Video Article

Systematic Analysis of *In Vitro* Cell Rolling Using a Multi-well Plate Microfluidic System

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Abstract

A major challenge for cell-based therapy is the inability to systemically target a large quantity of viable cells with high efficiency to tissues of interest following intravenous or intraarterial infusion. Consequently, increasing cell homing is currently studied as a strategy to improve cell therapy. Cell rolling on the vascular endothelium is an important step in the process of cell homing and can be probed *in-vitro* using a parallel plate flow chamber (PPFC). However, this is an extremely tedious, low throughput assay, with poorly controlled flow conditions. Instead, we used a multi-well plate microfluidic system that enables study of cellular rolling properties in a higher throughput under precisely controlled, physiologically relevant shear flow[1,2]. In this paper, we show how the rolling properties of HL-60 (human promyelocytic leukemia) cells on P- and E-selectin-coated surfaces as well as on cell monolayer-coated surfaces can be readily examined. To better simulate inflammatory conditions, the microfluidic channel surface was coated with endothelial cells (ECs), which were then activated with tumor necrosis factor-α (TNF-α), significantly increasing interactions with HL-60 cells under dynamic conditions. The enhanced throughput and integrated multi-parameter software analysis platform, that permits rapid analysis of parameters such as rolling velocities and rolling path, are important advantages for assessing cell rolling properties *in-vitro*. Allowing rapid and accurate analysis of engineering approaches designed to impact cell rolling and homing, this platform may help advance exogenous cell-based therapy.

Video Link

The video component of this article can be found at http://www.jove.com/video/50866/

Introduction

One of the major challenges in the successful clinical translation of cell-based therapy is the inefficient delivery or targeting of systemically infused cells to desired sites[3,4]. Consequently, there is a constant search for approaches to improve cell homing, and specifically cell rolling, as a strategy to improve cell therapy. Cell rolling on blood vessels is a key step in the cell homing cascade, classically defined for leukocytes that are recruited to disease sites[5]. This step is governed by specific interactions between endothelial selectins, *i.e.* P-and E-selectin (P-and E-sel), and their counter ligands on the surface of leukocytes[5,6]. Better understanding and improved efficiency of cell homing, and specifically the rolling step, are of great importance in the quest for new platforms to improve cell-based therapy. To date this has been achieved by using parallel plate flow chambers (PPFCs), comprising two flat plates with a gasket between them, with an inflow and outflow port located on the upper plate, through which a cell suspension is perfused by using a syringe pump[7,8,9]. The surface of the bottom plate can be coated with a relevant cell monolayer/substrates and the interaction between perfused cells and the surface under shear flow is then explored[7]. However, PPFC is a low throughput, reagent-consuming, and fairly tedious method, with bubble formation, leakage, and poorly controlled flow presenting major drawbacks.

An alternative technique to the traditional PPFC is a multi-well plate microfluidic system, permitting higher throughput performance of cellular assays (up to 10 times higher than PPFCs) under accurate, computer-controlled shear flow, with low reagent consumption[1,10]. Cell rolling experiments are performed inside the microfluidic channels, which can be coated with cell monolayers or engineered substrates and imaged using a microscope, with rolling properties readily analyzed using a suitable software. In this study, we demonstrate the capabilities of this multi-well plate microfluidic system by studying the rolling properties of human promyelocytic leukemia (HL-60) cells on different surfaces. HL-60 rolling on substrates like P-and E-sel, as well as on cell monolayers expressing different rolling receptors, was analyzed. In addition, antibody (Ab) blocking was used to demonstrate direct involvement of specific selectins in mediating the rolling movement of HL-60 on those surfaces.

Rolling experiments were performed with increased throughput, under stable shear flow, with minimal reagent/cell consumption, allowing efficient analysis of key rolling parameters such as rolling velocity, number of rolling cells, and rolling path properties.

Protocol

1. Cell Culture

1. Human promyelocytic leukemia (HL-60) cells
    1. Culture HL-60 cells in 75 cm^2 flasks with 15 ml of Iscove's Modified Dulbecco's Medium (IMDM), supplemented with 20% (v/v) fetal bovine serum (FBS), 1% (v/v) L-Glutamine and 1% (v/v) Penicillin-Streptomycin.
    2. Change media every 3 days by aspirating half of the cell suspension volume and replacing it with complete IMDM media.
    3. For carboxyfluorescein diacetate, succinimidyl ester (CFSE) staining, centrifuge HL-60 cell suspension (400 x g, 5 min), resuspend in a 1 µM CFSE solution (prepared in prewarmed PBS) and incubate for 15 min at 37 °C. Then centrifuge cells, aspirate supernatant and resuspend cells in fresh prewarmed medium for 30 min. Wash cells in PBS and then use for rolling experiments (see **Figure 1B** for representative image of CFSE-stained HL-60 cells on P-sel-coated surface).

Note: CFSE staining is optional, and is presented here to demonstrate the rolling phenomenon in the microfluidic channel. Analysis of rolling parameters presented in this manuscript was performed on unstained cells using standard brightfield imaging.

