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JoVE Editors
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Dear Editors,

I am writing to re-submit a manuscript for JoVE entitled, „A protocol for the analysis of shear flow-induced migration of murine marginal zone B cells (MZBs) in vitro“.

Our manuscript was returned to us with the reviewers' comments on July 24th and we were supposed to re-submit by August 7th. I apologize for submitting it late now, and I hope that you might still consider the submission.

The manuscript describes a protocol for imaging and quantifying migration under shear flow of a type of B cell called marginal zone B cells (MZBs). We will upload the following documents:

- A revised version of the manuscript
- A revised version of the manuscript with the “comparison/track changes” feature on to allow easy comparison with the previous manuscript
- A point-by-point response to the reviewer comments
- A Word doc containing the text for an ImageJ macro, “Threshold MZBs and run MTrack2kt”
- A word doc containing the text for a modified ImageJ plugin, “MTrack2 kt”
- An Excel spreadsheet listing the materials used in the protocol

If possible, could you please add a second corresponding author, the last author (Klaus-Dieter Fischer) to the protocol? I tried to do it online but I'm not sure if it worked.

Thank you for once again considering this manuscript, and I hope it will be of interest to JoVE.

Best regards,
Kerry Tedford

TITLE:

Analysis of Shear Flow-Induced Migration of Murine Marginal Zone B Cells *In Vitro*

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KEYWORDS:

B cells, marginal zone, migration, shear flow, integrins, adhesion, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1)

SUMMARY:

Marginal zone B cells (MZBs) respond to the force of shear flow by re-orienting their migration path up the flow. This protocol shows how to record and analyze the migration using a fluidics unit, pump, microscope imaging system, and free software.

ABSTRACT:

Marginal zone B cells (MZBs) are a population of B cells that reside in the mouse splenic marginal zones that envelop follicles. To reach the follicles, MZBs must migrate up the shear force of blood flow. We present here a method for analyzing this flow-induced MZB migration *in vitro*. First, MZBs are isolated from the mouse spleen. Second, MZBs are settled on integrin ligands in flow chamber slides, exposed to shear flow, and imaged under a microscope while migrating. Third, images of the migrating MZBs are processed using the MTrack2 automatic cell tracking plugin for ImageJ, and the resulting cell tracks are quantified using the Ibidi chemotaxis tool. The migration data reveal how fast the cells move, how often they change direction, whether the shear flow vector affects their migration direction, and which integrin ligands are involved. Although we use MZBs, the method can easily be adapted for analyzing migration of any leukocyte that responds to the force of shear flow.

INTRODUCTION:

Immune cells are the most motile cells in the human body and often must contend with shear force from blood and lymph flow. However, there are comparatively few studies on shear force-induced migration of leukocytes¹⁻⁵. We present here a reliable and quantitative protocol to analyze the response of an immune cell to flow *in vitro*. Performing the assay does not require

fabrication of components, and all equipment and consumables are commercially available. The protocol, including cell purification and migration analysis, can be performed in a single day. Finally, although we describe the migration of marginal zone B cells (MZBs), the protocol can be adapted to analyze migration against flow of other types of immune cells. Therefore, it is feasible to use this assay to systematically analyze a broad range of leukocytes with a comprehensive panel of conditions.

MZBs are a population of B cells that, in the mouse, are found only in the spleen and shuttle between the interior of follicles and the marginal zones⁶⁻⁹. The marginal zone is a layer of immune cells approximately 5-10 cells thick. The cell layer envelops the follicle and consists primarily of MZBs and macrophages but also invariant natural killer T (iNKT) cells, dendritic cells (DCs), and neutrophils, among others¹⁰. The cells in the marginal zone are exposed to unidirectional blood flow originating from splenic arteries that terminate in a marginal sinus surrounding the follicle. The blood flows from holes in the marginal sinus through the marginal zone and is then collected in venous sinuses in the red pulp and restored to the circulation¹¹. The free-flow of blood washes over the MZBs and exposes them to antigens carried in the blood. The MZBs carry the antigen into the follicle by shuttling automatically between the marginal zone and inside of the follicle, which is not exposed to blood. Thus, as MZBs shuttle towards the follicle, they must migrate up the shear force of the blood flow¹² (**Figure 1A**).

In this protocol, we describe how to quantitatively determine how immune cells such as MZBs respond to either no flow or high flow *in vitro*, in order to reveal how they are programmed to migrate *in vivo*. In the first step, MZBs are purified from a mouse spleen using magnetic beads coupled to antibodies from commercially available kits. The freshly isolated MZBs are introduced into the well of a flow chamber slide, allowed to settle onto integrin ligands, and exposed to the flow of migration buffer using a pump system (**Figure 2A**). The cells are imaged using a time-lapse video microscopy system. The images are then processed for analysis with a free ImageJ plugin, MTrack2^{13,14}, to automatically track the cells. Tracks can then be quantified with the free Ibidi Chemotaxis tool¹⁵ to determine various parameters including velocity, straightness, and migration index. These values can be used to determine the effects of migration inhibitors, cell stimulators, chemokines, and other migration-affecting chemicals on the shear-flow induced migration in order to understand the forces controlling immune cell movement *in vivo*.

PROTOCOL:

All experiments involving the use of animals have been previously approved by the Landesverwaltungsamt Halle (Saxony-Anhalt), Germany, in accordance with all guidelines of the medical faculty of the OVGU University of Magdeburg.

1. MZB Cell Purification

1.1. Isolate leukocytes.

1.1.1. Sacrifice an 8- to 16-week-old mouse and remove the spleen¹⁶. Dissociate the spleen in 5 mL of ice-cold cell buffer [phosphate-buffered saline (PBS) + 0.5% fatty acid-free bovine serum albumin (BSA)], by either using a tissue dissociator or gently mashing the spleen through a 70 μ m cell strainer mesh into a well on a 6-well plate with the plunger from a 5 mL syringe.

1.1.2. Transfer the cells in cell buffer into a 15 mL conical tube. Collect additional cells by adding another 5 mL of ice-cold cell buffer to the tissue dissociator tube or the nylon mesh in the well. Transfer the additional 5 mL of cell buffer plus cells to the conical tube. Centrifuge at 300 x g for 10 min at room temperature (RT, 20 °C) and discard the supernatant.

1.2. Perform red blood cell lysis.

1.2.1. Re-suspend the cell pellet in 1 mL of red blood cell (RBC) lysis buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA) that is pre-warmed to RT. Add an additional 1.5 mL of RBC lysis buffer and swirl to mix. Incubate at RT (20 °C) for a total of 2 min, including the time required to re-suspend the pellet.

1.2.2. Add 7.5 mL of ice-cold cell buffer to the cell suspension to stop the lysis. Centrifuge at 300 x g and 4 °C for 10 min and discard the supernatant.

1.2.3. Re-suspend the cell pellet in 1 mL of cell buffer and add an additional 9 mL of cell buffer. Pipette the 10 mL cell suspension through a 30 μ m pre-separation filter into a new 15 mL conical tube to remove cell debris.

1.2.4. Centrifuge cells at 300 x g and 4 °C for 10 min and discard the supernatant. Re-suspend the cell pellet in 500 μ L of cell buffer. Keep cells cold at all subsequent steps of the procedure until incubation in the flow chamber slides.

