Endothelial glycocalyx regulates cytoadherence in *Plasmodium falciparum* malaria

Supplemental data

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S1. Healthy red blood cell suspension

Human red blood cells (RBCs) were sedimented by centrifugation at 3000 rpm for 5 minutes and the resulting supernatant aspirated. Leukocytes (white cells) tend to form a band at the red cells/plasma interface. The red cells pellet was resuspended in wash medium, prepared by adding the following components to a 500 ml bottle of RPMI-1640 culture medium: 18.75 ml of 1M Hepes, 5 ml of 20% glucose solution, 3 ml of 1M NaOH solution, 1.25 ml of gentamicin sulphate solution, 5 ml of 200 mM glutamine solution, and 0.5 ml of 100 mM hypoxanthine solution (all chemicals were bought from Sigma-Aldrich). RBCs were isolated from any remaining leukocytes and platelets using Lymphoprep (STEMCELL Technologies). The blood was carefully layered on top of Lymphoprep: red cells sedimented to the bottom of the tube, leukocytes, in contrast, remained in the plasma layer as their sedimentation is only slightly affected. The resulting mixture was centrifuged at 3000 rpm for 15 minutes and the supernatant aspirated. Finally, the cell pellet was suspended in wash medium at a 1:1 ratio by volume to have a 50% haematocrit (Hct) suspension, and placed at 4 °C for storage for up to 7-10 days.

S2. HUVEC culture

Primary human umbilical vein endothelial cells (HUVECs, from Sigma-Aldrich) were cultured at 37 °C in a 5% CO_2 incubator on uncoated tissue-culture polystyrene flasks. Endothelial cell growth medium (ECGM) (Sigma-Aldrich) was changed every day. After reaching a cell confluency of 85%, HUVECs were sub-cultured using trypsin (0.25 % trypsin-EDTA, Sigma-Aldrich): after a gentle wash with PBS, 4-5 ml trypsin was added and distributed to the whole flask surface to ensure the solution covered all the cells. After 3-4 seconds, 4.5 ml of trypsin was removed and the flask was put in the incubator for 2-3 minutes. To verify the trypsinization progress, cells were monitored under the microscope until they become rounded. About 5 ml of ECGM was added to the flask to inhibit further tryptic activity. Cells were then centrifuged at 1200 RPM for 5 minutes and re-suspended in ECGM. Finally, cells were counted and subsequently divided into new flasks. ECGM was changed every 1-2 days and HUVECs discarded after 8 passages.

S3. Perfusion system and endothelial layer characterization

HUVECs were subjected to shear stress stimulation in a custom-made flow setup, placed in the incubator at 37 °C and at 5% CO_2. The flow circuit was a closed loop, and ECGM was fed by the action of a peristaltic pump. ECGM from a reservoir passed first through the Ibidi flow

chamber perfusing the HUVEC monolayer, then into the pump, and finally returned into the reservoir. To avoid bubble formation, the reservoir was placed upside down.

The hydraulic diameter of rectangular microchannels is 723 μm. In steady laminar flow and for h<<w, the wall shear stress can be obtained by using the following equation:

$$\tau = \frac{6\eta Q}{wh^2} \quad ; \qquad (1)$$

where τ is the wall shear stress (dyne/cm^2), η is viscosity of the culture medium (Pa·s), Q is the flow rate (ml/s), and h and w are the microchannel dimensions.

In the center of the channel, at the maximum flow rate achievable with our system, the entrance length was about 4.11 mm, pressure drop was 1.67 mbar, and the Reynolds number Re was 128. This value of Re ensures a laminar regime in the microfluidic device. The observations were carried out beyond the entrance length, and pressure drops were negligible, being of the order of 1 mbar.

Images of HUVECs in static and under flow conditions were captured by using an inverted phase-contrast microscope (Nikon) equipped with a 10x objective. The morphology of each cell was analysed automatically by image segmentation to infer several morphological parameters such as area, perimeter, length, and orientation angle. Segmented objects at the edges of the image or too small to be cells (area less than 50 pixel^2) were automatically excluded.

