Lymphatic endothelial cell calcium pulses are sensitive to spatial gradients in wall shear stress

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Supplemental Information

Supplemental Movies

Fluorescent micrograph frames displaying Ca\(^{2+}\) dynamics in HLMVECs are described in brief below:

Supplemental Movie S1. HLMVEC Ca\(^{2+}\) dynamics in the IFC, recorded for 30 minutes starting from the onset of flow at \(t = 50\) s. Regions corresponding to Rings 1 and 2 are shown, which have average WSSs of 32 and 65 dyn/cm\(^2\), respectively. The flow direction is radially outward and symmetric about the jet center at the center of Ring 1. Frames were recorded every 5 seconds. Scale bar, 100 \(\mu\)m.

Supplemental Movie S2. HLMVEC Ca\(^{2+}\) dynamics as in Movie 1 for Rings 2 - 6, which have average WSSs of 65, 53, 30, 17 and 11 dyn/cm\(^2\). The flow direction is radially outward and here is roughly from left to right. Frames were recorded every 5 seconds. Scale bar, 100 \(\mu\)m.

Supplemental Movie S3. HLMVEC Ca\(^{2+}\) dynamics for cells exposed to uniform WSS (parallel plate flow), recorded for 30 minutes from the onset of flow at \(t = 50\) s. Here, all HLMVECs experience a WSS of 50 dyn/cm\(^2\). The flow direction is from the bottom of the video to the top. Frames were recorded every 5 seconds. Scale bar, 100 \(\mu\)m.

Supplemental Movie S4. HLMVEC Ca\(^{2+}\) dynamics under no flow conditions, recorded for 30 minutes. Frames were recorded every 5 seconds. Scale bar, 100 \(\mu\)m.

Image Postprocessing
Following acquisition (Supplemental Movies S1 – S4), image pixels were smoothened using the **Smooth** function in ImageJ and binned 4 by 4 (2560 x 2160 to 640 x 540) to reduce file size.

**Raw Data Acquisition**

The cytoplasm of every individual cell within the field of view was manually approximated using the oval shape trace tool on ImageJ and stored locally using the **ROI Manager**. The cells did not display any significant centroid movement over the course of the experiment due to the small-time window relative to the timescale of migration. A custom macro was written to measure the x and y centroid position, approximate area, extracellular background fluorescence intensity and cellular integrated density for each cell.

**Data Analysis**

The fluorescence intensity of each cell was normalized by its own background (i.e. resting/baseline fluorescence intensity in the absence of a pulse) to standardize the parameters gathered across all cells. This was performed by determining the average cellular background intensity between pulses and correcting all values per cell for this background as in Equation 1:

\[
(1) I = \left(\frac{1}{Area}\right) \left[\text{Integrated Density} - \left(Area \times \text{Background Mean Gray Value}\right)\right]
\]

A representative plot for the result from Equation 1 for an example cell is presented in Figure 1.

\( \text{Ca}^{2+} \) pulses were determined through an amplitude threshold (1.5 times the normalized cellular background intensity), and local maximum (i.e. first derivative, positive to negative
sign change) test. This threshold was optimized through careful iteration and comparison both visually and numerically to minimize false positives and missed pulses.

Figure 1E demonstrates the calculation of the pulse count, peak-to-peak period, full-width-half-max (FWHM), and pulse onset time. The pulse count was defined as the number of peaks observed during the 30 minute recording time and is a measure of pulse frequency. The pulse onset time is the experimental time from \( t = 0 \) at the onset of flow required to reach the first observed pulse. The peak-to-peak period (\( \tau \)) is the time between the maximum intensities of consecutive pulses. The FWHM was determined through the difference of the experimental times \( T_1 \) and \( T_2 \) for a given pulse, where the intensities are half of the maximum observed. As a further check to minimize error, the first derivative at these time points was taken to ensure the proper positive to negative sign change. From this measurement, the duty cycle (DC; between 0 and 1) was determined using Equation 2,

\[
(2) \quad DC = \frac{FWHM}{\tau_{avg}}
\]

where \( \tau_{avg} \) is the average peak-to-peak period for a given cell. The DC for a given cell is determined across all of its observed pulses, and requires at least two pulses. The measured cumulative distribution function (CDF) values for cells in a given ring or flow condition were determined using the empirical CDF function \( ecdf \) on MATLAB. In a CDF plot, the \( x \)-axis represents the range of a variable of interest. The \( y \)-axis shows the probability of encountering a data point less than or equal to that \( x \)-value. The measured CDFs were compared to plausible probabilistic models as described in the main text.
For determination of the corrected total nuclear fluorescence (CNTF) in Figure S1, Equation 1 was used with a nuclear mask rather than a cell trace in order to determine the nuclear intensity for NFATc1.