2. Lung microvascular endothelial cells (LMVECs)
    1. Coat 100 mm Petri dishes with 0.1% gelatin solution (v/v in PBS) and incubate at 37 °C for at least 30 min.
    2. Culture LMVECs on gelatin-coated 100 mm Petri dishes in complete endothelial growth medium (endothelial basal medium-2 (EBM-2)), supplemented with a specific growth supplement kit, see REAGENTS). Change media every other day and sub-culture cells upon reaching 80-90% confluence.
    3. For sub-culture, wash cells with PBS and then detach cells with 4 ml of 1x Trypsin-EDTA for 3 min at 37 °C and neutralize in an equal volume of complete EBM-2 media. Transfer the cell suspension to a 15 ml tube and centrifuge (400 x g, 5 min). Following centrifugation, resuspend the pellet in 1 ml of complete endothelial media and count cells with a hemocytometer. Do not over-passage the cells, as this affects their morphology and function- use only cells under passage 7 for all experiments.

3. Chinese Hamster Ovary-P-selectin (CHO-P) Cells
    1. CHO-P cells, which are CHO cells stably transfected to express human P-sel, were provided by collaborators (Beth Israel Deaconess Medical Center, Harvard Medical School)[11,12].
    2. Culture CHO-P cells in T175 cm^2 flasks in 25 ml of F-12 media.
    3. For passaging, wash cells with 10 ml of PBS for 4-5 sec and then trypsinize in 10 ml of 1x Trypsin-EDTA for 3 min at 37 °C, followed by neutralization in full media.
    4. Centrifuge the cell suspension (400 x g, 5 min), carefully aspirate the supernatant, resuspend the cell pellet in 1 ml of full media and count the cells with a hemocytometer.

2. Operation of the Integrated Multi-well Plate Microfluidic System

1. Make sure all the equipment is properly connected and turn on the different modules: computer, controller, inverted microscope, and CCD camera.
2. Open the imaging software; make sure the multi-well plate module and the imaging module are properly presented on the screen.
3. Connect the tubes to the vapor trap (connected to the controller) and also connect them to the Pressure Interface.
4. Place the multi-well plate in the plate heater/adaptor. Add reagents to wells (described below) and attach interface on top of the plate. Place plate for imaging on automated stage.
5. The interface attaches to the top of the plate and applies a pneumatic pressure from the controller to the top of the wells, driving the fluid through the microfluidic channels at the defined flow rate, easily controlled using the multi-well plate module screen under Manual mode.
6. Reagents in the channel flow across an observation area, located between the wells. Microfluidic channel dimensions are 350 µm wide x 70 µm tall. The length of the linear channel is 1 mm and the bottom of the channels comprises a 180 µm coverslip glass, which is compatible with brightfield, phase, fluorescence and confocal microscopy.
7. Acquire videos using a CCD camera (stream acquisition, 11 frames/sec) and analyze via compatible software.

3. Coating of Microfluidic Channels with a Protein Substrate or a Cell Monolayer

1. Coating microfluidic channel with fibronectin or P-/E-selectin
    1. Prepare 1 ml of 20 µg/ml fibronectin solution in PBS. Alter volume based on the number of channels to be coated (use 25-50 µl of fibronectin per channel).
    2. Add 25-50 µl of fibronectin solution to each inlet well. Apply shear force of 2 dyn/cm^2 for 5 min to perfuse the channel. Please note the bead of liquid appearing in the outlet well. Incubate for 30-45 min at R.T.
    3. Aspirate the solution from wells (do not aspirate directly from the middle circle that feeds the channel)[1,13]. Add 200-500 µl of PBS into outlet well and wash channel with PBS by applying shear flow of 2 dyn/cm^2 for 5 min. The channel is now properly coated with fibronectin and ready to be used.

4. To coat with P- or E-sel, prepare a 5 µg/ml solution of the desired human recombinant protein in PBS, and coat the channels as described above, with 1 hr incubation at 37 °C to allow surface coating.

2. Creation of CHO-P or LMVEC monolayer inside the microfluidic channel
    1. Gently trypsinize cells from culture dishes for 3 min, quench using a 2-fold volume of full media and centrifuge (5 min at 400 x g). Resuspend cells with 10 ml of full media and centrifuge (5 min at 400 x g) again.
    2. Count the cells to determine cell concentration in the suspension. To ensure the formation of a confluent LMVEC monolayer inside the channel, bring cell concentration to 15-20 million cells/ml. For a confluent CHO-P cell monolayer, use 50-60 million cells/ml. Use 25-50 µl of cell suspension for each channel - determine initial cell number used for the experiment accordingly.
    3. Add 25-50 µl of cell suspension in the appropriate concentration to the inlet well. Place the plate on the microscope stage and introduce cells into the channel (2 dyn/cm^2) until cells are observed on the screen filling the entire channels, and then stop the flow.
    4. Fill both outlet and inlet with 200 µl of either full LMVEC or CHO media. Let the cells settle and adhere for 3 hr in the incubator (37 °C, 5% CO_2).
    5. Following the 3 hr incubation, wash the channel with full media (2 dyn/cm^2, 10-15 min) to remove unattached cells. Cells should now appear completely confluent and the channel is now ready for use. Depending on initial cell seeding density, additional 2-3 hr of settling time may be required to ensure complete coverage of the surface with the cells.

