ARTICLE

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

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ABSTRACT

Although the primary origin of sickle cell disease is a hemoglobin disorder, many types of cells contribute considerably to the pathophysiology of the disease. The adhesion of neutrophils to activated endothelium is critical in the pathophysiology of sickle cell disease and targeting neutrophils and their interactions with endothelium represents an important opportunity for the development of new therapeutics. We focused on endothelin-1, a mediator involved in neutrophil activation and recruitment in tissues, and investigated the involvement of the endothelin receptors in the interaction of neutrophils with endothelial cells. We used fluorescence intravital microscopy analyses of the microcirculation in sickle mice and quantitative microfluidic fluorescence microscopy of human blood. Both experiments on the mouse model and patients indicate that blocking endothelin receptors, particularly ET_B receptor, strongly influences neutrophil recruitment under inflammatory conditions in sickle cell disease. We show that human neutrophils have functional ET_B receptors with calcium signaling capability, leading to increased adhesion to the endothelium through effects on both endothelial cells and neutrophils. Intact ET_B function was found to be required for tumor necrosis factor α-dependent upregulation of CD11b on neutrophils. Furthermore, we confirmed that human neutrophils synthesize endothelin-1, which may be involved in autocrine and paracrine pathophysiological actions. Thus, the endothelin-ET_B axis should be considered as a cytokine-like potent pro-inflammatory pathway in sickle cell disease. Blockade of endothelin receptors, including ET_B, may provide major benefits for preventing or treating vaso-occlusive crises in sickle cell patients.

Introduction

Sickle cell disease (SCD) is a genetic hemoglobinopathy resulting from a unique mutation in the β-globin gene. SCD is characterized by hemolytic anemia, painful vaso-occlusive crises (VOC) and progressive organ failure. Although red blood cell dysfunction is the major contributor to disease development and progression, other types of cells, which are not affected by the genetic mutation (endothelial cells, leukocytes, platelets[1,2]), are also key actors in the pathophysiology of SCD. Several

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studies have highlighted the important role of polymorphonuclear neutrophils (neutrophils), both during an acute VOC[3] and in the associated long-term morbidity and mortality.[4] Interestingly, a high, steady-state, peripheral white cell count is a risk factor for both significant morbidity – stroke, pulmonary complications, nephropathy – and early SCD-related death.[4-8]

The central role of neutrophils in the pathophysiology of SCD has recently been explored.[3,9] *In vitro* studies have shown that, compared to neutrophils from healthy controls, neutrophils from SCD patients have an increased expression of adhesion molecules,[10-12] rendering them more susceptible to inflammatory stimuli.[13] A relationship between clinical manifestations of SCD and the expression of adhesion molecules on neutrophils has also been reported.[2,14] It is likely that activated neutrophils engage in a complex process of abnormal interactions between activated endothelial cells, platelets and circulating red blood cells contributing to decreased blood flow and to endothelial injury. This further accentuates erythrocyte sickling, neutrophil recruitment and tissue ischemia.[9] Targeting the mechanisms of neutrophil-endothelial cell interactions would, therefore, represent a novel and potentially important therapeutic opportunity in SCD.

Endothelin-1 (ET-1) is the most potent endogenous vasoconstrictor.[15] It is released by activated endothelial[16] and non-endothelial cells[17] in response to hypoxia and reduced nitric oxide bioavailability in several animal models.[18] The effects of ET-1 are mediated via two receptors, the ET_A and ET_B receptors.[15] We previously found that mixed $ET_A/_B$ receptor antagonism has profound effects on organ injury and mortality in a mouse model of SCD.[19] In addition to inhibition of tonic ET-1-dependent vasoconstriction during experimental VOC, we also observed an unexpected but powerful inhibition of neutrophil recruitment in the lungs and kidneys although we could not link this effect to a direct action of ET-1 receptors on neutrophil-endothelial interactions. We, therefore, hypothesized that activation of ET receptors might promote a pathogenic pro-inflammatory role for neutrophils in SCD.

In the present study, we combined intravital videomicroscopy of the microcirculation in a murine model of SCD with quantitative microfluidic fluorescence microscopy of human blood to investigate the involvement of ET receptors in the interaction of neutrophils with endothelial cells.

