

## The endothelin B receptor plays a crucial role in the adhesion of neutrophils to the endothelium in sickle cell disease

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## Supplementary Data

### Supplementary Methods

#### Intravital videomicroscopy experimental protocol

To characterize *in vivo* the effects of the ET<sub>A</sub> and ET<sub>B</sub> receptor antagonists on neutrophil recruitment, we used a protocol described in supplemental figure 1A. Male mice were anaesthetized by spontaneous inhalation of isoflurane and maintained at 37°C with a heating pad. The left jugular vein was cannulated to administer drugs and antibodies. The left cremaster muscle was exposed (1) and mounted for intravital microscopic observations of the cremasteric microcirculation and adjacent tissue. The muscle was superfused with 36°C warmed bicarbonate-buffered saline pH 7.4.

ET<sub>A</sub> antagonist (BQ123, A.G. Scientific), ET<sub>B</sub> antagonist (BQ788, Tocris Bioscience) or equivalent volume of vehicle (isotonic saline) were injected 10 min before (10 mg/kg) and 3h15 after (5 mg/kg) intrascrotal injection of 0.5 µg tumor necrosis factor (TNFα, R&D Systems). 2h after TNFα injection, the cremaster muscle was incised and neutrophils were monitored by injection of labeled Phycoerythrin-conjugated anti-Ly6G antibody (0.05 µg/g BW, clone RB6-8C5, BD Biosciences Pharmingen). Venues were visualized with an intravital microscope (Zeiss Examiner D1) equipped with a water-immersion objective (Zeiss Plan-Apochromat 20X / 1.0NA) and fluorescence excitation was made through a Lambda DG-4 high-speed wavelength switcher (Sutter Instrument). Images were collected with a 512 x 512 pixel back-thinned EMCCD camera (Evolve, Photometrics). Images were analyzed using SlideBook 6.0 (Intelligent Imaging Innovations). The neutrophil rolling flux fraction, adhesion density, adhesion efficiency and transmigration were measured using playback assessment of 3-min digital time-lapse videos and 3D z-stack recordings of single unbranched venues (3 venues per mouse, 20-40 µm) from 2.5 hours to 5 hours after the TNFα challenge at 30-min intervals. Vessel diameter and wall shear rate measure methods are described in Supplementary Methods. Blood samples used to determine blood counts were taken immediately after recording (Hemavet, Drew scientific).

#### Hemodynamic measurements

Venular diameter was measured with a video caliper. The vessel centerline red blood velocity ( $V_{RBC}$ ) was measured using green fluorescent microspheres (1 µm, Molecular Probes) injected via the jugular cannula. Velocity of the fastest free-flowing bead along the vessels' centerline measured using manual tracking function of Slidebook was used as  $V_{RBC}$ . Mean blood flow velocity ( $V_b$ ) was approximated by multiplying the centerline  $V_{RBC}$  by a factor of 0.625 (2).

Wall shear rate ( $\gamma_w$ ) was estimated as  $\gamma_w = 2.12 \times 8 \times [V_b/d]$ , where d is the diameter of the vessel and 2.12 is a median empirical correction factor obtained from velocity profiles measured in microvessels *in vivo* (3).

### **Image analysis**

All analyses were made with playback assessment of digital recordings. Vessel diameter was measured using electronic calipers. Each rolling neutrophil passing a line perpendicular to the vessel axis was counted, and neutrophil rolling flux was expressed as neutrophils per minute. Neutrophil rolling flux fraction was calculated by dividing neutrophil rolling flux by total neutrophil flux estimated as  $[\text{Neutrophils}] \times V_b \times \pi \times (d/2)^2$  where  $[\text{Neutrophils}]$  is the systemic neutrophils count,  $V_b$  is the mean blood flow velocity, and d is the venular diameter. A neutrophil was considered as adherent if it remained stationary for at least 30 s, and total neutrophil adhesion was quantified as the number of adherent cells within a 100  $\mu\text{m}$  length of venule over 3-min and expressed per unit area of inside surface area of the venule, assuming cylindrical geometry. Neutrophils adhesion efficiency was calculated by dividing the number of adherent neutrophils (adhesion density, neutrophils/ $\text{mm}^2$ ) by the estimated number of circulating neutrophils passing through the venule (neutrophils flux, neutrophils/min). Neutrophil emigration was defined as the number of cells in the extravascular space adjacent to the observed venule within a 256 X 256 X 400- $\mu\text{m}$  volume. Optical sectioning (3D z-stack acquisition of 80 planes spaced by 5  $\mu\text{m}$ ) followed by maximum fluorescence intensity projection to create a 2D output image (0.0655  $\text{mm}^2$  area) was used for emigrated neutrophil quantification (**Supplementary Figure 1**).