4. LMVEC Pro-inflammatory Activation and Antibody Blocking of P-/E-selectin

1. Prepare a TNF-α solution (10 ng/ml) in LMVEC basal media.
2. To induce inflammatory activation of LMVEC in the channels, add 100 µl of the TNF-α solution to the inlet well and introduce the solution into the channel by applying shear flow of 2 dyn/cm^2 for 5 min. For control channels (nonactivated ECs), add 100 µl of LMVEC basal media to the inlet well and introduce into the channel (2 dyn/cm^2 for 5 min). Channel is now ready for a rolling assay.
3. To block P-sel and E-sel on LMVECs and CHO-P cells, introduce neutralizing P-sel (clone AK4, 5 µg/ml in basal media) or E-sel (clone P2H3, 5 µg/ml in basal media) antibodies into the channel and incubate for 1 hr at 37 °C. Next, wash channels with basal media (2 dyn/cm^2 for 5 min). Channels are now ready for a rolling assay.

5. HL-60 Rolling Assay on Substrate/Cell Monolayer-Coated Microfluidic Channels

1. Carefully examine the channels under the microscope to confirm that channels are properly coated (in the case of coating with cells, a fully confluent cell monolayer should be observed).
2. To prepare HL-60 cell suspension for the rolling experiments, centrifuge HL-60 cell suspension (5 min at 400 x g) and wash once with basal media. Count the cells and resuspend in IMDM (basal media, containing Ca^{2+} and Mg^{2+}) to create a HL-60 cell suspension with 5 million cells/ml. Use 25-50 µl of cell suspension for each channel to perform the rolling assay.
3. Add 25-50 µl of the cell suspension to outlet well, place plate inside the temperature-controlled plate holder (37 °C) and place on the microscope stage. Next, introduce cells into the channel by applying shear force of 2 dyn/cm^2 (cells should be observed within 10-15 sec flowing from outlet to inlet).
4. To examine the rolling response as a function of shear stress, reduce shear to 0.25 dyn/cm^2 and acquire 20-30 sec videos (using "stream acquisition" function) in each desired shear (increase shear gradually from 0.25 up to 5 dyn/cm^2. It is also possible to use higher shears).
5. Acquire videos using a CCD camera (stream acquisition, 11 frames/sec) and analyze rolling paths and rolling velocities via compatible software.

6. Flow Cytometry to Detect Expression Of Surface Molecules

1. Following trypsinization, prepare a cell suspension (using 1-2 x 10^5 cells/sample) of desired cell type (HL-60, CHO-P or LMVECs) in PBS (-/-), supplemented with 2% FBS. Wash cells twice and bring sample volume to 50 µl (using the same buffer).
2. Incubate each sample with the desired fluorophore-conjugated Ab (see attached table for detailed information) at 4 °C for 20 min (cover with aluminum foil).
3. Wash the cells twice (same buffer) and bring final volume of stained cell suspension to 200 µl. Analyze samples using a flow cytometer to detect expression of surface molecules.

Representative Results

HL-60 cells roll on P- and E-selectin surfaces, but not on fibronectin

HL-60 cells are considered gold standard "rollers" as they express a variety of homing ligands, including the rolling ligands P-sel glycoprotein ligand-1 (PSGL-1) and Sialyl-Lewis X (SLeX)[5,14] (**Figure 1A**). The surface protein PSGL-1 acts as a scaffold for the tetra-saccharide SLeX, mediating specific interaction with P- and E-sel, which are up-regulated on the endothelium during inflammation[5,6,15]. To test the capabilities of the multi-well plate microfluidic system, numerous microfluidic channels were coated simultaneously with different substrates and rolling interactions of HL-60 cells with those surfaces were analyzed. HL-60 cells exhibited a robust rolling behavior on P-sel-coated surface, with cells first captured from flow, followed by a distinct rolling movement. As shown in **Figure 1C**, and consistent with the literature, HL-60 cells exhibit a

similar rolling behavior on E-sel surfaces, yet not on fibronectin-coated substrates[14,16-19]. Cell velocity, analyzed via compatible software, was plotted against shear stress, showing a robust rolling response of cells on P- and E-sel with an average velocity between 1-12 µm/sec.