The characterization of the glycocalyx layer was carried out with confocal laser scanning microscopy (Leica TCS SP5). All CLSM images (512x512 pixels, 8 bit pixel depth) were acquired using a Leica HCX PL APO CS 63× 1.4 NA oil immersion objective. HUVECs were washed twice with PBS and then incubated in a Wheat Germ Agglutinin (WGA-Alexa Fluor 488 (Molecular Probes)) (WGA) solution (0.01 mg/ml) for 30-40 min at 37°C.

The fluorescence dye was excited at 495 nm by an argon laser (λ=488 nm) with a long-pass filter (λ=505 nm) and emitted at 519 nm.

In order to estimate the contribution of glycocalyx on malaria-induced cytoadherence, HUVEC monolayer was incubated with 2.5 U/ml neuraminidase (Sigma-Aldrich) for 30 min at 37°C while the control group was kept in the ECGM. After incubation, HUVECs were washed twice in PBS.

S4. Microfluidic experiments

Reynolds number, entrance length, and pressure drop were calculated in the microchannel at the maximum flow rate used for the experiment with RBCs suspensions: Reynolds number was

153.4, entrance length 4.9 mm, and pressure drop 0.21 mbar. A custom-built temperature-control system was used to maintain the entire set-up at 37 ^{o}C. A Nikon Eclipse Ti-E inverted microscope (Tokyo, Japan) was used in bright field with a Nikon 20X (NA 0.75) objective. Motorized functions of the microscope were controlled via custom software written in-house. Images were acquired using a CMOS camera (model GS3-U3-23S6M-C, Point Grey Research/FLIR Integrated Imaging Solutions (Machine Vision), Ri Inc., Canada). Data were collected for about 2 hours to have sufficient events for the analysis, while maintaining the integrity of the HUVECs.

S5. Velocity profiles

A preliminary rheological characterization of our novel set up was performed by flowing HRBCs suspension at 0.5% Hct on bare and endothelialized microchannels. Velocity profiles a function of the normalized channel height (along z axis of Figure S1.A) are reported in Figure S1.B for different wall shear stress. For each wall shear stress, velocity was calculated by automatically tracking 20 healthy and infected cells for 3 different fields of view, and following them for at least 10 frames. The parabolic profiles are evident, and are is in agreement with the fluid dynamic theory being channel height << channel width. As expected, for each shear stress, the profiles for bare and endothelialized channels superimpose one to each other, indicating that the presence of HUVECs does not affect RBC velocity along z axis. Regarding velocity profiles along x axis, they are reported in Figure S1.C as function of the normalized channel width. For both cases (i.e. bare and with HUVEC layer) it is possible to observe a flat (plug-like) fluid velocity profile, the increasing wall shear stress inducing a shift upward of the velocity values. The average HRBCs velocity evaluated in the middle of the channel increases linearly with wall shear stress (Figure S1.D), and it is higher in uncoated channels with a percentage difference from 5.9% to 8.9% ($p<0.001$). The decreasing of HRBC velocity in coated microchannels could be related to the reduction of channel lumen due to the HUVEC layer, which leads a difference of the resistance to flow between bare and endothelialized channels of about 5%.

In Figure S1.E the percentage of HRBCs flowing on HUVECs in tank-trading, tumbling, and rolling motions is reported as function of the wall shear stress. HRBCs are in a tank-treading and tumbling motion before 0.06 Pa, and move from tumbling to rolling afterwards. There is no difference in the dynamics and the transition point from one motion to the other for HRBCs flowing on HUVECs with and without sialic acid.