1.3. Purify MZBs.

1.3.1. Add 50 μ L of biotin-coupled antibodies from a commercially available kit against all undesired cells, including CD43 (on non-B cells), CD4 (on T cells), CD93 (on immature B cells), and Ter119 (on erythrocytes), to the cell suspension in a conical tube. Shake or flick the tube gently to mix, but do not vortex the cells. Lay the conical tube on a bed of ice at an almost-flat angle and rock at 25 rpm for 15 min.

1.3.2. Add magnetic beads coupled to anti-biotin antibodies in a total volume of 100 μ L to the cell suspension. Continue to rock the cell suspension at 25 rpm on ice for 20 min. Wash the cells by adding 5 mL of cell buffer, centrifuging at 300 x g and 4 °C for 10 min, and discarding the supernatant. Re-suspend cells in 1 mL of cell buffer.

1.3.3. Deplete the cell suspension of the magnetic-bead-coupled cells by retaining them on a magnetic column. Discard labeled cells (all non-B cells and immature B cells). Collect the non-

labeled cells (mature B cells) in a 15 mL conical tube that has been pre-coated with a brief application of 5 mL of cell buffer, in order to prevent non-specific adhesion by MZBs.

1.3.4. Wash the cell suspension with 5 mL of cell buffer. Centrifuge at 300 x g and 4 °C for 10 min and discard the supernatant. Re-suspend cells in 90 µL of cell buffer and add 10 µL of anti-CD23-coupled beads. Rock cell suspension gently on ice for 20 min at 25 rpm.

1.3.5. Wash the cell suspension with 5 mL of cell buffer. Centrifuge at 300 x g and 4 °C for 10 min and discard the supernatant. Re-suspend cells in 1 mL of cell buffer.

1.3.6. Deplete the cell suspension of the magnetic-bead-coupled cells by retaining them on a magnetic column. Discard the labeled cells (follicular B cells). Collect the non-labeled cells (MZBs), in a 15 mL conical tube that has been pre-coated with a brief application of 5 mL of cell buffer. Remove 50 µL of the cells for counting and checking purity.

1.4. Stain the sorted MZBs to check for purity.

1.4.1. Add 10% volume (5 µL) of Fc block to 10,000 cells in 50 µL and incubate at 4 °C for 5 min. Add 50 µL of cell buffer containing 0.3 µL each of B220-FITC (0.15 µg), CD21-PE (0.06 µg), and CD23-APC (0.06 µg) in a total volume of 105 µL (each antibody diluted 1:350), or any other combination of fluorophores for distinguishing between follicular B cells and MZBs. Stain the cells at 4 °C in the dark for 15 min.

1.4.2. Wash the stained cells once with 1 mL of cell buffer. Centrifuge at 300 x g and 4 °C for 5 min and discard the supernatant. Resuspend in 300 µL of cell buffer and acquire samples using a flow cytometer and standard methods. Gate on live lymphocytes, then on B220⁺ cells, and finally on CD23^{low} CD21^{high} cells (**Figure 1B**).

Note: The procedure typically yields 500,000 MZBs per spleen from a C57B/6 mouse.

1.5. Prepare MZBs for flow migration.

1.5.1. Wash purified MZBs once in 1-2 mL of cold migration buffer [Hanks' balanced salt solution (HBSS) containing cations, 10 mM Hepes, 0.2% fatty acid-free BSA] to ensure that the cation-containing migration buffer is not diluted by residual cation-free PBS buffer. Centrifuge at 300 x g and 4 °C for 10 min and discard the supernatant. Re-suspend MZBs in cold migration buffer to a concentration of 50,000 MZBs per 30 µL (equivalent to the amount loaded in one well of a flow chamber slide).

Note: The freshly isolated MZBs should be used as soon as possible. However, they are still capable of migrating for up to 8 h or more after the start of the experiment for as long as they are kept on ice. Optimal temperatures should be tested for other cell types. For example, it may be better to keep cultured T cells at 37 °C until the start of migration.

2. Flow Experiment

2.1. Coat slides with integrin ligand.

2.1.1. On the day before the experiment, thaw an aliquot of ICAM-1 (400 µg/mL). Dilute ICAM-1 by a factor of 1:80 in PBS to reach a final concentration of 5 µg/mL. Add 30 µL of ICAM-1 to one chamber on a flow slide for every migration movie required. Incubate slide in a humid chamber at 4 °C overnight.

Note: Each slide contains 6 chambers and up to 8 movies in one session can be easily recorded.

2.2. Block the coated wells.

2.2.1. On the day of the experiment, wash the chamber by adding 100 µL of PBS to one well and withdrawing 100 µL from the other well of the chamber. Add 100 µL of blocking buffer (2% fatty acid-free BSA in PBS) to a chamber and incubate the slide in a humid chamber at RT for 1.5 h.

2.2.2. Wash the chamber by adding 100 µL of PBS to one well, withdrawing it from the other well, then adding 100 µL of migration buffer to the chamber. Store the slides in a humid chamber at RT until the experiment.

2.3. Prepare the fluidics unit.

2.3.1. Plug in a fluid pump, attach the fluidics unit, and turn on the pump software. Wash the tubing once with 5-10 mL of migration buffer, then add 11.7 mL of migration buffer equally to both reservoirs and remove the air bubbles.

2.3.2. Clamp the tubing about 10 cm from the connector piece. Place the fluidic unit in the heated incubation chamber on the microscope.

2.3.3. Set the slide choice to “µ-slide VI 0.4” and the tubing to “white” in the software. Set the desired shear stress (*e.g.*, 4 dyn cm⁻²). Set the time of imaging to 30 min.

2.3.4. Set the tubing calibration factor by determining the flow rate through the tubing in mL/min. Measure the time required for 2 mL of the buffer to flow from one reservoir of the other and divide by 2. Make at least 4 measurements for accuracy. Use the actual flow rate to set the desired flow rate and shear stress by entering the values into the pump software.

2.4. Prepare the imaging system.

2.4.1. Pre-warm the microscope incubation chamber, fluidics unit, and migration buffer aliquots to 37 °C (**Figure 2B**). Test the fluidic pump by ensuring that the migration buffer flows back and forth in the reservoirs and that there are no air bubbles in the system.

2.5. Load the cells on the slide.

2.5.1. Clean the bottom of the slide with an ethanol-dampened tissue. Add 30 μ L of the cell suspension into one well of the chamber and withdraw 30 μ L from the other well. Incubate the slide with a cover on to prevent evaporation at 37 °C for 30 min to allow the cells to attach.

2.5.2. Slowly add the pre-warmed migration buffer to each well of the flow chamber slide until a positive meniscus rises out of the well and remove any air bubbles with the pipette tip.

2.6. Attach the slide to the fluidics unit.

2.6.1. Clamp the slide to the microscope stage. Remove the unmarked side of the fluidic tubing from the connector piece and fill up the end with migration buffer until a positive meniscus without air bubbles rises out of the end.

2.6.2. Flip the end of the tubing over and insert it into the top well of the flow chamber, mopping up spilled buffer with a tissue. While keeping the tubing clamped, repeat the procedure for the marked end of the tubing.

2.7. Image the cells.

2.7.1. Switch the imaging system to “Live” to set the focus on the cells. Select a field of view that is in the middle of the slide. De-focus the cells slightly to enhance the black outline of the cell and produce a white interior, which can then be thresholded to a round black cell shape on a white background for use with an automated tracking program.