Methods

Animal model

Animals were used in accordance with the National Institutes of Health *Guide for the care and use of laboratory animals* (NIH publication n. 85-23) and the study protocol was approved by the French ministry of agriculture. SAD1 (SAD) Hbβ single/single hemizygous mice were used in this study. This strain harbors a recombinant hβ-globin gene construct expressing human hemoglobin SAD (A2β2SAD), which contains two mutations [Antilles (β23I) and D-Punjab (β121Q)] in addition to the βS6V mutation.[19,20] This strain is bred on the C57BL/6J genetic background (with more than 30 backcrosses).

Intravital videomicroscopy: experimental protocol

The complete protocol is described in the *Online Supplementary Methods* and illustrated in *Online Supplementary Figure S1*. ET_A antagonist (BQ123, A.G. Scientific®, San Diego, CA, USA) or ET_B antagonist (BQ788, Tocris Bioscience®, Bristol, UK) were injected 10 min before (10 mg/kg) and 3 h 15 min after (5 mg/kg) intrascrotal injection of 0.5 μg tumor necrosis factor (TNFα, R&D Systems, Minneapolis, MN, USA) and neutrophils were monitored by injection of labeled phycoerythrin-conjugated anti-Ly6G antibody (BD Biosciences Pharmingen, Sparks, MD, USA).

The neutrophil rolling flux fraction, adhesion density, adhesion efficiency and transmigration were measured from 2.5 h to 5 h after the TNFα injection at 30-min intervals.

Patients and healthy volunteers

The study was approved by the Ethical Evaluation Committee for Biomedical Research Projects (CEERB) of Robert Debré Hospital, France (n. 2014/155). All patients were included during a scheduled medical consultation, at steady state. Blood samples were collected from 2- to 18-year old patients with SCD (SS genotype), and from young healthy adult donors (AA genotype) from the local blood bank (*Etablissement Français du Sang*). Patients in a chronic transfusion therapy program, those having received blood transfusion in the 3 months preceding enrollment and those treated with hydroxycarbamide (hydroxyurea) were excluded. Exceptions were made for the quantitative polymerase chain reaction analyses, as mentioned below.

Human neutrophil preparation

Human neutrophils were isolated (98% purity) from fresh whole blood within 4 h after blood sampling with EDTA as anticoagulant, using a MACSxpress Neutrophil Isolation Kit followed by MACSxpress Erythrocyte Depletion Kit (Miltenyi Biotec, Paris, France). Details on the flow adhesion assays are provided in the *Online Supplementary Methods*.

Real-time quantitative polymerase chain reaction

ET-1, ET_A and ET_B mRNA levels were determined by real-time quantitative polymerase chain reaction as described previously.[21] Beta-2 microglobulin mRNA was used as a housekeeping gene The relative expression of ET-1, ET_A and ET_B mRNA in neutrophils was calculated following the ΔΔCt method,[22] with the values obtained in HMEC-1 as a reference.

Flow cytometry assessment of adhesion molecules and intracellular Ca^{2+} measurements in neutrophils

Three hours after treatment with TNFα with or without ET receptor antagonists, mice were anesthetized; blood was collected on EDTA by intracardiac puncture and immediately processed for flow cytometry staining for viability and CD11a, CD11b and CD62L surface expression on $Ly5G^+$ neutrophils (see *Online Supplementary Methods*).

Isolated human neutrophils were fluorescently labeled with fluo-4-acetoxymethyl ester (Fluo-4-AM; Invitrogen). Neutrophils were incubated with BQ123 or BQ788 (both from Sigma®, Saint-Quentin Fallavier, France) at 10 μmol/L for 15 min and analyzed immediately by flow cytometry (FACSCanto II, BD Biosciences, Sparks, MD, USA). After 2 min of signal acquisition, the analysis was interrupted for less than 10 s to add 100 nM of ET-1 (Sigma®) to the tube before the analysis was resumed.

Endothelin-1 immunoassay

Isolated neutrophils were incubated at 37°C in RPMI medium supplemented with TNFα (10 ng/mL) for 2 h. Supernatants were frozen at -80°C until assay. The concentration of ET-1 was determined using a Quantikine® enzyme-linked immunosorbent assay (R&D systems.