**Analysis of Cell Clustering**

A clustering analysis was conducted to determine whether the pulsing frequency of neighboring cells were spatially correlated. The x- and y- positions of each cell were determined as in the earlier analysis of Ca^{2+} dynamics (see *Supplemental Information* for further details). The k-means clustering algorithm as implemented in MATLAB (*kmeans*) was then used to determine cell clusters (Figure S2). As a practical assumption, the analysis assumed that only cells that were in direct contact could form a cluster. This clustering algorithm generated clusters of roughly 8 – 10 cells. The algorithm also identified the Voronoi center, a mathematical term identifying the centroid/center-of-mass of the cluster (represented as a bolded x on Figure S2), along with the cluster's boundaries (represented as the centroids of the cells within that cluster). Certain cells may exist on the fringes of multiple clusters (usually, two) and can thus be placed in either, resulting in a bi-, tri-, etc. phasic steady-state for cluster convergence. In our method, this had an insignificant effect on the Voronoi center (*data not shown*). The clustering algorithm was iterated at least 1,000 times, until the total Euclidean distance of all cells to their respective clusters’ Voronoi centers was minimized. We found that cluster dimensions and Voronoi centers were robust.

We next determined the fraction of P cells (PC > 1) in each cluster for each experimental condition examined in the study (Figure S4). For comparative purposes, the
analysis was repeated after randomly shuffling the assignment of the observed pulse counts amongst the cells for a given experiment. This generated virtual experiments in which any spatial correlation in the fraction of P cells would be lost (Figure S5).
Supplemental Figures

Figure S1. NFATc1 nuclear fluorescent intensity increases in response to impinging flow. (A) Fluorescent micrographs showing VE-Cadherin, actin, nucleus, and NFATc1 immunofluorescence for HLMVECs subjected to impinging flow for 30 min, or under no flow. Scale bar, 100 μm. Expanded image of NFATc1 fluorescent intensity panels in (A) are presented for (B) impinging flow or (C) no flow. Scale bar, 50 μm. (D) Quantification of corrected total nuclear fluorescence (CTNF) for HLMVECs subjected to impinging flow (N = 329 cells total, binned by ring number; N₁ = 28, N₂ = 81, N₃ = 84, N₄ = 76, and N₅ = 60 cells) or under no flow (N = 360 cells). Experiments were performed in duplicate. Error bars represent standard error on the mean. Asterisks indicate statistically significant differences in the distributions of the different ring numbers with the no flow condition as determined using the Wilcoxon singed-rank sum test (n.s., not significant; *, p < 0.05; **, p < 10⁻⁴; ***, p < 10⁻⁷).
Figure S2. Box-and-whisker plot comparing the pulse durations (FWHM) for cells exposed to impinging flow (Rings 1 – 6), parallel plate flow, or no flow. For each condition, the left (darker color) boxplot shows the pulse durations for NP cells (PC = 1). The right (lighter color) boxplot shows the pulse durations for the first pulse of P cells (PC > 1). Experiments were performed in triplicate. Asterisks indicate statistically significant differences in these distributions as determined using the Kolmogorov – Smirnov test (n.s., not significant; *, p < 0.05; **, p < 10⁻⁴; ***, p < 10⁻⁷).
Figure S3. Box-and-whisker plots comparing the pulse durations (FWHM) for repeatedly pulsing cells (PC > 3) exposed to impinging flow for Rings 1 – 6 (panels A – F), spatially uniform WSS (parallel plate chamber) (G), or no flow (H). Each boxplot represents durations of the first (purple), second (pink), third (green) or fourth (coral) pulses, where each data point corresponds to an individual cell. (I) Average pulse durations for each dataset in A – H. Experiments were performed in triplicate.
Figure S4. Example plots showing k-means clustering applied to cells from the (A) jet center (Rings 1 – 2), (B) far rings (Rings 3 – 6), (C) parallel plate, and (D) no flow experiments. The x- and y- axes represent the spatial positions of cells (black dots: EC locations; lines: cluster boundaries; bolded “x”: cluster centroid). The fraction of P cells (PC > 1) was determined and color coded for each cluster. Experiments were performed in triplicate.
**Figure S5.** Box-and-whisker plot comparing the fraction of P cells per cluster for cells exposed to impinging flow (Rings 1–6), parallel plate flow, or no flow. For each condition, the left (darker color) boxplot shows the experimentally observed values. The right (lighter color) boxplot maintains the same spatial position of cells for each experiment but randomly assigns each cell a pulse count from the experimental distribution (Figure 3). Experiments were performed in triplicate. Asterisks indicate statistically significant differences in these distributions as determined using the Kolmogorov–Smirnov test (n.s., not significant; *, p < 0.05; **, p < 10^{-4}; ***, p < 10^{-7}).
Figure S6. Box-and-whisker plots comparing peak-to-peak periods between consecutive pulses for repeatedly pulsing cells (PC > 3) exposed to impinging flow for Rings 1 – 6 (panels A – F), spatially uniform WSS (parallel plate chamber) (G), or no flow (H). Each boxplot represents either the time between the first and second pulse (purple), the second and third pulse (pink), or the third and fourth pulse (green) where each data point corresponds to the measurement for an individual cell. (I) Average peak-to-peak periods for each dataset in A – H. Experiments were performed in triplicate.