HL-60 cells roll on CHO-P monolayer coating the microfluidic channel

Next, we aimed to assess the feasibility of using this microfluidic system to efficiently test interactions between cells of interest and a cell monolayer coating the surface. To explore the interaction of HL-60 cells with a cell monolayer that expresses rolling markers, we used CHO-P cells, which are transfected to stably express P-, but not E-, sel (**Figure 2A**. Also see **Figure 2B** for representative image of HL-60 cells on the CHO-P monolayer coating the microfluidic channel)[11,12]. HL-60 cells displayed a strong rolling response on CHO-P cells (**Figure 2C**). To test whether this rolling movement is indeed mediated by P-sel, the CHO-P monolayer was preincubated with blocking antibodies for either P- or E-sel, prior to perfusion of HL-60 cells into the channel. As shown in **Figure 2C**, blocking the CHO-P monolayer with a P-sel Ab resulted in a significant decrease in the number of rolling HL-60 cells on the surface, demonstrating that P-sel indeed mediates HL-60 rolling as previously described[14,18]. Performing the assay in a microfluidic channel permits rapid screening of different conditions and efficient blocking of the receptors by using only small volumes, as little as 25 µl. Isotype control or Ab blocking E-sel (which is not expressed on CHO-P cells) did not affect the number of rolling cells on CHO-P cells, demonstrating the strength of this assay in accurately pin-pointing the direct involvement of specific surface markers in cellular rolling interactions.

Rolling of HL-60 cells on TNF-a-activated LMVECs is mediated by E-selectin

Endothelial cells are known to up-regulate adhesion surface markers, such as P- and E-sel, during inflammation, assisting in the recruitment of leukocytes to sites of inflammation[5,6]. However, while murine ECs express both P-and E-sel in response to inflammatory stimuli like interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), human ECs only express E-sel in response to these cytokines[20,21]. This was validated in our flow cytometry assay, showing expression of E-sel, but not P-sel, on lung microvascular endothelial cells (LMVEC) in response to TNF-α stimulation (**Figure 3A**). The multi-well microfluidic plate consists of numerous separate microfluidic channels, allowing higher throughput testing of multiple different conditions. We used this advantageous design to plate LMVECs inside the microfluidic channels (**Figure 3B**) to rapidly analyze the interactions of HL-60 cells with ECs under multiple conditions. To simulate inflammatory settings, LMVECs were pretreated with the pro-inflammatory cytokine, TNF-α. Interestingly, HL-60 cells did not interact with un-activated LMVECs, and cells were not observed to roll on this surface. On the contrary, HL-60 cells displayed a strong rolling behavior on TNF-α-activated LMVECs, with average velocity of 5-15 µm/sec (**Figure 3C**).

We then aimed to explore the involvement of P-sel or E-sel in the rolling interaction between the HL-60 cells and the activated LMVECs. For this, TNF-α-activated ECs were preincubated with P-sel or E-sel blocking antibodies, and the rolling of HL-60 cells was analyzed. As shown in **Figure 4A**, blocking E-sel, which was up-regulated on TNF-α-activated LMVECs, resulted in a significant decline in the number of rolling cells on the activated endothelial monolayer. In contrast, using an isotype control or an Ab against P-sel, which was not expressed on the activated ECs, did not have a significant effect on HL-60 rolling on the activated endothelial layer. This data demonstrates the direct involvement of E-sel in HL-60 rolling on TNF-α-activated ECs, consistent with previous reports[20,21]. From the acquired videos, the analysis software permits one to track the paths of individual cells interacting with the substrate. We used this capability to specifically track the path of individual cells that interacted with TNF-α-activated ECs w/wo E-sel blocking. As shown in **Figure 4B**, the number of rolling cells on unblocked activated LMVECs was significantly higher than on E-sel-blocked activated ECs. Furthermore, it appeared that the rolling movement of HL-60 on unblocked LMVECs is continuous and robust, while the rolling paths of cells on E-sel-blocked ECs was fragmented (each color represents a different cell, see for example cells a-f vs. g-l in **Figure 4B**). In consistence with this finding, the rolling velocity of HL-60 cells on unblocked TNF-α-activated ECs was significantly lower than their rolling velocity on E-sel-blocked ECs (**Figure 4C**).