Flow adhesion assays

The experimental protocol is described in the *Online Supplementary Methods*. Briefly, human neutrophils were stained in whole blood with anti-CD16 antibody together with BQ123 (1 μM) or BQ788 (1 μM). The time between drawing blood and starting the anti-CD16 staining was always less than 3 h and reproducible. If more than 3 h elapsed, samples were discarded. Whole blood samples were perfused through biochip channels containing HMEC-1 monolayers or coated recombinant proteins (P-selectin, VCAM-1 and ICAM-1).

Statistics

Results are presented as means plus or minus the standard error of the mean (SEM), Data were analyzed using GraphPad Prism 6 software by ANOVA with the Tukey test, Wilcoxon rank test and Mann-Whitney test. *P* values less than 0.05 were considered statistically significant.

Results

Acute ET_A and ET_B receptor antagonism does not alter leukocyte blood count or microvascular hemodynamics in wild-type and SAD mice

In keeping with clinical SCD, SAD mice (n=5) showed an increase in circulating leukocytes with a neutrophil predominance compared to wild-type (WT) littermate mice, both at baseline and after TNFα administration (*Online Supplementary Table S1*). Treatment of TNFα-challenged animals with either BQ123 or BQ788 did not affect any of the peripheral cell counts. As changes in microvascular wall shear stress may influence neutrophil adhesion, we determined the effects of acute ET_A and ET_B receptor blockade on wall shear rate in cremasteric venules. There were no significant differences in venular wall shear rates between groups treated with TNFα + vehicle, TNFα + BQ123 and TNFα + BQ788 (*Online Supplementary Table S2*).

ET_A and ET_B receptors control neutrophil rolling in inflamed venules.

In WT mice, neither BQ123 nor BQ788 affected neutrophil rolling flux throughout the time course of the experiment. As in WT mice, ET_A antagonism with BQ123 had no effect on neutrophil rolling flux in SAD mice. In sharp contrast, ET_B blockade with BQ788 profoundly inhibited neutrophil rolling flux in SAD mice, compared to that in the vehicle-treated SAD group, throughout the experiment (Figure 1A).

ET_A and ET_B receptor antagonism decreases neutrophil adhesion in inflamed venules from sickle mice

Overall, in WT mice, neutrophil adhesion density was not influenced by BQ123 or BQ788 treatment. Over the time course of the study, adhesion decreased in all three groups of WT mice from about 670 adherent neutrophils/mm^2 at 2 h 30 min after the inflammatory challenge to reach about 250 adherent neutrophils/mm^2 at 5 h. In SAD mice, the inflammatory challenge with TNFα induced a marked increase in neutrophil adhesion compared to that in the WT counterparts (1000 *versus* 586 adherent neutrophils/mm^2, $P<0.05$). The adhesion remained elevated and stable for 90 min and then decreased slightly. At the end of the experiment, adhesion density in SAD mice challenged with TNFα was still increased compared to that in WT challenged mice (731 *versus* 271 adherent neutrophils/mm^2, $P<0.05$). In SAD mice, both ET_A and ET_B receptor blockade markedly inhibited adhesion of neutrophils throughout the time course of the study with the degree of adhesion being similar to that observed in WT controls (Figure 1B).

To define the neutrophil adhesion better, we calculated the efficiency of neutrophil adhesion as the ratio between adhering neutrophils and those that are estimated to be available to adhere (Figure 1C). We found that adhesion efficiency was, overall, unaffected by BQ123 and BQ788 in WT mice. In contrast, adhesion efficiency was markedly and significantly decreased in SAD mice treated with BQ788 compared to that in the TNFα and vehicle-treated SAD group throughout the time course of the experiment; no significant differences were observed in SAD mice treated with BQ123. Taken together, these data show a differential effect of ET_A *versus* ET_B receptor antagonism on neutrophil adhesion (Figure 1C-F).

