (e.g. EC) or protein in isolation. However, these studies do not take into consideration the effects of the local tissue environment (i.e. the presence of stromal cells) on recruitment of leukocytes and their subsequent migration into the tissue. Here we describe two flow-based methods in which stromal cells, specifically mesenchymal stem cells (MSC), are co-cultured with EC[23]. Such models allow us to examine the effect of stromal cell on endothelial responses, in particular their ability to support leukocyte recruitment from flow.

PROTOCOL:

1. Isolation and culture of primary human endothelial cells and mesenchymal stem cells

1.1. *Isolation and culture of human umbilical vein endothelial cells (HUVEC)*

1.1.1. Put umbilical cord on a tray with paper towels and spray with 70% ethanol. Place in a tissue culture hood.Identify the vein and cannulate at both ends.Place a cable tie around the cannulated end to secure it.

1.1.2. Wash out venous blood with PBS using a syringe. Fill syringe with air and pass through the vein to remove and discard the residual PBS.

1.1.3. Thaw 10mg/ml collagenase type Ia and dilute 1:10 in PBS (with calcium and magnesium chloride) to a final concentration of 1mg/ml.Pass collagenase solution into the vein until both cannulae are filled. Close the clamps on cannulae at both ends.

1.1.4. Cover the tray with tissue and spray with 70% ethanol. Place the cord into an incubator for 20min at 37^{o}C and 5% CO_2.

1.1.5. Take the cord out of the incubator and tighten cable ties. Massage the cord gently for 1min. Flush out the cell suspension with 10ml PBS and collect in a 50ml centrifuge tube.

1.1.6. Fill syringe with air and pass through the vein to remove any residual PBS twice, collecting the PBS in a 50ml centrifuge tube used in 1.1.5. Centrifuge at 400x g for 5min at room temperature.

1.1.7. Aspirate supernatant and resuspend pellet in 1ml complete EC medium. EC medium consists of M199 supplemented with 35µg/ml gentamicin sulphate, 10ng/ml human epidermal growth factor, 1µg/ml hydrocortisone, 2.5µg/ml amphotericin B and 20% fetal calf serum.

1.1.8. Add 4ml of EC medium and the EC suspension to a 25cm^2 flask. Change the medium on the following day and then every 2 days.

1.1.9. EC exhibit a cobblestone-like morphology (**Figure 1Ai**). For adhesion assays, EC are generally seeded when they reach 100% confluence (see Section 1.4.).

1.2. *Isolation of wharton's jelly mesenchymal stem cells (WJMSC) from human umbilical cords*

1.2.1. Isolate Wharton's jelly-derived MSC (WJMSC) from fresh umbilical cords or from cords that have already been used to isolate EC. Cut umbilical cord into 5cm long pieces. Cut each piece longitudinally to reveal the blood vessels (2 arteries [white, rigid] and 1 vein [yellow, distended]).

1.2.2. Using sterile scissors and forceps remove the blood vessels and discard. Cut all the tissue into 2-3mm^3 pieces. Using forceps place 2-3mm^3 pieces into a 50ml centrifuge tube.

1.2.3. Thaw 100mg/ml stock collagenase type II and dilute 1:10 in 10ml PBS to a final concentration of 1mg/ml. Thaw 20,000U/ml stock hyaluronidase and dilute 1:400 to a final concentration of 50U/ml in the collagenase solution.

1.2.4. Add enzymatic cocktail to the centrifuge tube containing the tissue fragments. Incubate the tissue fragments for 5hr at 37°C on a slow rotator.

1.2.5. Dilute the cell suspension 1:5 in PBS. Place a 100µm pore filter into a new 50ml centrifuge tube. Pour the cell suspension onto the 100µm pore filter.

NOTE: Remaining tissue fragments will be retained on the filter and cells will be collected in the 50ml centrifuge tube.

1.2.6. Discard the filter. Centrifuge the cell suspension at 400 x g for 10min at room temperature. Aspirate supernatant and resuspend WJMSC pellet in 12ml complete WJMSC culture medium (DMEM Low Glucose, 10% FCS and 100U/ml penicillin/100µg/ml streptomycin

mix).

1.2.7. Seed all cells in a 75cm^2 tissue culture flask. Change the medium after 24hr with 12ml complete WJMSC culture medium. Replace medium every 2-3 days. Cells should reach 70-80% confluence within 2 weeks. Passage when WJMSC reach 70-80% confluence (see Section 1.4.).