2.7.2. Start the imaging sequence program and record 1 image every 5 s for 30 min using the 10X dry objective. Remove the clamp from the tubing and turn the pump on, then non-adherent cells will wash off and adherent cells will begin to migrate. Image for 30 min or longer using a 10X objective or higher, as desired. Label and save the movie.

2.8. Detach the fluidic unit from the slide.

2.8.1. At the end of the imaging program, reset the pump for the next experiment: re-attach the clamp to the tubing, remove the tubing from the slide, re-attach the connector piece, open the clamp, and use the manual control of the pump to push several mL of buffer from one reservoir to the other to remove the air bubbles. Re-attach the clamp and lay the clamped tubing on the microscope stage for the next movie.

Note: The protocol can be paused indefinitely here. If using an inhibitor or modifier of cell migration, depending on its mode of action, add it to either the cell suspension before settling in the migration chamber or to the migration buffer in the fluidic unit.

3. Migration Track Analysis

Note: Cells can be tracked automatically using the MTrack2 plugin or by hand using the Manual Tracking plugin¹⁷. Automatic tracking works well with MZBs because these cells are mainly round and remain this way while migrating, making it easy to threshold the image of the cells to black objects on a white background. Automatic tracking is more difficult if other cell types are used, such as cultured, activated CD8⁺ T cells, because these cells stretch out during migration and become somewhat transparent, making it difficult to define the edges. In this case, either (1) the cells can be stained with an intra-vital fluorescent dye to produce images that can be thresholded to show black objects on a white background, or (2) other programs to outline the cells such as image segmentation and/or edge detection can be used. Manual tracking is a useful option when producing a high-contrast image of cell outlines is not possible.

3.1. Perform manual tracking using the plugin.

3.1.1. Install the “Manual Tracking” plugin in ImageJ and open the movie in ImageJ. Under the “Manual Tracking” menu, select “add new track”. Click the center of a cell as the movie advances automatically frame-by-frame. When the cell has been selected in every frame, select “add new track” to start following a new cell.

3.1.2. After the tracks have been recorded, copy the output results into a data spreadsheet. Change the format for decimal display for all spreadsheet cells to zero places after the decimal. Save the file as a “tab stop-separated” .txt file for subsequent analysis.

Note: Typically, 50-100 cells are tracked, which takes about 1 h to complete for a 20-min movie of about 240 frames.

3.2. Perform automatic tracking using the MTrack2 plugin. Download and install the MTrack2 kt plugin according to instructions in the file (see **Supplemental Coding File**).

3.2.1. Threshold the images from the cell migration movie. Open the image stack in ImageJ. Process the images in ImageJ to convert the video from color to high-contrast images showing the cells as black objects on a white background (**Figure 3A**).

3.2.1.1. Sharpen the images twice, despeckle the images, and convert the images to 8 bit. Select the “Image | adjust brightness/contrast” sub-menu and put the contrast slider at maximum contrast, then the cells will appear as white objects on a black background. Select the “Image | adjust threshold” sub-menu to ensure that the cells appear as black objects on a white background.

3.2.2. Run the automatic cell tracking plugin “MTrack2 kt” (see **Supplemental Coding File**). Set the following parameters on the options screen.

3.2.2.1. Set the particle size minimum at 1 pixel and particle size maximum at 30 pixels for MZBs.

3.2.2.2. For velocity (maximum distance between 2 particles on successive frames of the movie), if the cell density on the slide is high, set this value low to avoid having the track “jump” from one cell to another. For MZBs, set this value at 10 pixels to capture outliers, although MZBs will generally not exceed 2 pixels on successive frames that are 5 s apart.

3.2.2.3. For the minimum number of frames that a particle has to be present to be tracked, set this value at the number of frames in the movie for MZBs (*e.g.*, 240) for a 20 min movie with 12 frames/min. However, if the cells are fast enough to leave the field of view before the end of imaging, then set the minimum number of frames at less than the duration of the movie. For example, with fast-moving T cells, set the minimum number at the equivalent of 5-10 min of imaging.

Note: Record a macro of these steps (thresholding and MTrack2-kt) to automate this part of the procedure and save time (see **Supplemental Coding File**). Using the macro to automatically track a movie in ImageJ takes less than a minute.

3.2.3. Copy the output results into a data spreadsheet. Change the format for decimal display for all spreadsheet cells to zero places after the decimal. Add an empty row at the top of the cell tracks in the spreadsheet and delete the “D” column. Save the file as a “tab stop-separated” .txt file for subsequent analysis in the Ibidi Chemotaxis tool.

Note: The MTrack2 plugin outputs the cell tracks in rows of 75 parallel columns. For analysis in the Ibidi chemotaxis tool, the cell tracks must be in a single column. To convert the output, the MTrack2 plugin was modified to output either in the original format or as a single column (**Figure 3B**) (see **Supplemental Coding File “MTrack2 kt”**, which is written in Java). Copy the file to a free java compiler (*e.g.*, JD-GUI), convert it to a java file, then install it in ImageJ.

3.3. To analyze cell tracks with the Ibidi Chemotaxis tool, download the Ibidi Chemotaxis and Migration Tool v2.0 (“stand alone”) and open the program.

3.3.1. Select “Import data” and navigate to the .txt cell track files. Multiple files can be selected at the same time. Imported files appear in the “Datasets” pane in red. Select all of them and enter the correct values in the “Initialisation” menu below to apply the following settings.

3.3.2. Enter either the exact number or a range of the number of slices (the number of frames in the movie).

3.3.3. In the Calibration menu (X/Y calibration), enter the size of a pixel. Locate this information in the properties file of the movie. For MZBs, a 10X objective was used, giving a pixel size of 1.14 μm . In “Calibration menu | Time interval”, enter the length of time between frames in the movie.

3.3.4. Apply the settings. Select “Plot data” to view the track plots and confirm that the track files will open. From this point, many options are available which are described in the documentation for the program, including marking the tracks with different colors based on different properties

and displaying the individual or average values for velocity, forward migration index, directness, and more (**Figure 3B**).

Note: The MZBs take about 10 min to detect and respond to the flow, presumably by polarizing their integrin complexes to the flow-forward point of the cell. A graph of migration index over time will show an initial drop below zero, corresponding to the first couple of minutes that the cell is exposed to flow, followed by a gradual rise upwards until the forward migration index values are positive. Thus, it may be optimal to exclude these first minutes in the final calculations, unless this process is of interest.

REPRESENTATIVE RESULTS:

We used the protocol outlined above to compare migration of MZBs on ICAM-1-coated slides without flow (0 dyn/cm²) and exposed to shear flow (4 dyn/cm²). Cells were tracked automatically with MTrack2, and the resulting track files were overlaid on the cell migration movies of no flow (0 dyn/cm²) and (4 dyn/cm²) to show the distribution and shape of the tracks (**Figure 4A**). Cell tracks were then imported into the Ibidi Chemotaxis tool (ICT) to generate track plots of each movie (**Figure 4B**). The average migration index (called “FMly” in the ICT), velocity, straightness (called “directness” in the ICT), and straight-line distance (called “Euclidean distance” in the ICT) of cell tracks from 3 movies each for both conditions were calculated in the Chemotaxis tool using the “measured values” command. These average values were then copied into GraphPad Prism for generating graphs and calculating statistical significance (**Figure 4C**).