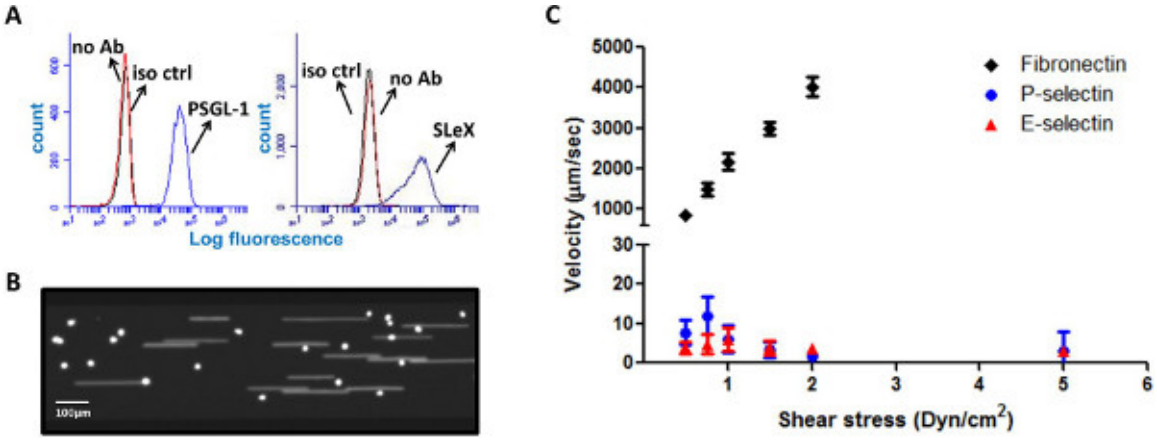

Figure 1. HL-60 cells roll on P- and E-selectin-coated surfaces. (A) HL-60 cells express the rolling ligands PSGL-1 and SLeX (Iso ctr - isotype control, no Ab - no antibody). **(B)** Representative snap-shot image of CFSE-stained HL-60 cells on P-sel-coated surface under flow. **(C)** HL-60 cells robustly roll on P- and E-sel-coated surfaces, but not on fibronectin-coated surface. Rolling velocity is plotted against shear stress (n=10-15 cells per data point, velocities were analyzed by compatible software). Click here to view larger figure.

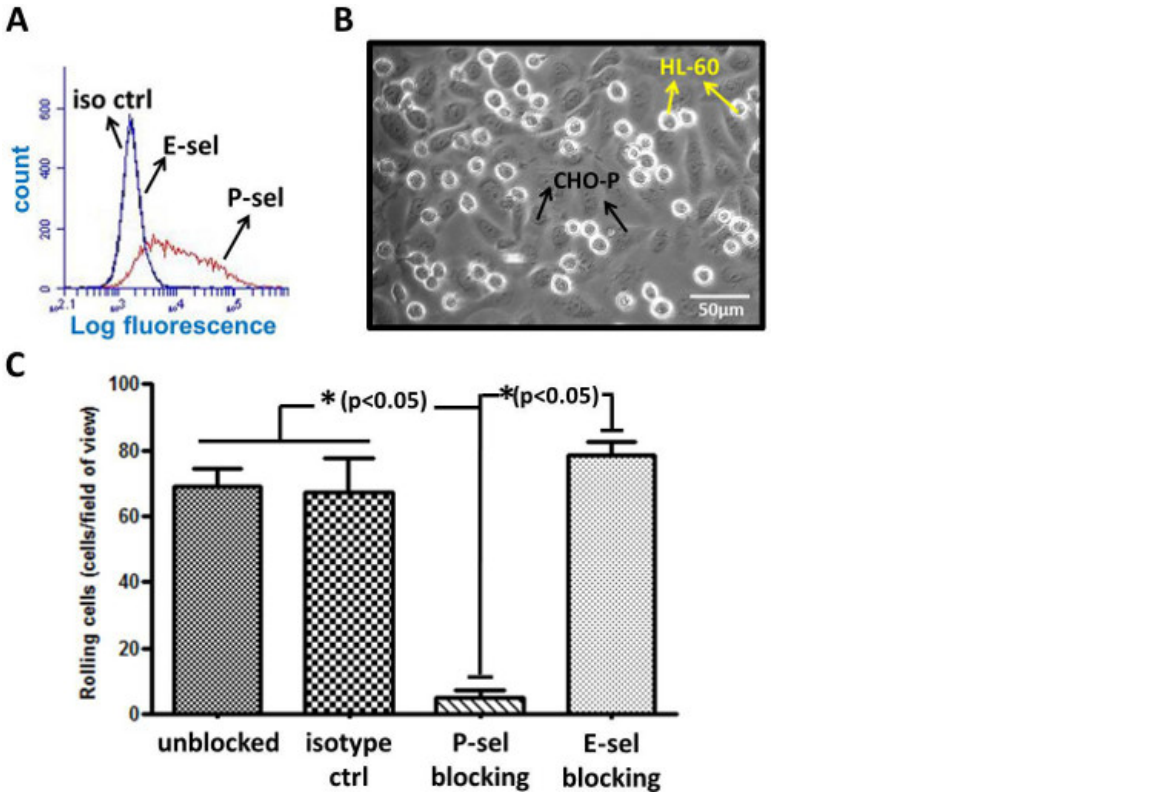

Figure 2. HL-60 rolling on CHO-P cell monolayer is directly mediated by P-sel. (A) CHO-P cells express P-sel, but not E-sel. **(B)** Representative image of HL-60 cells on the CHO-P monolayer coating the surface of the microfluidic channel (10X magnification). **(C)** HL-60 cell rolling on CHO-P monolayer is directly mediated by P-sel as demonstrated by Ab blocking assay (unblocked - CHO-P monolayer was not incubated with an antibody, isotype ctr-CHO-P monolayer incubated with an isotype control;*p<0.05, one-way ANOVA was used with Tukey's HSD post-hoc test, error bars represent SEM, n=3.