1.3. ***Expansion of bone marrow-derived MSC (BMMSC)***

1.3.1. Isolate human bone marrow-derived MSC (BMMSC) as previously described[24]. Add 10ml pre-warmed MSC growth medium (MSCGM) into a 15ml centrifuge tube.

1.3.2. Thaw a vial of p2 BMMSC by placing in a 37°C water bath for 2min. Add the BMMSC suspension to the centrifuge tube containing MSCGM. Mix well by pipetting.

1.3.3. Centrifuge at 400 x g for 5min at room temperature. Aspirate supernatant completely.

1.3.4. Resuspend cells in 1ml MSCGM and count the cells using a haemocytometer or a digital cell counter such as a cellometer.

1.3.5. Seed cells into 75cm^2 culture flasks at a density of 5,000-6,000 cells per cm^2 in 12ml MSCGM. Change the medium after 24hr with 12ml MSCGM. Feed cells with 12ml MSCGM every 2-3 days.

1.3.6. Passage when BMMSC reach 70-80% confluence (see Section 1.4.). Cells exhibit a fibroblastic morphology (**Figure 1Aii**).

1.4. ***Detachment of EC and MSC***

1.4.1. Aspirate medium from 25cm^2 culture flasks. Add 2ml of 0.02% EDTA for approximately 2min. Aspirate EDTA and add 2ml trypsin (2.5mg/ml). View under the microscope until the cells become round.

1.4.2. Tap the flask to detach the cells. Inactivate the trypsin by adding 8ml culture medium (dependent on cell type; EC medium for HUVEC, DMEM LG for WJMSC and MSCGM for BMMSC) to the culture flask and transfer suspension to a 15ml centrifuge tube.

1.4.3. Centrifuge at 400 x g for 5min at room temperature. Aspirate supernatant and resuspend the pellet as described below.

1.4.3.1. For passaging of MSC, resuspend pellet in 3ml culture medium. Add 11ml culture medium into three separate 75cm^2 culture flasks. Add 1ml cell suspension to each culture flask (1:3 split). Passage WJMSC and BMMSC 3 times (p3) before use in co-culture assays.

1.4.4. For seeding EC or MSC in adhesion assays – see Sections 2 and 3.

1.5. ***Freezing MSC***

1.5.1. At passage 3 detach MSC as described in Section 1.4. Aspirate supernatant and resuspend in 3ml ice-cold CryoSFM. Pipette 1ml aliquots of cell suspension into 1.5ml ice-cold cryovials. Put cryovials in a freezing container.

1.5.2. Store the container at -80°C overnight. Transfer to liquid nitrogen. Thaw vial of MSC (follow steps 1.3.1-1.3.6. in Section 1.3.). Resuspend MSC in 5ml culture medium (choose appropriate medium for WJMSC or BMMSC) and seed into a $25cm^2$ flask.

2. Establishing endothelial-mesenchymal stem cell co-cultures on Ibidi microslides

2.1. Trypsinise a confluent $25cm^2$ flask of EC (~$1.5x10^6$ cells; as Section 1.4.). Resuspend EC in 380µl MSCGM (1x$25cm^2$ flask will seed two 6-channel Ibidi microslides (~$1.25x10^5$/channel), for adhesion assays all cell types are cultured in MSCGM). Add 30µl of EC suspension to each channel (this will cover the growth area through capillary action). Incubate Ibidi microslide at 37^oC and 5% CO_2 for 1h.

2.2. Add 140µl MSCGM to each channel and then aspirate it off. Repeat twice for a total of three washes. Add 140µl MSCGM and place in the incubator at 37^oC and 5% CO_2 for 24hr.

2.3. For co-cultures detach MSC (Section 1.4.). Perform a cell count using a haemocytometer or a cellometer. Adjust the concentration of MSC to $1.5x10^5$ cells/ml.

2.4. Aspirate excess medium from the Ibidi channels (leaving only the growth area of the channel in medium).Add 30µl of MSC suspension to the Ibidi channels. Aspirate the medium that was ejected from the growth area of the channel and add another 30µl of MSC suspension. Repeat once more and then place the Ibidi microslide in the incubator at 37^oC and 5% CO_2 for 1h.

2.5. Add 140µl MSCGM to each channel and then aspirate it off. Repeat twice for a total of three washes. Add a final volume of 140µl MSCGM to each channel and place in the incubator at 37^oC and 5% CO_2 for 24h.