FIGURE LEGENDS:

Figure 1: MZB shuttling. (A) Model of MZB shuttling between the marginal zone and the follicle in the spleen. MZBs require internalization of S1PR1, a receptor for S1P, and functional CXCR5, a receptor for the CXCL13 chemokine, in order to enter the follicle. Additionally, to reach the follicle, MZBs must migrate against the force of blood flow emanating from pores in the marginal sinus that envelops the follicle. If a MZB loses adhesion to ICAM-1, the ligand for LFA-1 integrin, it is pushed into the red pulp by the force of the flow, where increased amounts of VCAM-1, the ligand for VLA-4, would not support migration. (B) Flow cytometry gating strategy to test purity of sorted MZBs using antibodies against B220, CD23, and CD21.

Figure 2: Setup of the fluidics system, pump, microscope, and incubation chamber. (A) Image of the pump system (Ibidi¹⁸) consisting of a pump with connected fluidics unit and laptop running the software to control the pump. Higher magnification image: the flow chamber slide with 6 flow chambers, the first of which is attached to the tubing of the fluidics unit. (B) Typical setup for measuring *in vitro* migration of MZBs against flow. A microscope equipped with a heating chamber (black box) containing the fluidics unit connected to a pump (blue square object to the lower right of the microscope) outside the microscope. Higher magnification image: the fluidics unit inside the microscope heating chamber.

Figure 3: Quantification of imaging data. (A) Representative images of a frame from a movie of migrating MZBs before (left) and after (right) thresholding the image to convert the cells to black objects on a white background. Scale bars in both low and high magnification images = 100 µm.

(B) Left panel: image of typical results output from the MTrack2 (single column output) plugin. Right panel: image of the Ibidi Chemotaxis and Migration 2.0 tool after input of MTrack2 results showing a track plot of migrating MZBs and a “measured values” display window showing the averages of parameters including velocity, migration index, and directness (green boxes), among others. Calibration settings shown include the pixel resolution of 1.14 μm per pixel and a time interval of 5 s (0.083 min) per movie frame.

Figure 4: Example of results using the protocol to measure MZB shuttling. (A) Representative stills of migrating MZBs with an overlay of tracks from the ImageJ plugin “MTrack2 kt”. Note: the track colors were arbitrarily set by the “Manual Tracking” plugin for ImageJ. Scale bars in both low and high magnification insets = 100 μm . (B) Representative track plots of migrating MZBs from the Ibidi Chemotaxis and Migration 2.0 tool. Red lines and black lines represent cell tracks that terminate below or above the horizontal axis, respectively. In (A) and (B): left, MZBs migrating with no flow (0 dyn/cm^2); right, MZBs migrating to a 4 dyn/cm^2 flow. (C) Migration index (FMly), velocity, straightness (directness), and distance for MZBs migrating with no flow (0 dyn/cm^2) or flow (4 dyn/cm^2) ($n = 4$ mice in 4 separate experiments). Error bars = mean \pm SD; Student’s t test, ** $p < 0.01$, *** $p < 0.001$.

DISCUSSION:

We describe here a method for analyzing the migration of cells that detect the force of shear flow and respond by altering their migration. An analysis of MZBs showed that MZBs migrate spontaneously on ICAM-1 and in the presence of flow, will migrate up the flow. In our previous work, we showed that MZBs do not migrate up the flow on VCAM-1 but instead remain fixed in place. The murine splenic marginal zone contains mainly ICAM-1, while the red pulp contains both ICAM-1 and VCAM-1. From these data, it could be inferred that MZBs would migrate up the flow while in the marginal zone but not in the red pulp. The analysis was validated *in vivo* using MZBs with defective adhesion that were mislocalized to the splenic red pulp by the force of the flow¹². For these reasons, MZBs represent a good positive control for testing the flow migration system described here, even if the system is used to study a different cell type.

The most critical aspect of the procedure is ensuring consistency in cell handling on the slide. Because the quantifiable aspects of cell migration depend on the degree to which the cells adhere to the slide, any decrease of cellular adhesion would make reproducible results difficult. Decreased cellular adhesion could result from inconsistency in multiple factors, including temperatures of the migration buffer or the heated incubation box (cold reduces adhesion), levels of cations in the cell suspension (cations affect integrin binding), slide handling (tapping or flexing the slide could dislodge cells), and cell density (collisions between cells could affect migration parameters). Other sensitive points during the procedure include not pipetting the buffer into the slide wells with too much force prior to attaching the tubing (as this could reduce cell adhesion) and keeping the time required for protocol steps consistent in every experiment (cell settling time could affect adhesion). In summary, any possible influence on cell adhesion must be kept consistent throughout repetitions to produce reliable cell migration data.

This method is easy to set up since it uses commercially available equipment and supplies and none of the steps require advanced instruction. For analysis of MZB, as well as other cell types such as cultured CD8⁺ T cells, coating the slides with various integrin ligands is sufficient to observe the migration up the flow. Studying flow-induced migration response to various integrin ligands leads to direct identification of active integrins on the cell surface, possibly revealing mechanisms relevant to *in vivo* functions as with MZB migration up the flow on ICAM-1 but not VCAM-1. However, it is also possible to add an endothelial cell layer to the flow chambers. One example of immune cell migration that is affected by shear flow is T cell extravasation through endothelial layers³. This procedure was used to unravel the activation of T cell adhesion *via* integrins, selectins, and chemokines, and to model lymphocyte migration through the blood-brain barrier. The only limitation to the cells that can be analyzed with this method is that they must be able to sense the force of flow as a directional signal.

Although the method outlined here is used to characterize cellular behavior such as velocity and turning, it can also be extended to analysis of the molecular aspects of migration. Molecular complexes relevant to flow-induced migration, including integrins such as LFA-1 and their cytoskeletal adaptors, would be located within 200 nm of the surface of the slide, amenable to visualization with TIRF microscopy. This addition to the method would be ideal for studying cells from mice with mutations in integrin- or cytoskeleton-related proteins. As many immune cells migrate at various stages in their development through blood or lymph flows, the *in vitro* migration described can be used to systematically test many kinds of cultured or primary leukocytes for response to flow and reveal how the cells are programmed to migrate in an immune response *in vivo*. In conclusion, the assay described here provides a reliable and straightforward method for analyzing flow-induced migration of MZBs that can also be extended to other cell types.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

