Factor H interferes with the adhesion of sickle red cells to vascular endothelium: a novel disease-modulating molecule

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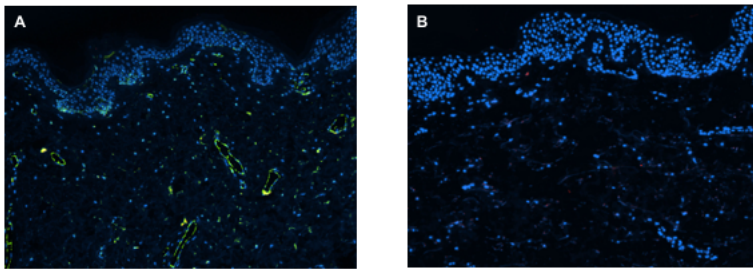
SUPPLEMENTARY FIGURES

Fig. 1S

Fig. 1S. A. CD31 staining showing positive vessels (green fluorescence) in normal skin of control samples. Nuclei were stained with Prolong Gold antifade reagent with DAPI (blue fluorescence). Direct immunofluorescence; original magnification x 100. **B.** Serial section of the same subject showing negative C5b9 expression on microvasculature (orange fluorescence). Direct immunofluorescence; original magnification x 100. One representative image of other 16 with similar results.
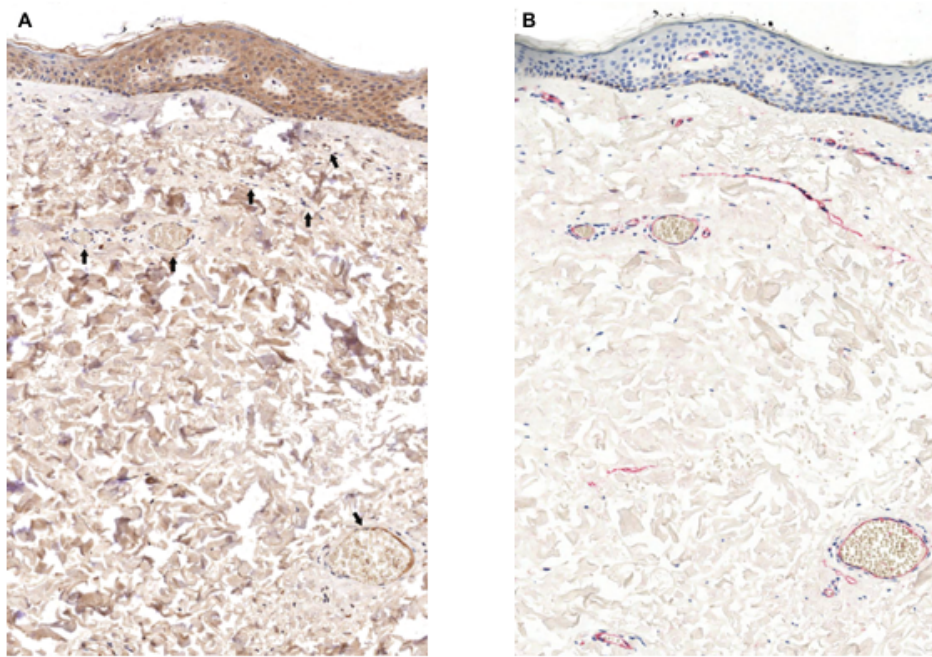
Fig. 2S