2.6. Thaw $1x10^5$U/ml stock TNFα and dilute 1:1000 in MSCGM to a final concentration of 100U/ml (equivalent to ~10ng/ml). Perform a serial dilution by diluting 100U/ml TNFα by 1:10 in MSCGM to obtain 10U/ml. Dilute 10U/ml TNFα by 1:10 in MSCGM to obtain 1U/ml.

2.7. Treat channels with TNFα at 37°C for 4hr prior to the assay. Temperature fluctuations during the cytokine treatment will alter the patterns of neutrophil recruitment observed. Add fresh MSCGM to untreated channels.

3. Establishing endothelial-mesenchymal stem cell co-cultures on filters

3.1. Detach WJ or BMMSC as described in Section 1.4. Resuspend the pellet in 1ml MSCGM. Perform a cell count cells using a haemocytometer or a cellometer.

3.2. Adjust volume so that the final concentration is $5x10^5$ MSC in 500µl of MSCGM.

3.3. Using sterile forceps invert 6-well, 0.4µm PET Transwell filters and place in a sterile box.

3.4. Seed $5x10^5$ MSC onto the outer surface of the filters. Incubate filters at 37°C and 5% CO_2 for 1h.

3.5. Collect media from the outer surface of the filter. Count the number of non-adherent MSC in the media. Re-invert filters using sterile forceps and place in a matching 6-well plate containing 3ml MSCGM.

3.6. Add 2ml of MSCGM onto the inner surface of the filter (**Figure 1B**). Place in an incubator at 37°C and 5% CO_2 for 24hr. Trypsinise a confluent $25cm^2$ flask of EC (**Figure 1Ai**; as Section 1.4.).

3.7. Resuspend EC in 8ml MSCGM (1x$25cm^2$ flask will seed four 6-well filters; ~$5x10^5$ EC/filter). Aspirate medium from the top and bottom of the porous filters. Add 3ml fresh MSCGM into the lower chamber (underneath the filter). Add 2ml EC suspension to the inner surface of each filter. Incubate for 1hr at 37°C and 5% CO_2.

3.8. Aspirate medium to wash off non-adherent EC and replace with fresh MSCGM. Set up parallel EC mono-culture filters by seeding cells on the inner surface without first seeding MSC. Incubate overnight at 37°C and 5% CO_2.

3.9. Check that the EC monolayer is confluent and contains no gaps. Sub-confluent monolayers cannot be used for adhesion assays (**Figure 1B**).

3.10. Treat the upper and lower chambers of the filters with 1, 10 or 100U/ml TNFα (equivalent to ~10ng/ml) at 37°C for 4hr prior to the assay (described in Section 2).

4. Isolation of leukocytes

4.1. Take venous blood from healthy volunteers and aliquot immediately into EDTA tubes. Invert tubes gently to mix.

4.2. Layer 2.5ml Histopaque 1077 onto 2.5ml Histopaque 1119 in a 10ml round bottomed tube. Layer 5ml whole blood onto the Histopaque gradient. Centrifuge at 800 x g for 40min at room temperature.

4.3. Harvest peripheral polymorphonuclear neutrophils (PMN) at the interface of Histopaque 1077 and 1119 (above the erythrocyte layer). Place in a 10ml round bottomed tube and make up to 10ml with PBSA. Gently invert tube and centrifuge at 400g for 5min at room temperature.

4.4. Dilute 7.5% BSA solution 1:50 in 100ml PBS (with Calcium and Magnesium Chloride) to a final concentration of 0.15% (w/v; PBSA). Aspirate supernatant and resuspend in 10ml PBSA. Centrifuge at 400 x g for 5min at room temperature.

4.5. Resuspend in 1ml PBSA. Take a 20µl aliquot of the cell suspension and add to 380µl PBSA (1:20 dilution). Count cells using a haemocytometer or a cellometer. Dilute to the required concentration ($2x10^6$/ml for Transwell filters and $1x10^6$/ml for Ibidi microslide) in PBSA. Maintain neutrophil suspension at room temperature until the assay.

5. Assembling the flow system

5.1. Set up the flow system as shown in **Figure 1C**. Turn on the heater and set to 37^{o}C. Attach a 20ml syringe (remove the plunger) and a 5ml syringe to a 3-way tap. Attach the tap to the Perspex chamber using Micropore tape.

5.2. Measure and cut a long piece of silicon 2/4mm (thick) tubing that is approximately the distance between the valve and the 3 way tap. Cut an 8-10mm long piece of 1/3mm (thin) tubing and insert into one end of the thick tubing. Attach the thick tubing side onto the 3-way tap.