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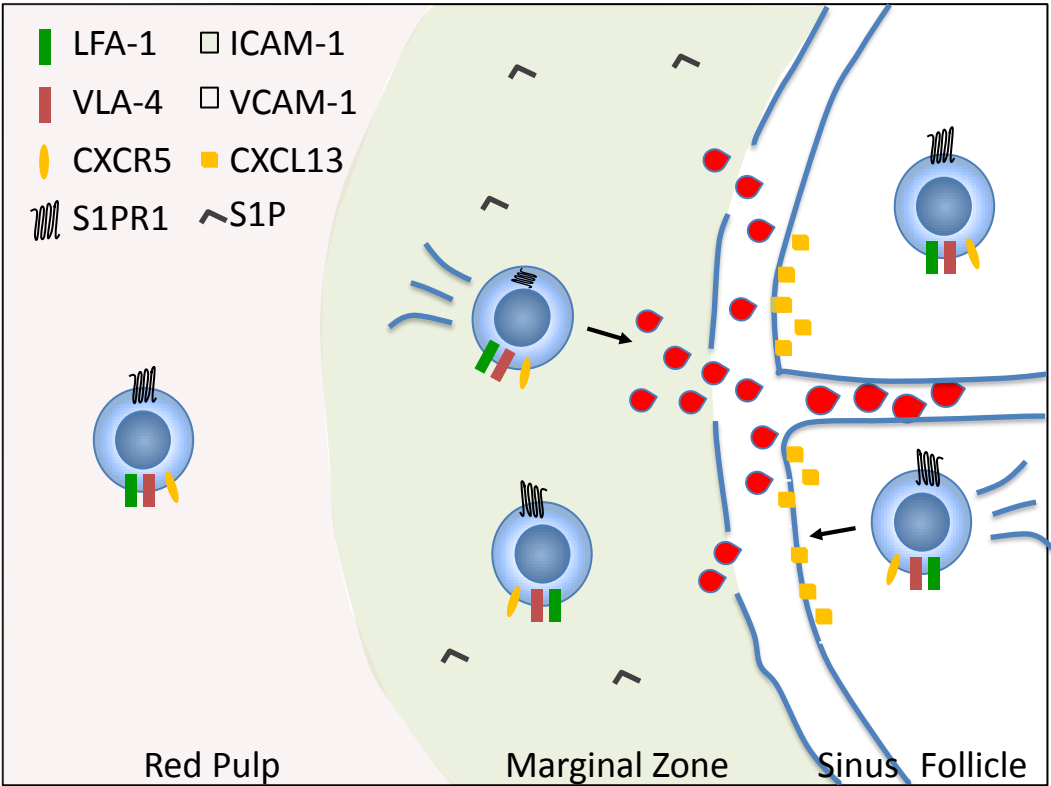
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508

Figure 1

A



B

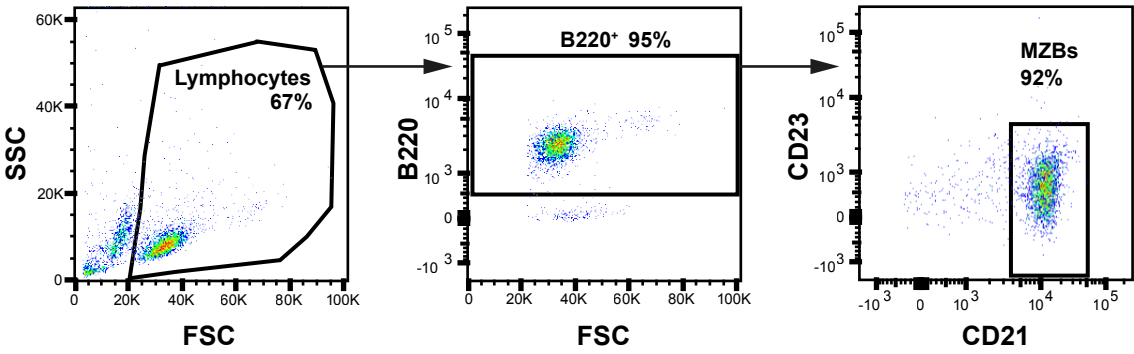
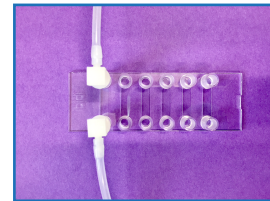
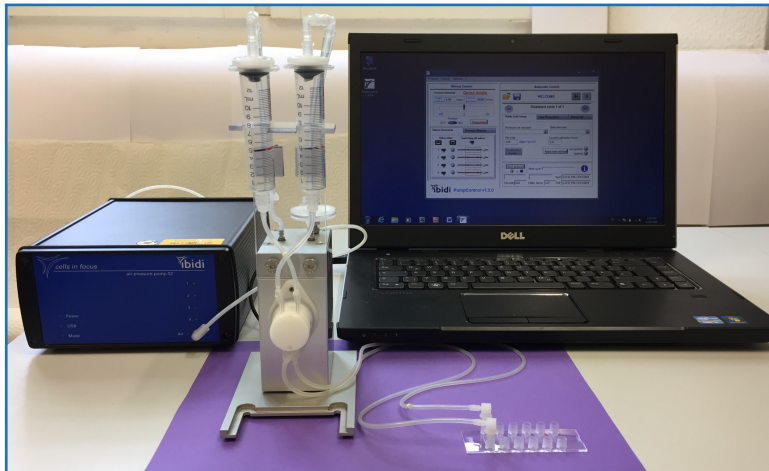
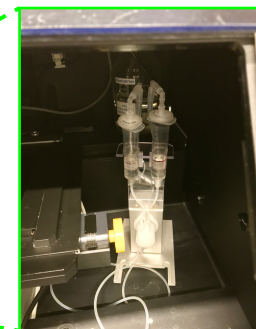
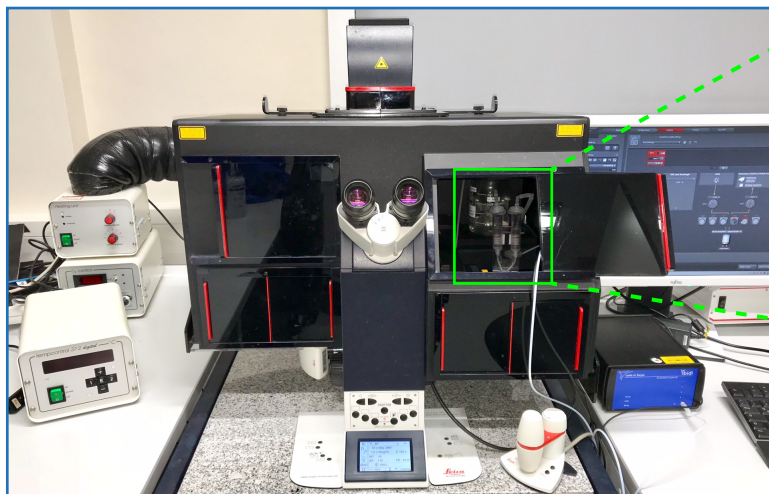
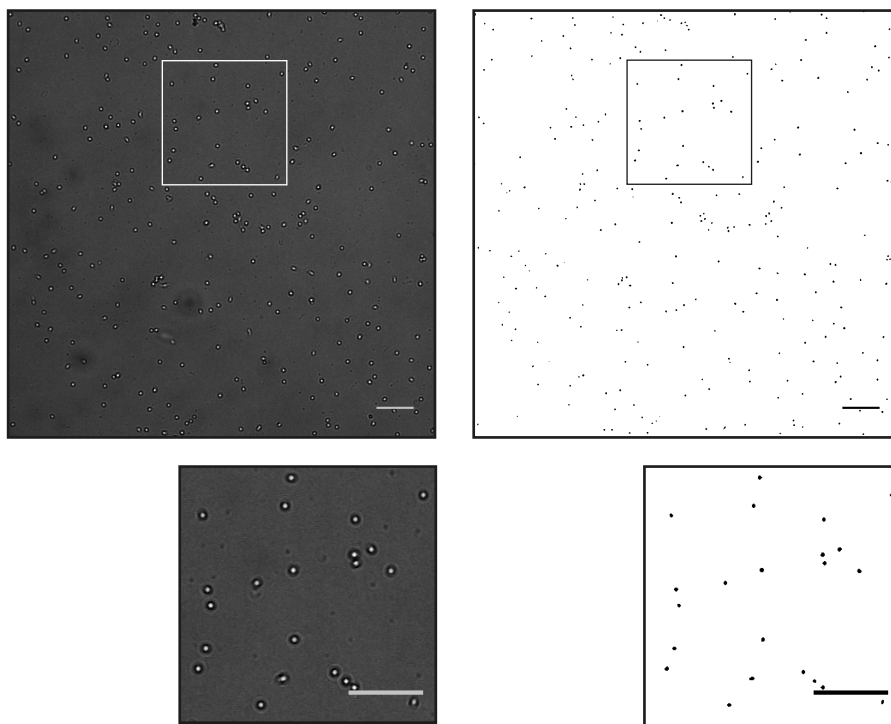