Fig. 2S. Representative immunohistochemical image of skin biopsy from healthy control, showing **(A)** all dermal vessel completely negative for C5b9 (arrow) and **(B)** the presence of vessels highlighted with CD31 staining (red) on the same field of panel A, from a consecutive histological section.
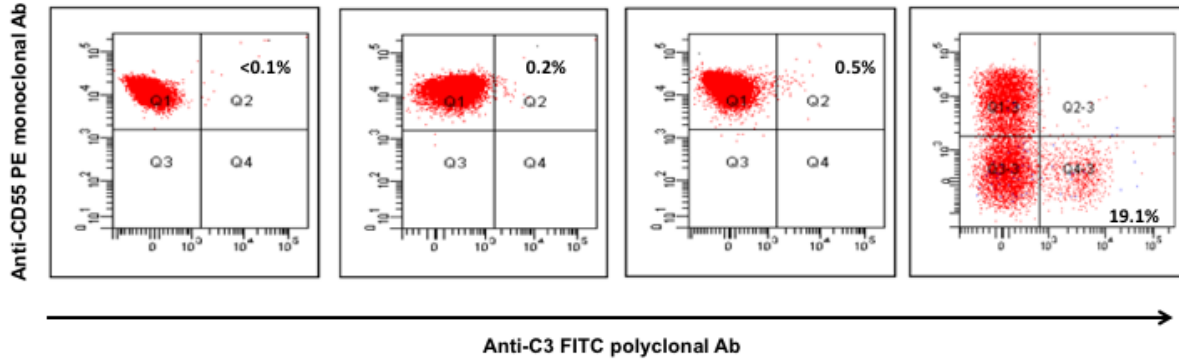
Fig. 3S

Fig. 3S. Flow cytometry of red blood cells from (from the left to the right): i. healthy subject (negative control); ii. and iii. sickle cell disease (SCD) patients; iv. paroxysmal nocturnal hemoglobinuria (PNH) patient on eculizumab (Risitano et al, Blood 2009). In each scatter-plot graph quadrants from Q1 to Q4 (Q1-1 to Q1-4 for the PNH patients, only viable erythrocytes are gated) represent respectively CD59+/c3-, CD59+/C3+, CD59-/C3- and CD59-/C3+ red blood cells. In healthy individuals, only CD59+/C3- cells are found (negative control); in PNH (positive control) a large population of C3+ cells is found, but only within the CD59- (PNH) red cell population (i.e., CD59-/C3+ cells). In SCD, a small population of C3+ cells is found, of course within CD59+ cell (no CD59, PNH-like cell can be detected). One representative of independent experiments with similar results; all data are presented in Fig. 1 scatter-plot graph.
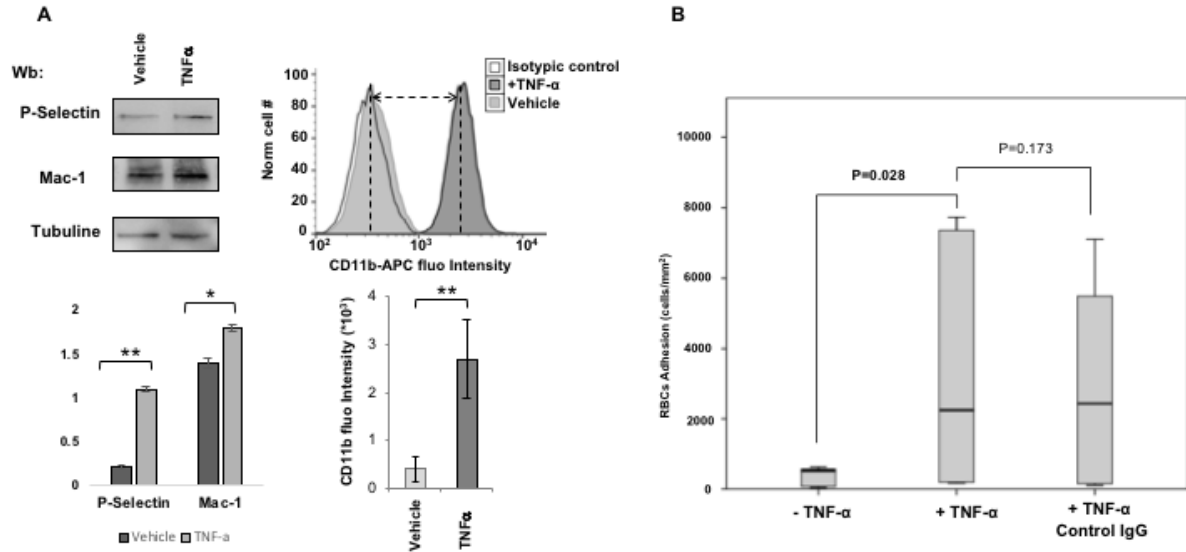
Fig. 4S. **A.** Western-blot (Wb) analysis of P-selectin and MAC-1 on activated (TNF-α) or non-activated vascular endothelium cells (Vehicle). Tubulin was used as protein loading control. **Lower panel.** Densitometric analyses of the immunoblot bands similar to those shown are presented at right (DU: densitometric Unit). Data are shown as means ±SD (*n*=3; *p<0.05; **p<0.02 compared to Vehicle). **Right panel**. Flow cytometry of activated (TNF-α) or non-activated vascular endothelium cells (Vehicle) stained with CD11b. Isotopic control is shown. One representative of 3 independent experiments with similar results.**(bottom)** Data are presented as means±SD (n=3; ; **p<0.02 compared to Vehicle).
B. Sickle red blood cell adhesion after 6 minutes of perfusion on activated or non-activated endothelium (+/- TNF-α) pre-coated with either vehicle or control IgG2 anti aCD20 antibody. Data shown are representative of other 6 independent assays with similar results. Wilcoxon test: * indicates the corresponding significance. All calculations were performed using the IBM SPSS 20.0 (IBM Inc., Armonk, NY) statistical package. Adhesion is expressed as median value with minimum-maximum range and shown by means of box plots. Data were analyzed with non-parametric tests, Mann-Whitney *U* test for unpaired samples and Wilcoxon signed-rank test for paired samples. A value of P<0.05 was considered statistically significant.