5.3. Connect the thin tubing end onto a port of the electronic 3-way microvalve. This is the "wash reservoir". Cut a 6-8mm long piece of thick and thin tubing.

5.4. Insert the thin tubing into one end of the thick tubing. Attach the thick tubing end onto a 2ml syringe (remove the plunger).

5.5. Connect the thin tubing end of the 2ml syringe onto a port on the electronic microvalve to make the "sample reservoir". Connect the valve to the filter flow chamber by measuring and cutting a long piece of thin tubing that is the distance between the microvalve and the centre of the microscope stage.

5.6. For the microslide model, attach an 8-10mm piece of thick tubing to the end of the thin tubing. Place an L shaped connector to the end of the thick tubing. This will connect to the microslide. For the filter model, attach an 8-10mm piece of Portex Blue Line Manometer connecting tubing to the end of the thin tubing.

5.7. Place the thin tubing end onto the microvalve. This is the common output for the wash and sample reservoirs. Fill reservoirs with PBSA. Prime tubing by flowing PBSA through them to remove any air bubbles.

5.8. Attach Manometer tubing to a 29mm (50ml) glass syringe. Prime the syringe by filling with 10ml PBSA. Invert the syringe so that the end connected to the tubing faces upwards and push out all air bubbles. Refill with 5ml PBSA.

5.9. For the microslide model only, attach a 10-12mm piece of thick tubing to the end of the Manometer tubing leading to the glass syringe. Place an L shaped connector onto the end of the thick tubing. Place the glass syringe into a syringe pump for infusion/withdrawal.

5.10. Calculate the refill flow rate (Q) required to generate the desired wall shear stress (τ_w in Pascal, Pa) of 0.1Pa (Transwell filters) or 0.05Pa (Ibidi microslides) using the following formulae:

$\gamma_w = (6.Q)/(w.h^2)$
$\tau = n.\gamma$

Where w = internal width and h= internal depth of the flow channel. n = viscosity (n) of the flowing solution, PBSA is n=0.7mPa.s. For the parallel plate filter flow chamber, the width (w) is 4mm and the depth (h) is 0.133mm. The depth of the chamber can vary slightly due to differences in the thickness of the parafilm gasket used. For the Ibidi microslide, the width is 3.8mm and the depth is 0.4mm.

NOTE: Due to differences in the dimensions of the flow channel, and capture dynamics we use different shear stresses for the microslide model compared to the filter model[6].

6. Setting up the parallel plate flow chamber incorporating filters

6.1. Cut a piece of parafilm (the same size as the glass coverslip) using a metal template. Cut out a 20x4mm slot in the parafilm (to create the flow channel) using a metal template. Use the gasket to mark the flow channel on the glass coverslip.

6.2. Align the edges of the 6-well filter on the glass coverslip. Ensure that the filter covers the flow channel markings. Carefully cut out the filter using a type 10A scalpel.

6.3. Carefully cover the filter with a parafilm gasket, ensuring that the flow channel slot is in the middle of the filter (**Figure 1D**). Use a piece of clean tissue and push out any bubbles.

6.4. Place the coverslip in the recess of the bottom plate of the Perspex flow chamber. Position the top Perspex plate over the top of the gasket and screw the plates together (**Figure 1D**).

6.5. Turn the 3-way tap to allow wash buffer (PBSA) to flow through the valve. Connect Manometer tubing to the inlet port of the top Persex plate. Run PBSA through the flow channel to allow bubbles to pass through.

6.6. Connect the Manometer tubing from the syringe pump into the outlet port of the Perspex plate. Set the syringe pump to refill and press run. Clean any PBSA that has dripped onto the upper plate of the chamber.

6.7. Place the chamber on the stage of an invert phase-contrast microscope. Adjust focus to

visualize the EC above the filters (**Figure 1B**).

7. Setting up microslides for flow

7.1. Place the microslide onto the stage of an invert phase contrast microscope. Connect the L shaped connector into the inlet port of a channel. Run PBSA through the flow channel.

7.2. Place the L shaped connector from the syringe pump into the outlet port of the channel. Set the syringe pump to refill and press run. Clean any PBSA that has dripped onto the microslide. Adjust focus to visualize the EC monolayer.

8. Perfusion of leukocytes over endothelial cells.

8.1. Put 2ml of purified neutrophils into the sample reservoir and leave to warm for 2min. Wash the endothelium with PBSA for 2min.

8.2. Turn the valve ON to perfuse neutrophils over endothelium. Deliver the neutrophil bolus for 4min. Turn the valve OFF and perfuse PBSA from the wash reservoir for the remainder of the experiment.