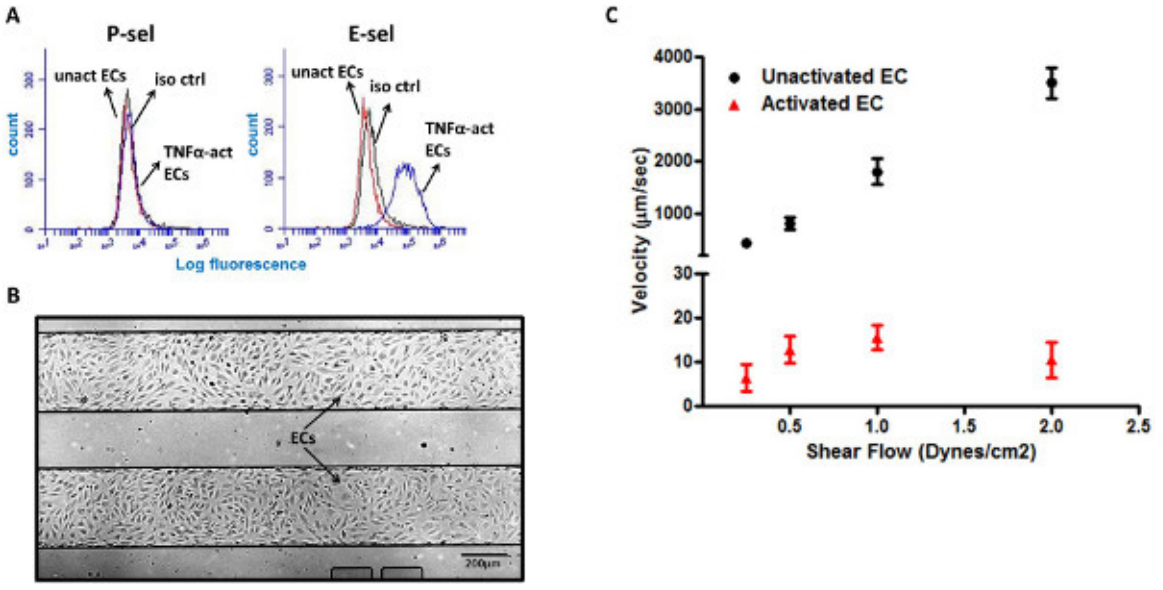

Figure 3. HL-60 cells roll on TNF-α-activated LMVECs. (A) TNF-α activation of LMVECs induce surface expression of E-sel, but not P-sel. **(B)** Representative image of confluent LMVEC monolayer in two microfluidic channels (4X magnification). **(C)** HL-60 cells exhibit a robust rolling response on TNF-α-activated LMVECs, but not on un-activated LMVECs. Iso ctr - isotype control, unact ECs - unactivated endothelial cells, TNF-α-act ECs - TNF-α-activated endothelial cells. Click here to view larger figure.