SUPPLEMENTARY VIDEO LEGENDS

Video 1. Red blood cells (RBCs) adhesion assay. Adhesion of healthy (AA) and sickle cell (SCD) RBCs to TNF-α-activated endothelium in presence of vehicle. Quantification data and statistical analysis are shown in Fig. 2A.

Video 2. Effects of FH (18 nM) on adhesion of sickle red (SCD) cells on vascular endothelial cells in presence (+) or absence (-) of TNF-α. Quantification data and statistical analysis are shown in Fig. 4E.

SUPPLEMENTAL METHODS

Evaluation of C5b-9 complement deposition on fixed skin biopsy

Immunofluorescent staining assay. Paraffin-embedded tissue blocks were cut into 2 to 3 µm sections and mounted on adhesion microscope glass slides. After the sections were dewaxed and rehydrated. Antigen retrieval was performed in prewarmed citrate buffer (pH 6, at 95°C) for 30 minutes. The sections were cooled to room temperature and then incubated with a protein-blocking serum free solution for 15 minutes at room temperature (RT) to block non-specific binding. In healthy controls, we stained dermal vessels in serial sections by anti-CD31 as an endothelial marker (mouse monoclonal antibody at 1: 50 dilution, Clone JC70A, Dako, Denmark; green fluorescence) and C5b9 (anti-human SC5b-9 at 1:100 dilution; Quidel, San Diego, CA; orange fluorescence) staining in healthy controls (Figure 1SA). Since Sprott et al. previously reported C5b9 deposition to be detectable only in the vessel walls of pathologic skin with no involvement of other cutaneous compartments, we also analyzed anti-C5b9 staining in skin biopsies from SCD patients (32, 33). Slides were then incubated with the corresponding Alexa 488-conjugated antibody or Alexa 546-coniugated antibody (1:800; INVITROGEN Molecular Probes). Nuclei were stained with Prolong Gold antifade reagent with DAPI (INVITROGEN Molecular Probes, blue staining). Slides were examined with an Olympus BX61 microscope.

Immunohistochemical staining assay. Paraffin-embedded tissue blocks were cut into 2-3 µm sections and mounted on adhesion microscope glass slides. For immunohistochemical staining, sections were stained in an auto-stainer Leica Bond System with anti-C5b9 (AbCam clone aE11) and CD31(mouse monoclonal antibody at 1: 50 dilution, Clone JC70A, Dako, Denmark) as previously reported (34).