8.3. Ensure that air bubbles do not pass through the flow channel at any point during the assay as this will disrupt the EC monolayer and cause detachment of adherent neutrophils.

9. Recording neutrophil capture and behavior

9.1. Record Neutrophil recruitment either during neutrophil flow or post-perfusion.

9.2. Make all digital recordings of at least 5-10 fields in the center of the flow channel. Identify the center of the channel by moving the objective to the edge of the channel at the inlet port and identifying the middle of the port.

9.2.1. For recording during neutrophil flow, take images of a single field every 10sec for 1min. Move along the channel and record another field for 1min. Repeat for duration of bolus.

9.2.2. For recording post-perfusion, make 10sec recording of 5-10 fields down the centre of the flow channel for assessing leukocyte behavior (typically 2min after the end of the neutrophil bolus). Take images every second within the 10sec interval. This allows sufficient time for capture from flow and the behavior of adherent neutrophils to be analyzed.

9.2.3. Record a single field containing at least 10 transmigrated neutrophils for 5min, taking images every 30sec. This can be used to calculate the velocity of migrated cells (either above or underneath the endothelium).

9.2.4. Record another series of 10sec fields (typically 9min post-perfusion). This allows

neutrophils time to migrate through the endothelial monolayer.

9.2.5. Stop the syringe pump and remove the tubing. Disassemble the flow chamber and rinse the sample reservoir and tubing. Repeat for subsequent filters/microslide channels.

10. Analysis of leukocyte recruitment and behavior

10.1. Count the number of neutrophils in each field during the 10 second recordings at the 2min time point. All cells must be present throughout the 10 second recording to be counted; as such cells that enter or leave part way through (e.g. at 9sec) are not included. Cells that are partially in the field on 2 sides (e.g. top/right hand border) of the field of view are included in the counts, as long as they remain in the field for the full 10 seconds.

10.2. Calculate the mean number of adherent neutrophil per field. Measure the length and width of the recorded field. Calculate the area of the field. Calculate how many fields there are in $1mm^2$.Multiply the mean neutrophil count by the number of fields in $1mm^2$.

10.3. Calculate the total number of neutrophils perfused by multiplying the amount of neutrophils perfused (e.g. $2x10^6$/ml*Q [e.g.0.0999ml/min for parallel plate flow chamber]) by the duration of the bolus (e.g. 4min).

10.4. Divide the neutrophil count/mm^2 by the total number of neutrophils perfused to determine the total number of cells that have adhered (adherent cells/mm^2/ 10^6 perfused).

10.5. Assess whether the adherent neutrophils are rolling, firmly adherent or transmigrated (**Supplementary Video 1 and 2**).

10.5.1. A rolling neutrophil is phase bright and will slowly move along the endothelial monolayer (1-10μm/s; **Supplementary Video 1**).

10.5.2. Firmly adherent cells are phase bright and bound to the EC surface, either remaining stationary (i.e. not moving during recording) or have undergone shape change and are migrating over the EC surface (**Figure 1Ei**).

10.5.3. A transmigrated neutrophil is phase dark and below the EC layer (**Figure 1Eii**).

10.6. Calculate the percentage of adherent cells that are rolling, stationary and transmigrated. Alternatively, neutrophil behavior can be expressed as total cell numbers that exhibit the different behaviors by applying the same formula used to calculate total adhesion (as described in 10.6-10.7).

10.6.1. Mark the leading edge of a rolling neutrophil. Mark the leading edge of the same cell at the end of the 10sec sequence. Draw a line between the two points and measure the distance that the cell has travelled.

10.6.2. Divide this value by the duration of the recording in which the cell is rolling (i.e. 10sec). Try and select neutrophils that are in the field for the entire 10sec interval.

10.7. To calculate the velocity of neutrophils migrating over the surface (shaped changed phase bright) or underneath the endothelium (phase dark) use the 5min recording (**Supplementary Video 2**).

10.7.1. Draw an outline of migrated cells at the beginning of the sequence and track their movements throughout the sequence. Make note of the X and Y positions of the centroid at each 30sec interval for each cell. Subtract values of the X and Y position from the first image of the sequence from the values in the second image. This is based on Pythagoras' theorem.

10.7.2. Subtract the values from the second image from the third image. Do this for all images in the sequence. Square the X and Y values and add them together.

10.7.3. Square root the resulting value. Calculate the velocity for each cell by averaging the velocities calculated at each minute interval. Track 10 migrated neutrophils and calculate the mean velocity.