### **Flow cytometry**

After 3h of treatments, mice were anesthetized; blood was collected on EDTA by intra-cardiac puncture and immediately processed for flow cytometry staining.

Neutrophils were analyzed using the following antibodies: BV785-conjugated anti Ly6G (1A8, Biolegend), PE-Cy7-conjugated anti-CD11b (M1/70, Ebioscience), AlexaFluor 647 conjugated anti-CD11a (M17/4, BD Pharmingen) and PerCPCy5.5-conjugated anti-CD62L (MEL-14, BD Pharmingen). Viability was assessed using the LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit, an amine reactive dye (ThermoFischer Scientific). After staining, blood was lysed and fixed using BD FACS™ Lysing Solution (BD Pharmingen) according to manufacturer's instructions. Cells were analyzed using a flow cytometer (LSR II, BD Biosciences) and data were analyzed using FlowJo software.

## Flow adhesion assays

HMEC-1 (Human Microvascular Endothelial Cell line) monolayers were grown in Vena8 Endothelial+ Biochips (Cellix Ltd, Dublin, Ireland) as previously described (4) and activated by TNF $\alpha$  (10 ng/ml) for 24 h prior to the adhesion assay. In brief, adhesion was measured under flow conditions using Vena8 Endothelial+TM biochips (internal channel dimensions: length 20 mm, width 0.8 mm, height 0.12 mm) and ExiGoTM Nanopumps (Cellix Ltd, Dublin, Ireland). We quantified the neutrophil adhesion with the mean fluorescence of 11 pictures for each condition, 895.26 $\mu$ m x 670.80  $\mu$ m (0.6 mm<sup>2</sup>) each. For experiments with protein substrates, a mix of human P-selectin (2  $\mu$ g/ml), VCAM-1 (2  $\mu$ g/ml) and ICAM-1 (10  $\mu$ g/ml) recombinant proteins (R&D Systems) was coated in Vena8 Endothelial+ Biochips as previously described (5). For adhesion assay, the anticoagulant used was lithium heparin. The length of time between blood draw moment and the adhesion experiments was less than 3 hours. If this length of time was more than 3 hours, samples were discarded. PMNs were stained in whole blood with anti-CD16 alexa 488-conjugated mouse monoclonal antibody (Biolegend, San Diego, USA) for 1 hour at 37°C. Together with this staining, BQ123 (1  $\mu$ M) and/or BQ788 (1  $\mu$ M) were added. Whole blood samples were perfused for 45 min at 1 dyn/cm<sup>2</sup> through the biochip channels containing HMEC-1 monolayers or coated proteins. PMN adhesion was monitored using AxioObserver Z1 microscope and ZEN software (Carl Zeiss, Le Pecq, France). Images were taken in 11 representative fields at the centerline of each channel at 10 min intervals throughout the assay. Adhesion levels were quantified in the last images set, by measuring the surface area of fluorescent patches using ImageJ Software. Adhesion in each channel is defined as the median value of fluorescence of the 11 fields.

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**Supplementary Tables and Figures:**