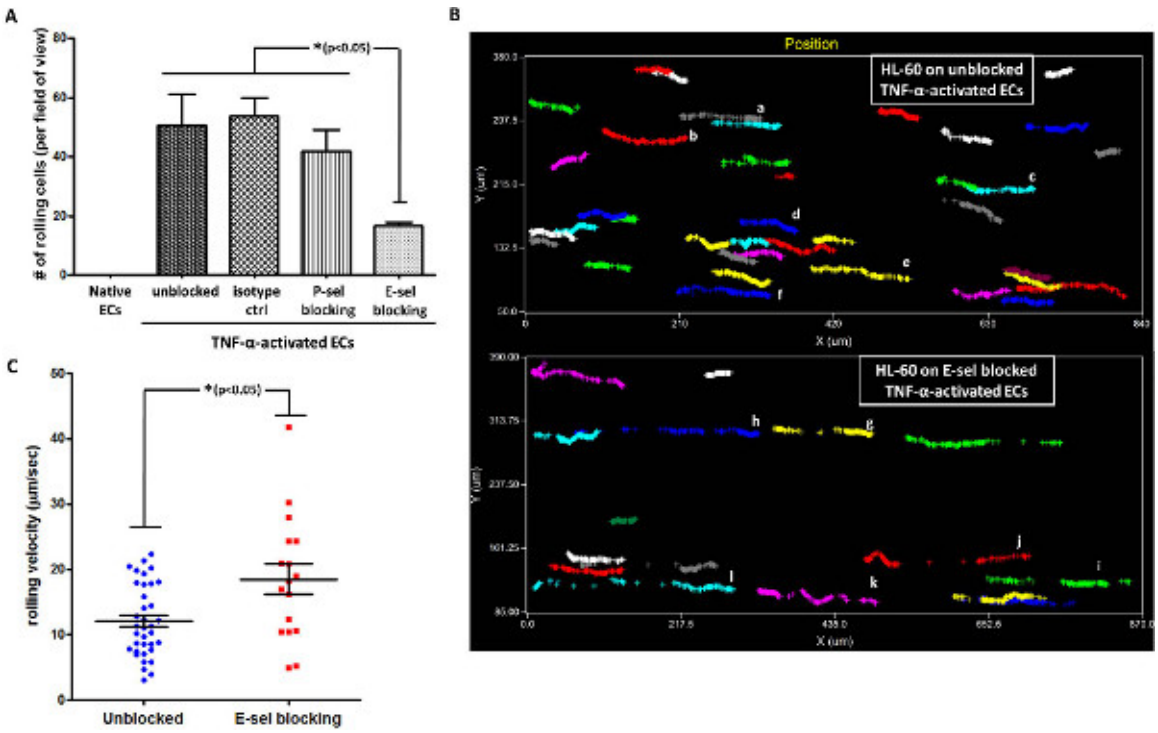

Figure 4. HL-60 rolling on TNF-α-activated LMVECs is mediated by E-selectin. (A) HL-60 rolling on TNF-α-activated LMVECs is mediated by E-sel, rather than P-sel, as demonstrated by Ab blocking assay (isotype ctr-EC monolayer incubated with an isotype control; *p<0.05, one-way ANOVA was used with Tukey's HSD post-hoc test, error bars represent SEM, n=3). **(B)** Cell path analysis reveals continuous and robust rolling of HL-60 cells on unblocked activated ECs compared to fragmented, weak rolling observed on E-sel-blocked activated ECs (every color represents a different cell. Analysis was performed via suitable software). **(C)** HL-60 rolling velocity on TNF-α-activated ECs is slower on unblocked activated ECs compared to E-sel-blocked activated ECs (Shear stress used: 2 dyn/cm^2. *p<0.05, unpaired two-tailed t-test, error bars represent SEM, n=17-36 cells per group). Click here to view larger figure.

Discussion

One of the major challenges in successful translation of exogenous cell-based therapy is the inability to efficiently deliver cells to sites of injury and inflammation with high engraftment efficiency[3]. Cell rolling represents a critical step in the process of cell homing, facilitating the deceleration of cells on the walls of blood vessels, eventually leading to their firm adhesion and transmigration through the endothelium into the tissue[5]. Better understanding of the rolling process for candidate cell types may lead to development of techniques to enhance cell homing and contribute significantly towards improving cell-based therapy.

PPFC is a widely used tool to explore cell rolling, as well as other cellular behaviors under shear flow. The application of PPFC for examining neutrophil adhesion on the endothelium was first studied by Lawrence *et al.* in 1987, and since then commercially available products have been developed[8,9]. PPFCs consist of two flat plates separated by a gasket that controls the dimensions of the chamber[7]. The upper plate contains an inflow and outflow port, which through the use of a syringe pump enables the perfusion of the cell suspension through the chamber[7,8]. There is also an additional port that applies negative pressure (vacuum) to keep the plates pressed together[7]. Although parallel plate flow chambers have been effectively used to investigate cellular responses, including cell rolling, under shear flow conditions, there are numerous limitations that reduce its effectiveness. A major limitation of flow chambers is the high number of cells and large volumes of reagents that are required due to the large dead volume within the flow system[7]. Another critical issue is the presence of air bubbles, which can easily arise during flow chamber assembly, potentially hampering the cell monolayer and substrate coating on the bottom plate[22]. However, further modifications have been made to traditional PPCFs to incorporate an additional port on the upper plate to act as a bubble trap to facilitate the removal of air bubbles[23]. Setting up the PPFC experiment is also tedious, and the flow cell is susceptible to leakage if the gasket is damaged or not assembled carefully. Finally, there is a difference between the desired and experimentally applied shear rates, which results in a narrow range of uniform flow rates. By theoretically comparing four PPFCs with varying inlet and outlet positions, it was found that two of the configurations modeled shear rates that deviated from the calculated shear rates by up to 75%[24]. To reuse the same chamber requires time-consuming washing steps, and combined with the above described challenges, this makes the PPFCs fairly tedious and low throughput.

In our study, we used a fully integrated multi-well plate microfluidic system, relying on accurately controlled shear flow[1,2,13]. This 48 well microfluidic plate includes 24 microfluidic channels, with each pair of adjacent wells connected with a microfluidic channel[1,25]. 10-12 rolling assays can be performed in 1 hr, allowing rapid screening of several dozen conditions in a single day. The microfluidic channels can be easily coated with a protein substrate or cell monolayers, and the interaction between cells of interest and the surface can be imaged using a

microscope, acquired by a CCD camera and analyzed by compatible software. Key parameters such as cell quantification, calculation of rolling velocity and specific track path analysis can be easily obtained to efficiently analyze rolling behavior on multiple substrates[13]. In this study, we assessed the efficiency of this system in studying cell rolling by using HL-60 promyelocytic leukemia cell line, well established "rollers" that express key rolling ligands, such as PSGL-1 and SLeX, which together act as the counterpart ligands for P-and E-sel[15,26]. In correlation with previous reports, HL60 cells indeed exhibited a robust rolling behavior on P-and E-sel-coated microfluidic channels, with slow rolling velocities of 1-12 μm/sec[14,17-19]. HL-60 cells did not roll on fibronectin surface, consistent with previous reports showing that undifferentiated HL-60 cells do not exhibit adhesive interactions with fibronectin[16]. The design of the multi-well plate microfluidic system, allowing up to 10-12 assays/hr compared to only 1-2 assays/hr that can be tested using PPFCs, significantly increases throughput by at least 5 times. Moreover, PPFCs need to be reassembled and washed before each reuse, further slowing down performance rate. In addition, the easily controlled flow enables rapid performance of the shear-dependent experiments, which can then be readily analyzed via suitable software.