REPRESENTATIVE RESULTS:

Initially, we analyzed the effect of stimulating EC with TNFα on the recruitment of neutrophils from flow using the Ibidi microslide model (Section 7-9). In the absence of TNFα, little if any neutrophils adhered to the endothelial monolayer (**Figure 2A**). This was expected, as untreated/resting EC do not express the necessary adhesion molecules (selectins) or chemokines to support binding[25,26]. In contrast, cytokine-stimulation significantly increased neutrophil adhesion to the endothelium in a dose-dependent manner (**Figure 2A**). Adhesion normally remains stable over the course of the assay. Binding of leukocytes to untreated endothelium indicates that either the EC are activated (i.e. contaminated with LPS during the culture process) and/or the neutrophils were activated during the isolation process. Indeed, LPS has been shown to increase the expression of E-selectin, ICAM-1 and VCAM-1[25-27] on the surface of EC, allowing them to bind neutrophils.

When analyzing the behavior of the recruited neutrophils we typically observe a dose-dependent decrease in the percentage of neutrophils rolling (**Figure 2B**) with a concomitant dose dependent increase in the percentage of neutrophils migrating through the endothelial monolayer (**Figure 2C**). At the lower doses of TNFα-stimulation (1U/ml) a larger proportion of neutrophils appear phase bright indicating that they are attached to the apical surface of the endothelium (**Figure 2B**). In contrast, at 10 and 100U/ml (higher doses) approximately 40% of the recruited neutrophils appear phase dark at 2min indicating that these cells have migrated through the endothelial monolayer and are underneath the endothelium (**Figure 2C**). Neutrophils are able to migrate through the EC within 1-2min, with transmigration reaching maximal levels at ~10min post-perfusion[28]. Here we observed an increase in neutrophil transmigration from 40% at 2min to 60% by 9min post perfusion (**Figure 2C**). We observed no effect of TNFα concentration on the velocity of rolling (~3μm/sec) or migrating (~10-12μm/min)

neutrophils.

In the filter based model, TNFα-stimulation increased neutrophil adhesion in a dose-dependent manner similar to that seen using the Ibidi microslide model (**Figure 3A**). In terms of behavior, neutrophil rolling was unaffected by TNFα dose (**Figure 3B**), whilst a dose-dependent increase in percentage transmigration was observed (**Figure 3C**). In this series of experiments we observed no significant effect of time on neutrophil transmigration (**Figure 3C**).

We provide methods on how to generate two different co-culture constructs, each of which is devised to answer specific questions. In the ibidi microslide model, EC and MSC are cultured in a single monolayer in direct contact with one another. This model is useful for examining the effect of therapeutic injection of MSC into the blood and their subsequent integration into the EC monolayer. In contrast in the filter-based model, EC and MSC are cultured on opposite sides of the filter in close proximity but not necessarily in direct contact. This more closely resembles tissue, with endothelial cells forming a monolayer representing the blood vessel, and MSC residing in the subendothelial compartment. This allows us to examine the effects of tissue-resident MSC on the response of EC to cytokine stimulation.

Based on our experiences, we observe that maximal neutrophil recruitment and transendothelial migration occurs when EC are stimulated with 100U/ml TNFα. As such, we have used this concentration to examine the effect of MSC co-culture on endothelial recruitment of neutrophils from flow. Here, we present data for BMMSC in co-culture with EC using the microslide and filter-based models however, other types of MSC can also be examined e.g. WJMSC. In both models the presence of BMMSC significantly reduced neutrophil adhesion to the EC when compared to EC cultured alone (**Figure 4A**). Co-culture had no effect on the behavior of recruited neutrophils, with similar levels of rolling and transmigration observed on EC cultured alone or with MSC (**Figure 4B and C**). Thus, MSC can modify the EC response to cytokine stimulation, which suppresses their ability to support neutrophil recruitment from flow.

FIGURE LEGENDS:

Figure 1: Establishing EC-MSC co-culture and analyzing neutrophil recruitment using a flow-based adhesion assay.

(A) Micrograph of **(i)** primary EC and **(ii)** passage 3 BMMSC grown on tissue culture flasks. **(B)** Micrograph of EC and MSC cultured on 6-well Transwell filter inserts. **(C)** Diagram of the perfusion system used to generate flow. **(D)** Schematic representation of the parallel plate filter flow chamber. **(E)** Micrograph of **(i)** firmly adherent (FA) and **(ii)** transmigrated (TM) neutrophils following recruitment from flow to EC stimulated with 100U/ml TNFα. *Images C and D are taken from Figures 2 and 3 in Methods in Molecular Biology: T-cell Trafficking, 2010, pages 53-4*[28] *with kind permission from Springer Science and Business Media.*

Figure 2: Neutrophil recruitment from flow to TNFα-stimulated EC using Ibidi microslides.