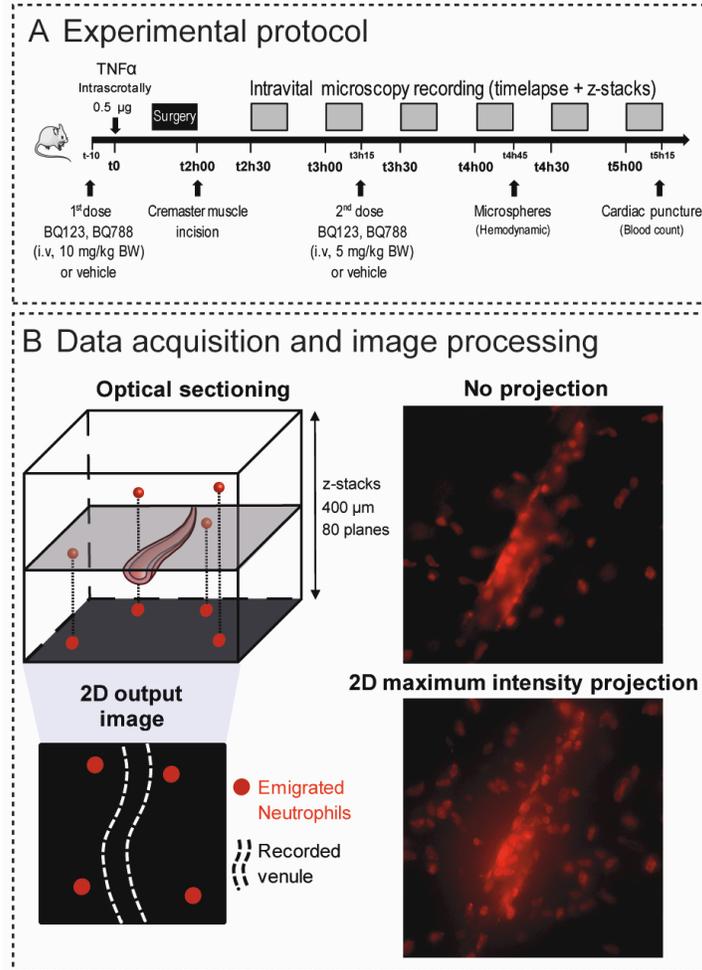
	RBC (cells/ $\mu$ l)	Hemoglobin (g/dL)	Hematocrit (%)	WBC (cells/ $\mu$ l)	Neutrophils (cells/ $\mu$ l)	Platelets ( $10^3 \times$ cells/ $\mu$ l)
<b>Basal state</b>						
WT mice	9730 $\pm$ 334	14.0 $\pm$ 0.6	44.5 $\pm$ 1.5	8995 $\pm$ 786	1933 $\pm$ 226	887 $\pm$ 56
SAD mice	9000 $\pm$ 191	13.1 $\pm$ 0.3	42.6 $\pm$ 0.9	15550 $\pm$ 991***	3206 $\pm$ 191**	1208 $\pm$ 82*
<b>Intravital study</b>						
<b>WT mice</b>						
PBS	7840 $\pm$ 75	11.2 $\pm$ 0.4	34.1 $\pm$ 0.6	3940 $\pm$ 277	1307 $\pm$ 458	928 $\pm$ 76
TNF $\alpha$	8295 $\pm$ 336	11.5 $\pm$ 0.3	37.2 $\pm$ 1.2	1745 $\pm$ 256	517 $\pm$ 48	929 $\pm$ 24
TNF $\alpha$ + BQ123	8673 $\pm$ 968	11.2 $\pm$ 1.3	40.5 $\pm$ 5.2	1550 $\pm$ 37	642 $\pm$ 201	784 $\pm$ 20
TNF $\alpha$ + BQ788	7685 $\pm$ 526	10.4 $\pm$ 1.0	35.7 $\pm$ 3.3	2225 $\pm$ 785	843 $\pm$ 356	625 $\pm$ 87
<b>SAD mice</b>						
PBS	7277 $\pm$ 1223	10.0 $\pm$ 1.7	30.7 $\pm$ 5.5	4893 $\pm$ 1230	2173 $\pm$ 768	872 $\pm$ 403
TNF $\alpha$	7794 $\pm$ 260	10.9 $\pm$ 0.3	33.6 $\pm$ 1.3	4884 $\pm$ 463*	1338 $\pm$ 263	1039 $\pm$ 168
TNF $\alpha$ + BQ123	8194 $\pm$ 119	10.5 $\pm$ 0.4	35.1 $\pm$ 1.1	3128 $\pm$ 611	964 $\pm$ 206	1073 $\pm$ 39
TNF $\alpha$ + BQ788	8418 $\pm$ 512	11.4 $\pm$ 0.5	34.7 $\pm$ 2.6	4752 $\pm$ 690*	1890 $\pm$ 280**	902 $\pm$ 107

**Supplementary Table 1: Blood cell counts at basal state:** Data are presented as mean  $\pm$  SEM of 8 mice per group. Student t-tests were performed. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$  for SAD vs. WT groups.