We then aimed to test the efficiency of this microfluidic system in exploring interactions between cells in suspension and a cell monolayer coating the channel. For this purpose, we used CHO-P, CHO cells which are stably transfected to overexpress P-sel (**Figure 2A**)[11,12]. HL-60 cells exhibited a significant rolling response on CHO-P cells, demonstrating the ability to use this system to efficiently explore cell-cell interactions under shear flow given a design that prevents the formation of bubbles that may jeopardize the integrity of the cell monolayer, which often occurs when using PPFCs[22,23]. We then blocked the CHO-P cells with P- or E-sel antibodies to explore their potential involvement in the rolling process. P-sel blocking significantly reduced the number of rolling cells on the CHO-P monolayer, indicating a direct involvement of P-sel in mediating the HL-60 rolling, consistent with previous reports[14,18,20]. E-sel Ab and isotype control had no effect on the rolling on HL-60 on CHO-P, demonstrating that Ab blocking can be used in this microfluidic system to efficiently detect specific markers mediating cell-cell interactions. Importantly, each channel requires only minimal volume of costly Abs or cell suspension for this assay, as little as 25-50 μl, unlike the reagent-consuming PPFC and its typical large dead volumes[7].

We next tested this multi-well plate system by analyzing interactions between HL-60 cells and ECs coating the microfluidic channel. ECs are known to express P- and E-sel during the inflammatory process[5]. To provide ECs with an inflammatory stimulus, we pretreated them with TNF-α. While E-sel was up-regulated, P-sel was not, consistent with the literature showing that human ECs express E-sel, but not P-sel, in response to TNF-α activation since the primate P-sel promoter lacks TNF-α response elements, resulting in transcriptional induction of only E-sel[20,21]. Inflammatory conditions were then simulated inside the channel by incubating the EC monolayer with TNF-α, followed by perfusion of HL-60 cells to explore its interactions with the EC surface. While HL-60 did not interact with unactivated ECs, they did display a robust rolling response on TNF-α-activated ECs (**Figure 3C**), correlated with their reported response in the literature[22]. Ab blocking experiments (**Figure 4A**) then showed that E-sel, but not P-sel, blocking resulted in a significant reduction in HL-60 rolling on activated ECs. These data demonstrate the direct involvement of E-sel, and not P-sel, in mediating the rolling of HL-60 on TNF-α-activated human ECs[20,21]. The Ab blocking experiments, conversely showing E-sel-mediated rolling on activated ECs vs. P-sel-mediated rolling on CHO-P, further validates the feasibility and relevance of Ab blocking experiments rapidly performed in this microfluidic system. Interestingly, this inhibition of rolling was not complete (about 70% reduction) suggesting that other surface receptors, such as VCAM-1, also participate in HL-60 rolling on activated ECs[27]. Track path analysis further revealed another interesting phenomenon - while the rolling of HL-60 on unblocked activated ECs was continuous and robust, the low number of cells that still rolled on E-sel blocked activated ECs displayed a fragmented rolling path on the blocked ECs (**Figure 4B**). This phenomenon was not observed in ECs incubated with P-sel blocking or isotype control (data not shown). This strongly suggests that while a rolling response is possible via other markers when EC E-sel is blocked, this rolling relies on weak, partial HL-60-EC interactions, supporting only a loose, partial rolling response with the activated ECs[5,22,27-29]. This is supported by data shown in **Figure 4C**, demonstrating significantly slower rolling of HL-60 cells on unblocked activated ECs compared to their rolling on E-sel-blocked activated ECs, indicating a weaker rolling response on E-sel-blocked ECs. Using the multi-well plate microfluidic system instead of PPFC allowed us to conduct the delicate HL60-ECs experiments rapidly, while accurately controlling shear flow, avoiding bubbles and using only minimal volumes of Abs and cells. Advantageous use of microfluidics for cell rolling applications was previously demonstrated[30-33], and improving throughput of microfluidics via the multi-well plate system presented here, further highlights the potential of this technology for efficient and precise analysis of key rolling properties towards improved therapeutic applications.

This study focused on examination of HL-60 cell rolling on protein substrates and cell monolayers under physiologically relevant and accurately controlled shear flow using a multi-well plate microfluidic system. Similarly, other cell types and other substrates/cell monolayers can readily be used to study their rolling properties. Ease of use and simple analysis permits a precise analysis of important rolling properties and Ab blocking or activation with different growth factors or inhibitors can be used to explore potential involvement of molecular markers in the rolling response. This may be specifically useful towards studying stem cell rolling and homing, which may be engineered to improve stem cell-based therapy[34-36]. Importantly, multiple conditions can be tested with improved throughput (5-10 times higher vs. PPFCs), permitting rapid and efficient study of rolling properties due to the plate design. Other assays relevant for cell homing, such as cell adhesion, chemotaxis and transmigration may also be studied using this system[1,10,13]. Overall, this microfluidic system emerges as a powerful technique to study cell rolling and should serve as a useful tool to aid in the clinical translation of exogenous cell-based therapy.

Disclosures

Authors declare no conflict of interests.

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