EC were stimulated with increasing concentrations of TNFα (0-100U/ml) for 4hr. A 4min bolus of neutrophils was perfused over the EC monolayer at 0.05Pa. **(A)** Neutrophil adhesion assessed

at 2min. ANOVA showed a significant effect of TNFα treatment on neutrophil adhesion, $p<0.01$. Neutrophil behavior was assessed at 2 and 9min and expressed as a percentage of adherent cells that were **(B)** rolling or **(C)** transmigrated. ANOVA showed a significant effect of TNFα treatment on the behavior of the adherent neutrophils, $p<0.001$. In C, ANOVA showed a significant of time on transmigrated neutrophils $p<0.01$. Data are mean ± SEM from n=3 experiments. *$p<0.05$ and **$p<0.01$ compared to the unstimulated (0U/ml) EC control by Dunnett post-test. $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ compared to the 1U/ml EC at the same time point by Bonferroni post-test.

Figure 3: Neutrophil recruitment from flow to TNFα-stimulated EC using filter-based assay.
EC were stimulated with increasing concentrations of TNFα (0-100U/ml) for 4hr. A 4min bolus of neutrophils was perfused over the EC monolayer at 0.1Pa. **(A)** Neutrophil adhesion assessed at 2min. ANOVA showed a significant effect of TNFα treatment on neutrophil adhesion, $p<0.001$. Neutrophil behavior was assessed at 2 and 9min and expressed as a percentage of adherent cells that were **(B)** rolling or **(C)** transmigrated. In C, ANOVA showed a significant effect of time and cytokine treatment on neutrophil transmigration, $p<0.05$. Data are mean ± SEM from n=3 experiments. **$p<0.01$ and ***$p<0.001$ compared to the unstimulated (0U/ml) EC control by Dunnett post-test. $^{\#}p<0.05$ compared to the 1U/ml EC at the same time point by Bonferroni post-test.

Figure 4: Neutrophil recruitment from flow to TNFα-stimulated EC-BMMSC co-cultures.
BMMSC were co-cultured with EC for 24hr prior to stimulation with 100U/ml TNFα for 4hr. A 4min bolus of neutrophils was perfused over the EC monolayer at 0.05Pa for **(A, C, E)** microslides and 0.1Pa for **(B, D, F)** filters. **(A-B)** Neutrophil adhesion was assessed at 2min. Neutrophil behavior was assessed at 2 and 9min and expressed as a percentage of adherent cells that were **(C-D)** rolling or **(E-F)** transmigrated. In C and D, ANOVA showed a significant effect of culture conditions on neutrophil rolling, $p<0.05$. In E and F, ANOVA showed a significant effect of time on neutrophil transmigration, $p<0.05$. However, no significant differences were observed in transmigration between individual treatments by Bonferroni post-test. Data are mean ± SEM from n=5 experiments. *$p<0.05$ compared to the EC monoculture by paired t-test or Bonferroni post-test.

Supplementary Video 1: Analysis of neutrophil rolling velocities.
EC cultured on a Transwell filter were stimulated with 100U/ml TNFα for 4hr. A bolus of neutrophils was perfused over the EC for 4min. Representative digitized sequence of a single 10sec field taken 2min post-perfusion. The change in position of a single rolling neutrophil from the beginning to the end of the 10sec sequence can be used to calculate the velocity at which the neutrophil is rolling.

Supplementary Video 2: Analysis of neutrophil migration velocities.
EC cultured on a Transwell filter were stimulated with 100U/ml TNFα for 4hr. A bolus of neutrophils was perfused over the EC for 4min. Representative digitized sequence of a single 5min field to track the movement of transmigrated neutrophils. This can be used to calculate the velocity.