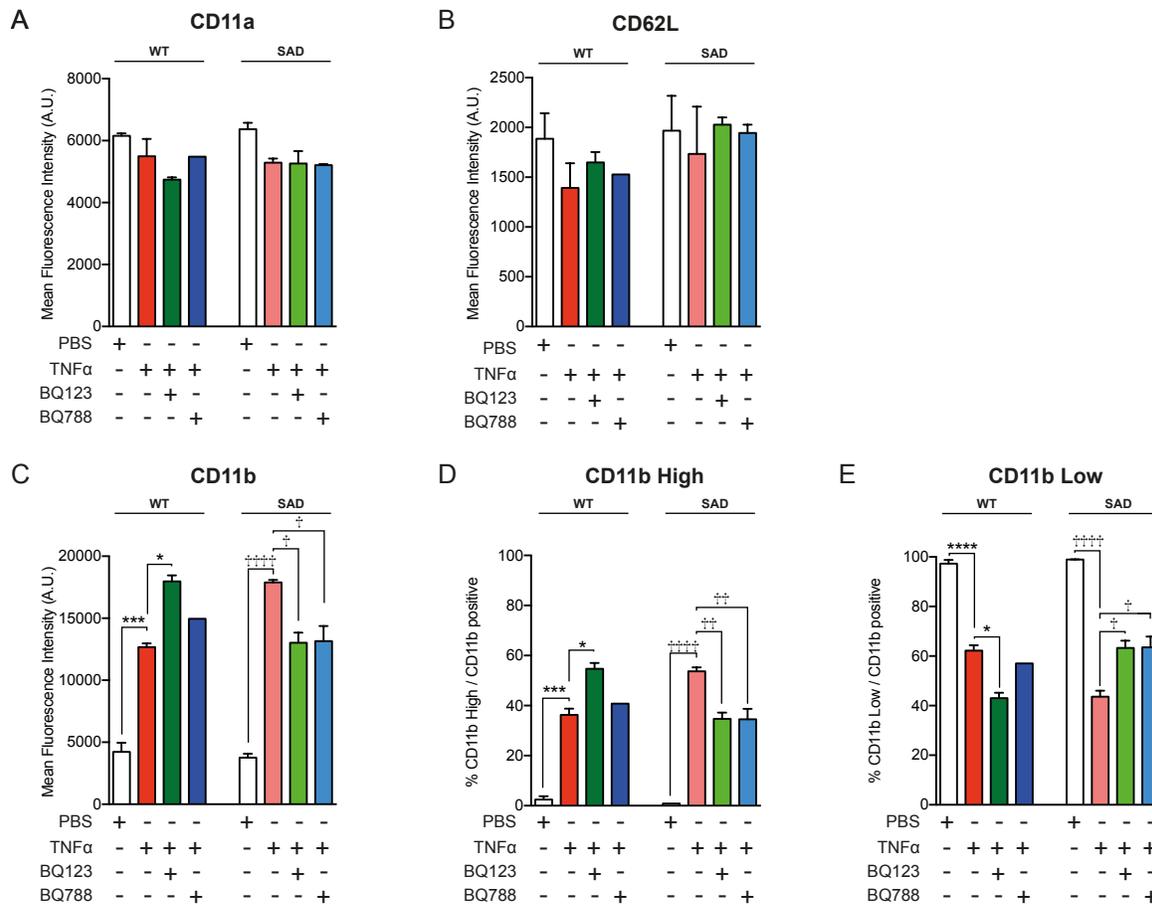
**Hematological parameters 5h after TNF $\alpha$  administration, at the end of the intravital imaging procedure.** Data are presented as mean  $\pm$  SEM of five mice per group. One-way ANOVA with Tukey's multicomparison test were performed. \*:  $p < 0.05$  compared with WT-TNF $\alpha$ , \*\*:  $p < 0.01$  compared with WT-TNF $\alpha$ . No differences were observed between SAD mice groups.

	Number of mice	Number of venules	Average diameter ( $\mu\text{m}$ )	Centerline blood flow velocity ( $\mu\text{m}/\text{sec}$ )	Wall shear rate ( $\text{s}^{-1}$ )
<b>WT mice</b>					
PBS	3	12	20.5 $\pm$ 1.6***	1440 $\pm$ 181	801 $\pm$ 100
TNF $\alpha$	5	16	31.7 $\pm$ 1.5	1689 $\pm$ 461	550 $\pm$ 153
TNF $\alpha$ + BQ123	5	14	30.1 $\pm$ 1.3	1068 $\pm$ 449	373 $\pm$ 150
TNF $\alpha$ + BQ788	5	14	26.8 $\pm$ 1.0*	1026 $\pm$ 208	427 $\pm$ 100
<b>SAD mice</b>					
PBS	3	12	26.5 $\pm$ 1.7	2452 $\pm$ 468	1039 $\pm$ 219*
TNF $\alpha$	5	15	27.4 $\pm$ 1.3	1095 $\pm$ 219	424 $\pm$ 82
TNF $\alpha$ + BQ123	5	14	30.0 $\pm$ 1.8	2249 $\pm$ 461	833 $\pm$ 177
TNF $\alpha$ + BQ788	5	14	29.5 $\pm$ 1.6	982 $\pm$ 312	324 $\pm$ 84

**Supplementary Table 2: Hemodynamics parameters.** Data are presented as mean  $\pm$  SEM of five mice per group. One-way ANOVA with Tukey's multicomparison test were performed. \*:  $p < 0.05$ ; \*\*\*:  $p < 0.001$  vs. TNF $\alpha$  with vehicle only (TNF $\alpha$ ).