Measurements of phosphatidylserine+ and C3d+ RBCs

The deposition of C3-derived opsonins (i.e. C3b, iC3b, C3dg) on RBCs was measured by flowcytometry using an antibody (monoclonal antibody anti C3 - EIA Quidel Corp., San Diego, CA; USA) recognizing the C3d fragment and is reported as C3d+ cells (11, 12). In brief, RBCs were obtained from fresh peripheral blood after centrifugation and three washings in saline buffer; washed RBCs were resuspended in saline at approximately at the concentration of 10^4 RBCs/µL, and then incubated with a phycoerythrin (PE)–conjugated anti-CD59 (59PE Valter Occhiena) diluted 1:10, and with a fluorescein isothiocyanate (FITC)–conjugated anti-C3 polyclonal

antibody (Ab14396 Abcam; a 1:20 working solution from the original tube was used at a final dilution of 1:50). Samples were incubated at RT for 1 hour, and then analyzed with a FACScan cytometer (Becton Dickinson Italia), after dilution with 10 volumes of saline (or FACS flow, Becton Dickinson Italia).

Red blood cell adhesion assay

Blood samples were collected in ACD anticoagulant and RBCs were separated from the plasma, washed in phosphate buffer (PBS), re-suspended at 2% haematocrit (Hct) in RMPI serum-free Roswell Park Memorial Institute medium (RPMI, Gibco) medium (containing 1% bovine serum albumin, Sigma Aldrich), and then were incubated with 70uM Carboxyfluorescein Diacetate Succinimidyl Ester (CFDA-S; Life Technology) for 1 hour at 37°C. Labelled RBCs first were washed three times and then were re-suspended at 0.2% Hct in serum-free RMPI medium, containing 1% bovine serum albumin. The RBCs suspension, depleted of white blood cells (WBC), was then incubated for 10 minutes at 37°C with either full-length FH or its recombinant segments encompassing domains 6-8 (FH6-8) or 19-20 (FH19-20), which were produced as previously described (41), or with calcium and magnesium-free PBS as a negative control, then perfused to generate a wall shear stress of 30 sec^{-1}. Prior to the adhesion assays, haemolysis was determined using CN-FREE HGB reagent on ADVIA 120/2120 system (Siemens); no hemolysis was detected after the incubation time, prior to the adhesion assay (data not shown). A monolayer of immortalized endothelial cells (EA 926.hy cell line, ATCC® CRL-2922™) was grown at the bottom of the perfusion chamber, then incubated with TNFα (Sigma Aldrich-Merk KGaA, Darmstadt, Germany) for 4 hours at 37°C in 5% CO_2 in serum free medium. In preliminary experiments, primary TNFα-activated endothelial cells (HUVEC, ATCC® CRL-1730™) were used in the presence or absence of Factor H, and the results obtained were similar to those obtained with immortalized cells. RBC suspension perfused in the flow chamber was monitored for 10 minutes, during which time 10 different locations along a line oriented to the flow were examined every 3 min. in order to study cell adhesion and aggregation. To discriminate and count cells on flow we set-up a new software called Cell Counter-BL The software exploits the characteristics of long exposure photographs, in which the moving parts tend to disappear, while the adhering cells are imprinted on the image. Blocking antibodies against P-selectin (Anti-CD62P [AK4]; Abcam) or Mac-1 (CD11b/ CD18 [CBRM1/5]; Biolegend) were used at final concentration of 25 ug/ml.

Immortalized human endothelial cell EA926.hy were grown as monolayer in DMEM 10% FCS medium. This cell line was obtained from HUVEC (Human Umbelical Vein endothelial cell) cell line and maintain key characteristic of HUVEC. Endothelial cell passage was accomplished with trypsinization, as required. For flow experiments EA 926.hy cells were plated at a density sufficient to reach confluence. EA 926.hy cells were treated with 20nM TNFα for 4hours at 37 °C to create a cytokine mediated activation mimicking inflammatory state, non-activated endothelium was used as a control. In some experiment HUVEC cells were used as a control to compared and confirm results obtained with immortalized endothelial cells.

Flow chamber position and optical area of analysis

The flow chamber was positioned on the stage of an inverted microscope equipped with epifluorescent illumination (Diaphot-TMD; Nikon Instech, Shinagawa-ku, Japan), an intensified CCD videocamera (C-2400-87; Hamamatsu Photonics, Shizuoka, Japan), and appropriate filters. The total area of an optical field corresponded to approximately 0.007 mm^2. Blood cells were aspirated through the chamber with a syringe pump (Harvard Apparatus, Hollistone, MA) at a flow rate calculated to obtain the desired wall shear rate at the inlet. Experiments were recorded in real time on videotape at the rate of 25 frames/s, which resulted in a time resolution of 0.04 s.