DISCUSSION:

Here we describe two *in vitro* "vascular" models for studying the recruitment of circulating neutrophils by inflamed endothelium. A major advantage of these models is the ability to analyze each step in the leukocyte adhesion cascade in order, as would occur *in vivo*. We have previously observed a dose-dependent increase in neutrophil adhesion to and transmigration through TNFα-stimulated EC[9,29]. We also describe how both models can be adapted to study the effects of stromal cells on leukocyte recruitment. Here, MSC were co-cultured with EC in an Ibidi microslide or on opposite sides of a porous filter. This allows both cell types to communicate with one another, thereby modifying each other's phenotype and response. We have shown here, and in previous studies[23], that the presence of MSC suppressed neutrophil adhesion to TNFα-stimulated EC. This indicates that stromal cells modify the EC response to cytokines and subsequently alters the recruitment of circulating leukocytes.

In addition to the models described above, Cellix Biochips, Bioflux plates and Glyotech parallel plate flow chambers are commercially available flow channel systems that provide a surface for culturing endothelium and observing recruitment. In all systems, certain parameters should be considered whilst establishing endothelial cultures and performing the flow-based adhesion assays, some of which are highlighted below and in previous reports[30,31]. Any anti-inflammatory agents, such as hydrocortisone, that may affect the cytokine responses should be omitted from the medium for the duration of the culture and assay[18,29]. When using the Ibidi microslide ensure that there are no air bubbles present in the flow channel during the culture of the EC as these will disrupt the EC monolayer. The integrity of the endothelial monolayer should be confirmed prior to cytokine-treatment, as neutrophils will bind to the gaps in the monolayer where the BSA has coated the microslide/filter. It is also essential to ensure that EC are maintained at 37°C throughout the cytokine-stimulation because TNFα is temperature sensitive and only has the maximal effect at 37°C. For the flow assay itself, select an appropriate wash buffer, we typically use PBSA for short assays (less than 30mins) and M199 medium supplemented with BSA (0.15%) for longer assays (1-48h)[30,31]. Finally ensure that there are no air bubbles present in the flow channel during the assay as this disrupts the flow rate, damages the EC monolayer and activates adherent neutrophils.

One of the major advantages of the multi-cellular *in vitro* models described here is their ability to replicate the *in vivo* interactions between EC and stromal cells. It is difficult to isolate the effects of specific stromal components *in vivo* and to modify them in a controlled manner. In our models, stromal cells can be manipulated to elucidate how they communicate with EC and influence the inflammatory process in health and disease. For example, using siRNA technology we have previously shown that production of IL-6 by MSC during co-culture was necessary for their immunosuppressive effects[23]. Each model can be used to address specific questions i.e. the effect of tissue-resident stromal cells (filter-based model) or therapeutically administered stromal cells (Ibidi microslides and other commercially available systems) on leukocyte recruitment. In both cases we have titrated different stromal cell types to ensure their viability and to establish a suitable ratio of stromal cell to EC for assessing effects on recruitment[15;25]. Similarly culture medium must be compatible with each cell type incorporated into the model.

In our hands co-cultures are typically performed in the stromal cell medium[11,18,23,31].

Using the filter-based model, we have previously shown that various stromal cells modulate the ability of EC to support adhesion and migration of leukocytes in a tissue-specific manner and that these effects become altered in chronic diseases[3]. This led to the concept that stromal cells establish tissue-specific "address codes" which actively regulate the recruitment of leukocytes to inflamed tissue[24]. These models are specifically designed to examine the initial stages of recruitment in great detail but are unable to study the subsequent migration within the subendothelial space (i.e. away from the endothelium into the tissue). Multi-cellular, multilayered 3D constructs such as a static collagen gel assay[12,32] would be more appropriate for studying these latter phases of recruitment.

Our flow-based adhesion models are highly versatile. We have described their use in the context of neutrophil recruitment, but other leukocyte subsets can be investigated in a similar manner. We have also used the ibidi microslide system to investigate the recruitment of circulating MSC by EC[23], and whether this occurs through the same adhesion cascade reported for leukocytes. Likewise these models could be used to examine the recruitment and incorporation of metastatic tumor cell lines, and their subsequent effects on endothelial responses and leukocyte recruitment. Alternatively, the filter-based model could be adapted to incorporate different types of stromal cells (e.g. fibroblasts, podocytes, smooth muscle cells) from healthy tissues[13,21] and sites of disease[11,18,20]. This would enable the study of tissue-specific regulatory pathways acting at the level of on EC and/or leukocytes. In all cases the disruption of normal regulatory processes in a range of disease conditions can be examined to identify key regulatory mediators (such as IL-6 and TGFβ) and potential new therapeutic targets. In the context of chronic inflammation these agents might be used to switch off the recruitment process, whilst in cancer biology one could imagine their use to turn on recruitment to target the tumor.

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DISCLOSURES:
The authors declare that they have no conflicts of interest.

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