Figure 2

A**B**

A

After thresholding



B

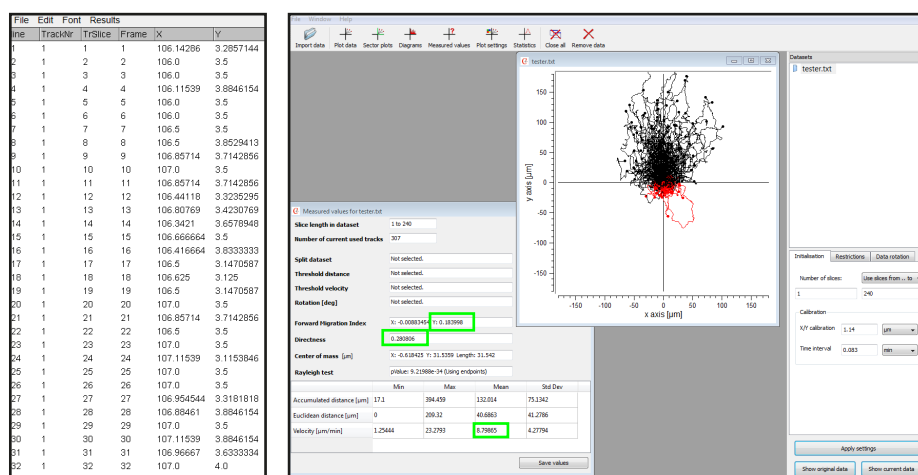
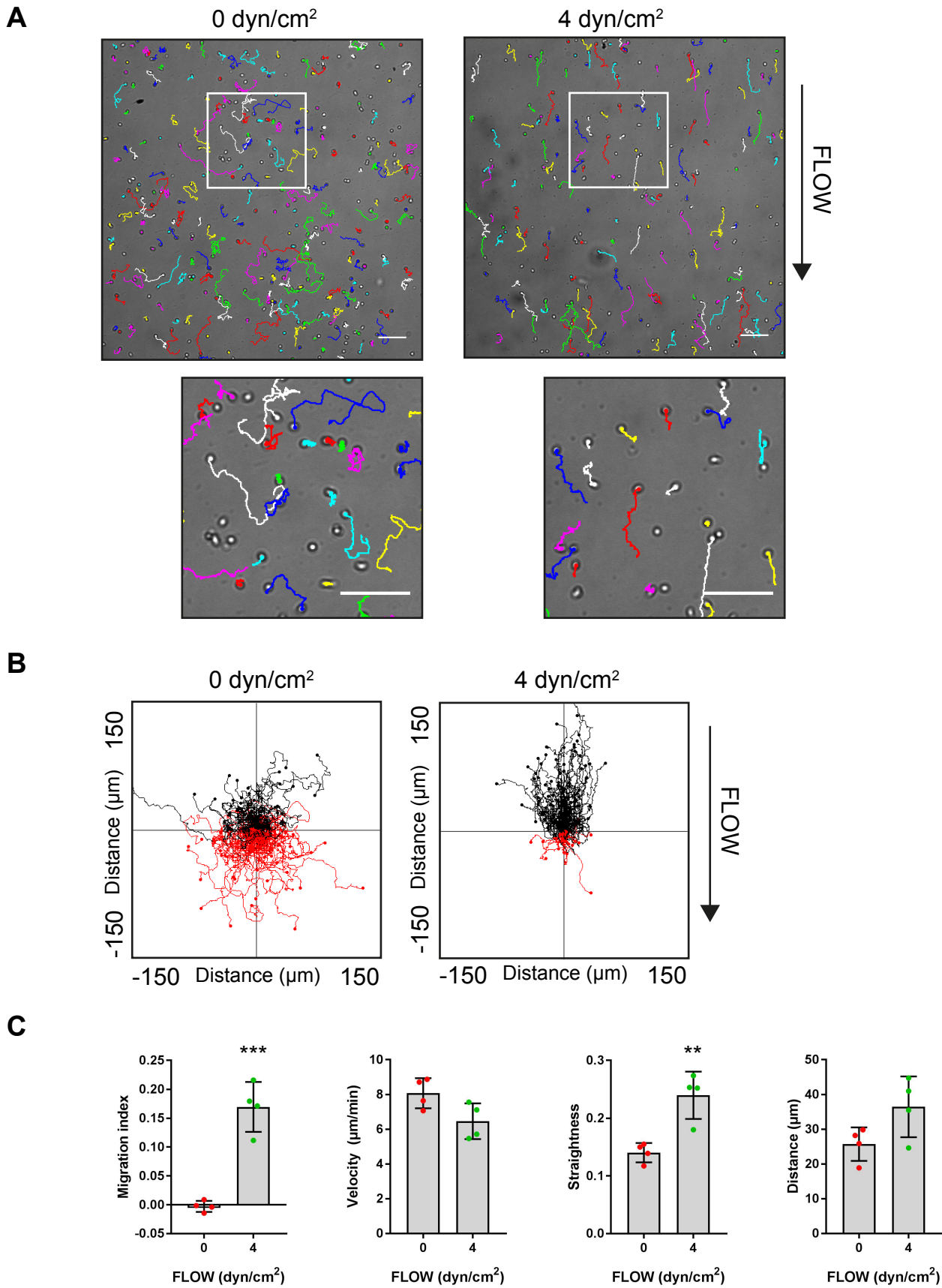


Figure 4



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
VWR Cell Strainer, 70 µm	VWR	10199-656	
Pre-Separation Filters, 30 µm	Miltenyi	130-095-823	
MZB and FOB cell isolation kit	Miltenyi	130-100-366	
B220 CD45R, clone RA3-6B2, FITC	Biolegend	103206	
CD21 / CD 35, clone 7G6, APC	BD Biosciences	558658	
CD23, clone B3B4, PE	Biolegend	101608	
HBSS	Biochrom	L2035	
D-PBS 1x	Gibco by Life Technologies	14190-094	
BSA albumin fraction V, fatty acid-free	Roth	"0052.3"	
ICAM-1	R&D Systems	796-IC-050	
Ibidi µ-slides VI 0.4, hydrophobic, uncoated	Ibidi	80601	
Perfusion set, white, 50 cm, 0.8 mm	Ibidi	10963	
Ibidi Pump system	Ibidi	10902	



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Comment 1:

There is a 2.75 page limit for filmable content (including headings and spacing). After you have made all the changes, please ensure that the highlighted content meets this page limit.