Quantification of red blood cell adhesion: Cell Counter-BL Software

It is based on computational sum of frames that constitute a field of view. This is then divided by the number of frames obtaining an effect similar to a long exposure. The first image has non-homogeneous light background because of presence of a bright spot. For this reason, it was necessary to perform a background subtraction by calculation of the dynamic background on each image. An interpolating function of degree 4 was applied for all rows of the image. For the part of the cell count has exploited an algorithm known in the literature (http://en.wikipedia.org/wiki/Flood_fill).

Development of an algorithm to determine RBC transit and flux trajectory

Experiments were performed on an inverted fluorescence microscope and recorded in real time on videotape at the rate of 25 frames/second, resulting in a time resolution of 0.04 sec. Endothelial cells activated with TNFα were grown on the coverslip of the flow chamber and experiments were run as reported above. Selected video sequences were digitalized using VirtualDub software (www.virtualdub.org; open source software). Image analysis was accomplished using the Volocity software (Perkin Elmer) and by programming codes in MatLab R2014b (The

MathWorks). The trajectory of each individual RBC was followed with Volocity from first appearance of the particle in the field of view to its disappearance, considering the field in question as a fixed region of 125 x 375 pixels, which was equivalent to an area of 50 x 150 μm, (using a conversion factor of 0.4 μm/pixel).

For each RBC, fixing the x axis as the flow direction and the y axis as its perpendicular coordinate, the absolute values for instantaneous speed ($|v|_t$) and for the instantaneous transverse speed ($|v_y|_t$, both in μm/second) were quantified with MatLab, according to their definition, as:

$$|v|_t = sqrt(v_x^2 + v_y^2)_t = sqrt((\Delta x / \Delta t)^2 + (\Delta y / \Delta t)^2)_t$$

$$|v_y|_t = |\Delta y / \Delta t|_t = |(y_t - y_{t-1}) / (t - (t-1))|$$

where t is the time instant in which the calculation was performed and t-1 the preceding instant before. All computational analyses were performed frame by frame, for each observation time.

P-selectin and Mac-1 expression *in vitro* in vascular endothelial cells

Immunoblot analyses. Vascular endothelial cells were lysed with iced lysis buffer (LB containing: 150mMNaCl, 25mM bicine, 0.1% SDS, 2% Triton X-100, 1mM EDTA, protease inhibitor cocktail tablets [Roche] and 1mMNa$_3$VO$_4$ final concentration) followed by centrifugation at 4°C for 30 min at 12,000 g. Proteins were quantified and analyzed by mono-dimensional SDS polyacrylamide gel electrophoresis. Gels were transferred to nitrocellulose membranes for immunoblot analysis with specific antibodies: anti-P-Selectin (both from BD, Biosciences, San Jose, CA, USA)). Anti-tubulin was used as a loading control (Developmental Studies Hybridoma Bank [DSHB], Department of Biology, University of Iowa, Iowa City, IA, USA). Secondary donkey anti-rabbit IgG and HRP-conjugated were from GE Healthcare Life Sciences. Blots were developed by using the Luminata Forte Chemiluminescent HRP Substrate from Merck Millipore (Darmstadt, Germany), and images were acquired with an Image Quant Las Mini 4000 Digital Imaging System (GE Healthcare). Densitometric analyses were performed by using an ImageQuant TL software (GE Healthcare Life Sciences) (42).

Cytofluorimetric analysis. EA926.hy endothelial cells were seeded on a cover-sleep in order to obtain a cell monolayer. Endothelial cells were activated with

20ng/ml of TNFα in serum free medium for 4 hours before and then they were analyzed for surface CD11b (with anti MAC-1 APC, ICRF44 Biolegend). As controls, untreated cells and an isotypic antibody (MOPC-21 mouse IgG1k; Biolegend) were used. Analysis was performed with a FACScan cytometer (FORTESSA, Becton Dickinson).