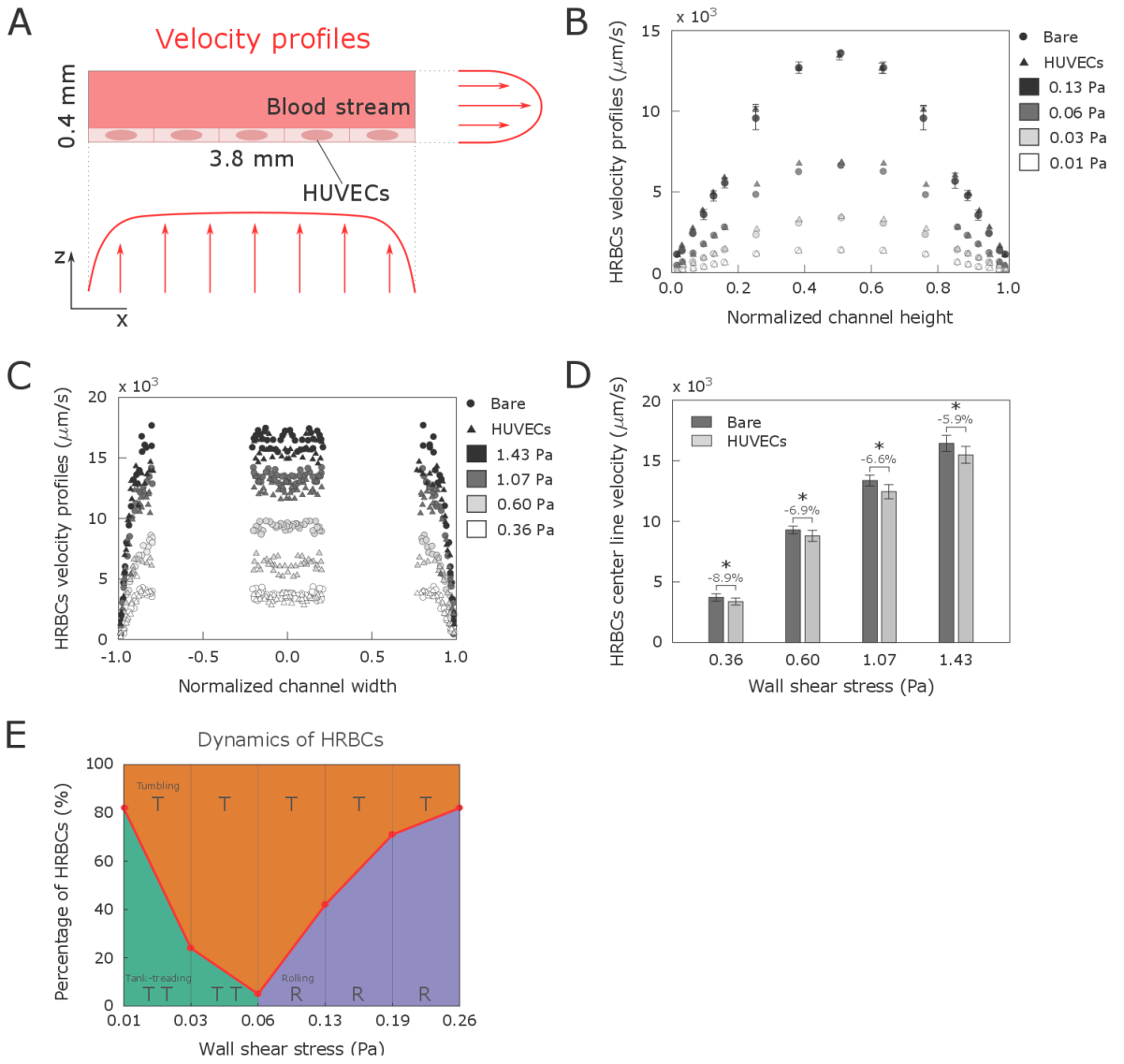

Figure S1. *Velocity profiles for HRBCs in bare and endothelialized microchannels. (A) Sketch of velocity profiles along the channel cross section. Experimental velocity profile along channel height (B) and width (C) at increasing wall shear stress with (triangle) and without (circle) HUVECs. Each point on the panels B and C represents the mean and standard deviation calculated from 20 HRBCs. (D) Average HRBCs velocity in the middle of the channel as a function of wall shear stress. HRBCs velocity is higher in uncoated channels with a percentage statistical significant difference from 5.9% to 8.9% ($p<0.001$). (E) Dynamics of HRBCs in flow is the same in the presence and absence of sialic acid of HUVEC glycocalyx for all the considered wall shear stresses. Most of HRBCs undergo a tank-treading to tumbling transition between 0.01 and 0.06 Pa and then a tumbling to rolling transition between 0.06 and 0.26 Pa.*