ET_A and ET_B receptor antagonism decreases neutrophil transmigration in inflamed venules from sickle mice

We counted neutrophils that had migrated into the extravascular space adjacent to the observed venules by optical sectioning (*Online Supplementary Figure S1*, Figure 1G,H). TNFα-induced emigration of neutrophils was significant in both groups of mice compared to the emigration in animals treated with vehicle (phosphate-buffered saline) but distinct kinetics were observed in SAD and WT animals. While neutrophil emigration reached a plateau at 3 h 30 min in the WT cremasteric microcirculation and subsequently resolved to baseline values, sickle SAD mice experienced a more sustained and more intense increase in emigration which was double that measured in WT after 4 h and lasted until the end of the experiments (Figure 1 G,H). In WT mice, TNFα-induced emigration was significantly decreased by BQ123 and BQ788 administration but only at early time points (~2.3-fold decrease at 2 h 30 min, $P<0.01$ for BQ123, ~3.5-fold decrease at 2 h 30 min and ~2.1-fold decrease at 3 h for BQ788, $P<0.001$ *versus* vehicle-treated mice). In SAD mice that displayed more intense and prolonged emigration of neutrophils both ET_A and ET_B blockade significantly prevented trans-endothelial migration of neutrophils to tissues, especially at late points [BQ123: ~2.3-fold decrease at 2 h 30 min ($P<0.01$ *versus* TNFα only-treated mice); BQ788: ~3.5-fold decrease at 2 h 30 min ($P<0.001$ *versus* TNFα-treated mice) and ~2.1-fold decrease at 3 h ($P<0.01$ *versus* TNFα-treated mice)] (Figure 1H). Representative images indicating less neutrophil recruitment in the presence of ET_A and ET_B, particularly in SAD mice, are shown in Figure 2.

All these experiments suggest that blocking ET receptors influences neutrophil recruitment in mice, especially in the context of sustained and intense neutrophil recruitment specific to experimental SCD.

ET_A and ET_B receptor antagonism reduces tumor necrosis factor α-induced high CD11b/Mac1 expression on neutrophils and does not alter cell viability.

CD11b, also known as Mac-1α or integrin αM chain, is part of the CD11b/CD18 heterodimer, also known as the C3 complement receptor. Increased adhesion has been linked to engagement of CD11b membrane expression on neutrophils from sickle mice.[23] We, therefore, measured