Figure S7. HLMVECs were treated with either 10 μM SKF96365, 5 μM cyclosporin A, or left untreated (control) under no flow. CDF plots of Ca²⁺ (A) pulse count (B) peak-to-peak period (C) DC (D) FWHM for NP cells (single pulse; pulse count = 1) are presented for each condition. Insufficient pulses were recorded under these conditions for cells treated with 10 μM SFK96365. Cells in each tested condition came from a
confluent well. The average of each measured distribution is presented in the bottom right corner of each graph. Asterisks indicate statistically significant differences in CDFs as determined using the Kolmogorov–Smirnov test (n.s., not significant; *, p < 0.05; **, p < 10^{-4}; ***, p < 10^{-7}).

**Figure S8.** HLMVECs were treated with either 10 μM SKF96365, 5 μM cyclosporin A, or left untreated (control). CDF plots of Ca^{2+} pulse count for HLMVECs exposed to impinging flow are presented for Rings 1 – 6 (panels A – F). The colored dots indicate the measured CDF and the bold lines indicate a fit of the CDF to a Γ-Poisson distribution. The average of each measured pulse count distribution is presented in the bottom right corner of each graph. Experiments were performed in duplicate. Asterisks indicate statistically significant differences in CDFs as determined using the Kolmogorov–Smirnov test (n.s., not significant; *, p < 0.05; **, p < 10^{-4}; ***, p < 10^{-7}).
Figure S9. HLMVECs were treated with either 10 μM SKF96365, 5 μM cyclosporin A, or left untreated (control). CDF plots of peak-to-peak periods for HLMVECs exposed to impinging flow are presented for Rings 1 – 6 (panels A – F). The average of each measured distribution is presented in the bottom right corner of each graph. Experiments were performed in duplicate. Asterisks indicate statistically significant differences in CDFs as determined using the Kolmogorov – Smirnov test (n.s., not significant; *, p < 0.05; **, p < 10^-4; ***, p < 10^-7).
Figure S10. HLMVECs were treated with either 10 μM SKF96365, 5 μM cyclosporin A, or left untreated (control). CDF plots of Ca\(^{2+}\) average FWHM (pulse duration) for P cells exposed to impinging flow are presented for Rings 1 – 6 (panels A – F). The average of each measured distribution is presented in the bottom right corner of each graph. Experiments are performed in duplicate. Asterisks indicate statistically significant differences in CDFs as determined using the Kolmogorov – Smirnov test (n.s., not significant; *, p < 0.05; **, p < 10\(^{-4}\); ***, p < 10\(^{-7}\)).
Figure S11. HLMVECs were treated with either 10 μM SKF96365, 5 μM cyclosporin A, or left untreated (control). CDF plots of Ca$^{2+}$ average FWHM (pulse duration) for NP cells (single pulse) exposed to impinging flow are presented for Rings 1 – 6 (panels A – F). The average of each measured distribution is presented in the bottom right corner of each graph. Asterisks indicate statistically significant differences in CDFs as determined using the Kolmogorov – Smirnov test (n.s., not significant; *, $p < 0.05$; **, $p < 10^{-4}$; ***, $p < 10^{-7}$).