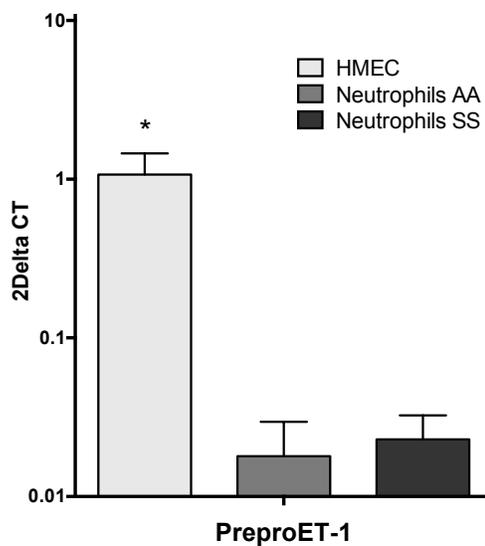
**Supplementary Figure 1: Experimental protocol, data acquisition and image processing.** (A) Experimental scheme. After anaesthesia and surgical preparation, WT or SAD mice were intravenously injected with selective  $\text{ET}_A$ ,  $\text{ET}_B$  receptor antagonist or vehicle. After 10 min, mice were intrascrotally injected with murine  $\text{TNF}\alpha$  (t0). To ensure activities of the endothelin receptor antagonists during the entire course of the study, mice received a second dose of antagonists or vehicle 3h25 after the first injection. Images of the cremasteric venules under intravital microscopy were recorded between the time points of t2h30 and t5h00 min. During filming, the hemodynamic parameters, including centreline velocity, venular diameter, and shear rate, were measured. (B) Acquisition protocol and image processing for emigrated neutrophils quantification. Optical sectioning (3D z-stack acquisition of 80 planes spaced by  $5 \mu\text{m}$ ) followed by maximum fluorescence intensity projection to create a 2D output image ( $0.0655 \text{ mm}^2$  area) allowing detection and quantification of emigrated neutrophils in the extravascular space adjacent to the observed venule. Arrows indicate examples of detected emigrated neutrophils.



**Supplementary Figure 2: Effect of ET-1 receptors antagonists on TNF $\alpha$ -induced alteration in CD11a, CD11b and CD62L expression on mouse neutrophils.**

Effect of TNF $\alpha$  administration with or without systemic infusion of ETR antagonists BQ123 and BQ788 on CD11a, CD62L and CD11b expression on neutrophils from SAD and WT mice. No effect of either TNF $\alpha$  or of the ETR antagonists was observed on CD11a and CD62L neutrophils surface expression (**A**, **B**).

By contrast, TNF $\alpha$  challenge induced a very significant upregulation of CD11b expression in PMNs from both in wild type and SAD animals (**C**, **D**). This effect was markedly increased in neutrophils from SAD mice compare that measured in WT mice (+40 %,  $p < 0.05$ ). Both ET receptors antagonists limited CD11b-associated mean fluorescence surface intensity (**C**) and the proportion of neutrophils with high CD11b expression in neutrophils (**D**) from TNF $\alpha$ -challenged SAD animals. Moreover, anti ET<sub>A</sub> and ET<sub>B</sub> antagonists displayed no effect on CD11b expression in normal mice (**C**, **D**, **E**).  $n = 5$  mice per condition, \* or † :  $p < 0.05$ ; †† :  $p < 0.01$ ; \*\*\* or ††† :  $p < 0.001$ ; \*\*\*\* or †††† :  $p < 0.0001$ .



**Supplementary Figure 3: Prepro-endothelin-1 (preproET-1) mRNA relative expression in human microvascular endothelial cells (HMEC) and polymorphonuclear neutrophils from healthy controls (AA), and patients with SCD (SS). Results are means of eight independent samples in duplicate for controls and patients (\*:  $p < 0.05$  vs. neutrophils).**