Done ✓ We have now highlighted only 2.75 pages.

Comment 2:

Please provide a reference for how to perform this procedure.

Done ✓

Comment 3:

This step is vague. Please either provide more specific details for the settings or unhighlight it.

We have now corrected this step to include the specific settings.

Comment 4:

Please include all relevant details that are required to perform the step in the highlighting.

We added one explanatory sentence with a link to the relevant file that contains more information on how to install it.

Comments 5, 7:

Because the weblinks are quoted as references now, please indicate the specific reference number here.

We changed the “[LINK]” to “[Supplemental Code Files]” and marked the relevant files as such in the JoVE submission system.

Comment 6:

The Note is highlighted for filming while the steps above are not. Please include all relevant details that are required to perform the step in the highlighting. 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.

We corrected the highlighting to limit it to 2.75 pages and have fixed this as a result.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. [Done ✓](#)
2. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .svg, .eps, .psd, or .ai file. [Done ✓](#)
3. Figures 2 and 3: Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate figure Legend. [Done ✓](#)
4. Please rephrase the Introduction to include a clear statement of the overall goal of this method.
[Done ✓. We re-wrote the Introduction a bit to answer both this comment and one below. We re-ordered the paragraphs, emphasized the main goal of the protocol, and removed some redundant phrasing.](#)
5. Please spell out each abbreviation the first time it is used. [Done ✓.](#)
6. Please use SI abbreviations for all units: L, mL, μ L, h, min, s, etc. [Done ✓.](#)
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10. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution. [Done ✓.](#)
11. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. [Done ✓.](#)

12. In the JoVE Protocol format, “Notes” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section. [Tried to do: we removed some notes, but others seemed important and we had a hard time moving them. Please advise if the remaining Notes are acceptable. If not, we will delete or move to the Discussion.](#)

13. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Some examples: [Tried to do.](#)

For culture media and buffer, please spell out at first use and provide composition. If they are purchased, please cite the materials table. [Done ✓.](#)

Line 102: Please specify the filter type, pore size, etc.

[Done ✓. New line 99: We specified “70 µm cell strainer mesh” in the text and put the specific company and order number in the Excel table.](#)

Line 103: Additional 5 mL of what? Please specify. [Done ✓. New line 103.](#)

What happens after centrifugation? Is supernatant discarded? Please specify throughout. [Done ✓.](#)

1.3: Is 2 min set for incubation or the whole lysis process? Please specify. [Done ✓. New line 109.](#)

1.3.1 and 1.3.2: Please specify the buffer used in these steps. [Done ✓. New lines 132, 136.](#)

1.4, 1.5: Please ensure that the protocol here can stand alone. As currently written, users must refer to another protocol and refer back and forth in order to complete this protocol. [Done ✓. We rewrote the “MZB purification” section to remove mention of the Miltenyi kit and added a more complete description of the procedure in steps 1.3.1 to 1.3.5. We now describe it a “commercially available kit” \(new line 123\).](#)

1.5.2: Please describe how analyze using flow cytometry. [Done ✓. New lines in section 1.4.1 describing how to gate on the MZBs and added a figure \(Figure 1B\) to show the FACS gating strategy.](#)

14. Lines 146-150: The Protocol should contain only action items that direct the reader to do something. Please either write the text in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.), or move the solutions, materials and equipment information to the Materials Table. [Done ✓.](#)

15. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-

steps as necessary. Please move the discussion about the protocol to the Discussion.
Done ✓.

16. Please remove the weblink and use a superscripted numbered reference instead.
Done ✓.

17. Lines 311-323: Please remove these commands from the protocol and instead include them in a supplemental file. Done ✓.

18. For in-text references, the corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text (before punctuation but after closed parenthesis). The references should be numbered in order of appearance. Done ✓.

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20. References: Please do not abbreviate journal titles. Done ✓.

Reviewers' comments:

Reviewer #1:

Application of interest to a broad area of scientists.....could be improved by adding suggestions of complementary applications (ie effect of added chemokines/ blocking reagents etc...).....data if available....

Done ✓. We added this sentence (Line 80-83): "These values can be used to determine the effects of migration inhibitors, cell stimulators, chemokines, and other migration-affecting chemicals on the shear-flow induced migration in order to understand the forces controlling immune cell movement in vivo."

could use CD1d as an additional MZ B cell marker.....LFA-1, ICAM_1 expression?

Done ✓. We "or any other combination of fluorophores for distinguishing between follicular B cells and MZBs" (Line 152).

please mention source of ICAM protein....

Done ✓. We listed the ICAM-1 in the Excel Materials table.

Reviewer #2:

Manuscript Summary:

In this manuscript, Tedford et al. describe a method for visualizing and quantifying shear flow-induced cell migration (flowtaxis) using a simple flow chamber method. The authors use marginal zone B cells as a cell model for testing this method given the fact that the lab's previous research elegantly showed MZ B cell migration against flow in vitro and in vivo. The manuscript is well-written and detailed, and likely to be useful to a

broader scientific community. A few issues and minor typos were found throughout the manuscript that should be corrected.

Major Concerns:

None

Minor Concerns:

I wouldn't call it concern but there are a few minor details that need to be clarified.

These are described below:

Line:

67 Authors should describe cell isolation methods and may mention a particular kit but the manuscript should avoid over-advertisement of specific companies. For example, authors may write "cells were magnetically enriched with commercially available kits".
Done ✓. We rewrote the "MZB purification" section to remove mention of the Miltenyi kit in section 1.3. We now describe it a "commercially available kit" (new line 121).

69-74 Authors may leave the details for the main protocol and just mention that they will be explaining the use of an automated tracking tool (M2track) vs manual tracking in Image J . Done ✓. We re-wrote the Introduction a bit to address this comment and the one above. We re-ordered the paragraphs, emphasized the main goal of the protocol, and removed some redundant phrasing. We removed the reference to the manual tracking plugin from the introduction as it is not critical to the protocol.

130 The last step of the magnetic isolation is not well described. This reviewer had to look up the protocol online to understand which fraction they were referring to.
Done ✓. We rewrote the "MZB purification" section and added a more complete description of the procedure in steps 1.3 to 1.3.5. We specified which cells are in the labeled and unlabeled fractions in the two sorting steps.

133/134 When explaining antibody stains use quantity of antibodies/ X number of cells, not volumes or dilutions of antibodies. Different vendors may sell antibodies at different concentrations. Done ✓. Lines 149-152: We added the necessary information. The sentence now reads: "Add a 10 % volume (5 µl) of Fc block to 10,000 cells in 50 µl and incubate for 5 min at 4°. Add 50 µl of Cell Buffer containing 0.3 µl each of B220-FITC (0.15 µg), CD21-PE (0.06 µg), CD23-APC (0.06 µg), in a total volume of 105 µl (each antibody diluted 1:350)."

139 Describe ages for mice used.

Done ✓. Line 97: We added this: "...a mouse aged 8-16 weeks old..."

163 Correct ICAM-1 concentration to 400µg/mL. Done ✓. New line 179.

163 This is not a 100% clear. Do I understand correctly that only 1 chamber of the IBIDI slide is coated? It would be helpful to have a schematic of the flow chamber with the IBIDI slide as an additional figure (maybe even if it is shown clearly in the movie). I assume that only one chamber is used for the experiment and all reagents (coating, blocking, cells) are applied to this one chamber.

191 As mentioned above, a schematic representation of the flow chamber slide and the fluidics setup would be very helpful in addition to the video.

Done ✓. New line 179-181: We clarified that the slides have 6 chambers each and the researcher can coat as many as there are planned movies: "...and add 30 µl of it [ICAM-1] to

one chamber on a flow slide for every migration movie required. Each slide contains 6 chambers and a researcher can easily record up to 8 movies in one session.” We also added a new Figure 1A that shows an image of the flow chamber slide with tubing attached to one chamber, up close in a high magnification image, as well as an image of the pump, fluidics unit, and attached slide.

222 Which resolution is used? What are the pixel dimensions in micrometer?

Done ✓. New line: 324. We added “..For MZB, a 10x objective was used, giving a pixel size of 1.14 μm .”. Also in Figure Legends, new line 389: “..Calibration settings shown include the pixel resolution of 1.14 μm per pixel and a time interval of 5 sec (0.083 min) per movie frame.”

237 Correct typo. Done ✓.

276 How exactly is the threshold determined? If automated thresholding is used in ImageJ, which algorithm is the automated thresholding based on?

Done ✓. We re-wrote section 3.2.1, “Threshold images from the cell migration movie:” to explain how to turn the grayscale or color image of migrating cells into a black and white movie. We write that we simply boosted the contrast of the images to the maximum setting to get white cells on a black background. We then used the threshold command to convert this to black cells on a white background, as this is required by the MTrack2 plugin.

292 What is the size distribution of the MZB cells? It may be useful to show a histogram of MZB size as an additional figure.

We wrote that the MZB sizes are fairly uniform and they would therefore all be contained within the range we chose (from 1 to 20-30 pixels). We made a histogram of MZB size for about about 600 thresholded MZBs in pixels (see below). The sizes range from about 2 to 26 pixels, therefore the value chosen in MTrack2 for the upper size limit (30 pixels), is appropriate. We did not add this additional figure to the manuscript for 2 reasons: one is that the sizes are not precise outlines of the MZBs because they are measured on cells that are slightly defocused and therefore a bit larger; two, we think it may be sufficient to specify the values we used in the text (1-30 pixels) for MZBs, and a researcher using the protocol for a different kind of cell would in any case measure the sizes of those cells. However, we can add the figure to the manuscript if necessary.

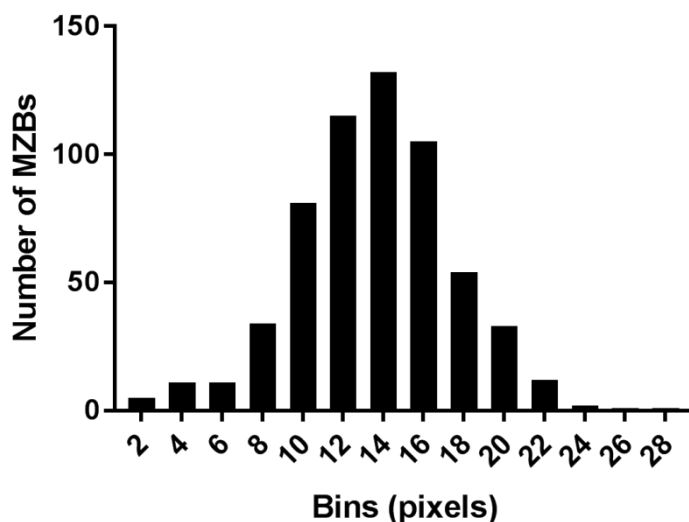


Figure 1R: Size distribution of MZBs used in the movies shown in Figure 3B. 1 Pixel = 1.14 μm .

401 Close-ups of the microscopy images should be shown - it difficult to see the cells and to judge the quality of the binary image. Scale bars are missing.

We now include macro views of a portion of the microscope images and scale bars for all images in Figures 3 and 4.

404 I don't think this figure 2B is necessary if the macro is already included in the protocol.

Agreed, we removed the figure. We will upload the macro text as a separate file.

407 A colored frame could be used to highlight the window / or the values in the screenshot that are mentioned in the figure legend.

We added a bright green boxes to Figure 3b to the three values specified in the legend.

410 What are the red tracks in comparison to the black tracks? Please state in the figure legend.

We added a sentence to the legend for Figure 4b explaining that the red tracks terminate below the horizontal axis while the black tracks terminate above the axis. The color difference is to help to roughly determine the direction of the migrating cells with a quick glance.

416 Which statistical test was used here?

We added the missing information: Student's *t*-test.

423 Is VCAM1 blocking migration against the flow in the presence of ICAM1? Please state this more clearly.

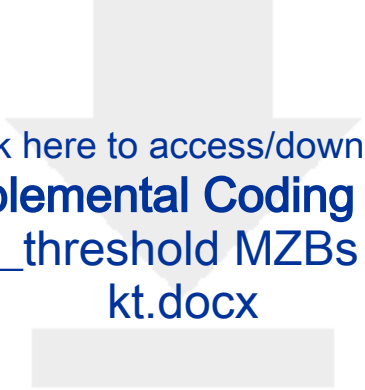
We apologize for not stating this clearly enough. The MZBs under flow on ICAM-1 can migrate relatively long distances up the flow but on VCAM-1 they are mostly stuck fast. On ICAM-1 alone, about 80% of MZBs move more than a cell length but on VCAM-1 only 30% of MZBs move more than a cell length. We believe that if the flow is strong enough, the weak MZB migration on VCAM-1 translates to being pushed down the flow. Otherwise, the MZBs stand fast on VCAM-1, and not because it specifically blocks them from migrating up the flow, rather that it just does not support migration up the flow. We now state this in the legend for Figure 1A, the model of MZB shuttling (New Line 366).

For the discussion of the method, it is important to discuss how comparable the in vitro measured data is to the in vivo situation. For example, how does 4 dyne/cm² apply to fluid flow in the MZ 3-D environment?

It is difficult to discuss the in vivo flow situation in terms of specific shear stress numbers and to compare it to the 4 dyn cm⁻² flow that we used in vitro. The early publications that described the existence of flow through the marginal zone, using corrosion casts or injections of labelled particles, did not determine specific numbers for pressure or flow rate in this area. However, it is clear that the blood flows through the marginal zone. We observed flow using injections of labeled antibodies, and the Cyster group observed flow using 2-photon microscopy showing fast movements of B cells caught in flow streams in the marginal zone. There are studies on the rates of blood flow entering the spleen in humans

and mice but to our knowledge there is nothing known on the rates of blood flow or pressure specifically in the marginal zone. It's possible that it's not easy or technically feasible to measure flow just in this area.

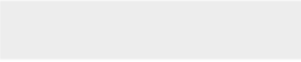
What we can say, is that we showed that on ICAM-1, MZBs migrate in all directions at a flow rate of 1 dyn cm^{-2} but migrate directionally at a flow rate of 3 dyn cm^{-2} . They maintain the directional migration up to $15\text{-}20 \text{ dyn cm}^{-2}$ without much change in their behavior, but we have not investigated what happens at higher shear flow levels. We therefore assume that the flow through the marginal zone is equivalent to at least the 3 dyn cm^{-2} flow that we used, but may be even higher.



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Supplemental Coding Files

Tedford_MACRO_threshold MZBs and run MTrack2
kt.docx





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