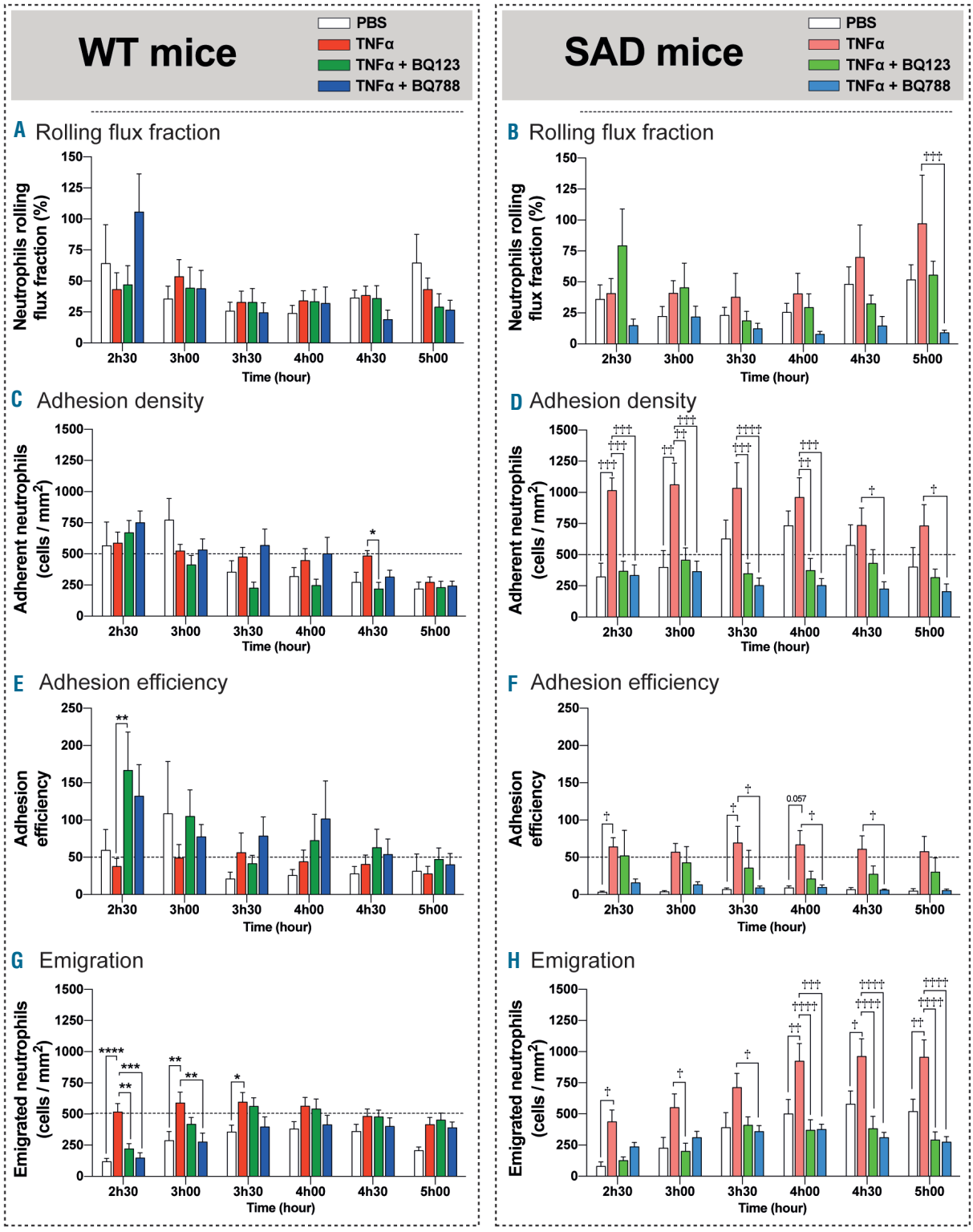

Figure 1. The rolling, adhesion and emigration of neutrophils in inflamed cremasteric venules are attenuated in tumor necrosis factor-alpha challenged wild-type and SAD mice treated with the selective endothelin receptor inhibitors BQ123 and BQ788. (A, B) Rolling neutrophil flux fraction in TNFα inflamed venules of WT and SAD mice. The number of rolling neutrophils per minute (flux) was counted and the neutrophil rolling flux fraction was determined by dividing the rolling neutrophil flux by the total neutrophil flux (all neutrophils passing through the venule). (C, D) Neutrophil adhesion density, defined as the number of adherent neutrophils per square millimeter, in TNFα inflamed venules after BQ123 and BQ788 treatment in WT and SAD mice. (E, F) Neutrophil adhesion efficiency, defined as the number of adhered neutrophils per square millimeter normalized by the number of circulating neutrophils passing through the venule. (G, H) Neutrophil emigration, defined as the number of emigrated neutrophils per square millimeter of extravascular space in inflamed venules after BQ123 and BQ788 treatment in WT and SAD mice. Emigrated neutrophils were visualized and quantified by optical sectioning and two-dimensional maximum intensity projection. Fifteen venules (5 mice) were analyzed in each group [12 venules and 3 mice for control with phosphate-buffered saline (PBS)]. Data are presented as mean ± SEM. Two-way ANOVA with the Tukey multiple comparison test. **P*<0.05 compared with WT-TNFα; ***P*<0.01 compared with WT-TNFα; ****P*<0.001 compared with WT-TNFα; *****P*<0.0001 compared with WT-TNFα. †*P*<0.05 compared with SAD-TNFα. ††*P*<0.01 compared with SAD-TNFα. †††*P*<0.001 compared with SAD-TNFα. ††††*P*<0.0001 compared with SAD-TNFα.

CD11b expression on neutrophils from SAD and WT mice without or after TNFα administration. In the latter case, we evaluated the effect of BQ123 and BQ788 antagonists 3 h after injection of TNFα or vehicle (phosphate-buffered saline), when ET receptor antagonists displayed inhibitory effects on neutrophil adhesion in intravital imaging experiments.

TNFα challenge induced a very significant upregulation of CD11b expression in neutrophils from both WT and SAD animals within 3 h (*Online Supplementary Figure S2*). This effect was markedly increased in neutrophils from SAD mice compared to that in WT mice (+40 %, $P<0.05$). Both ET receptor antagonists limited CD11b-associated mean fluorescence surface intensity and the proportion of

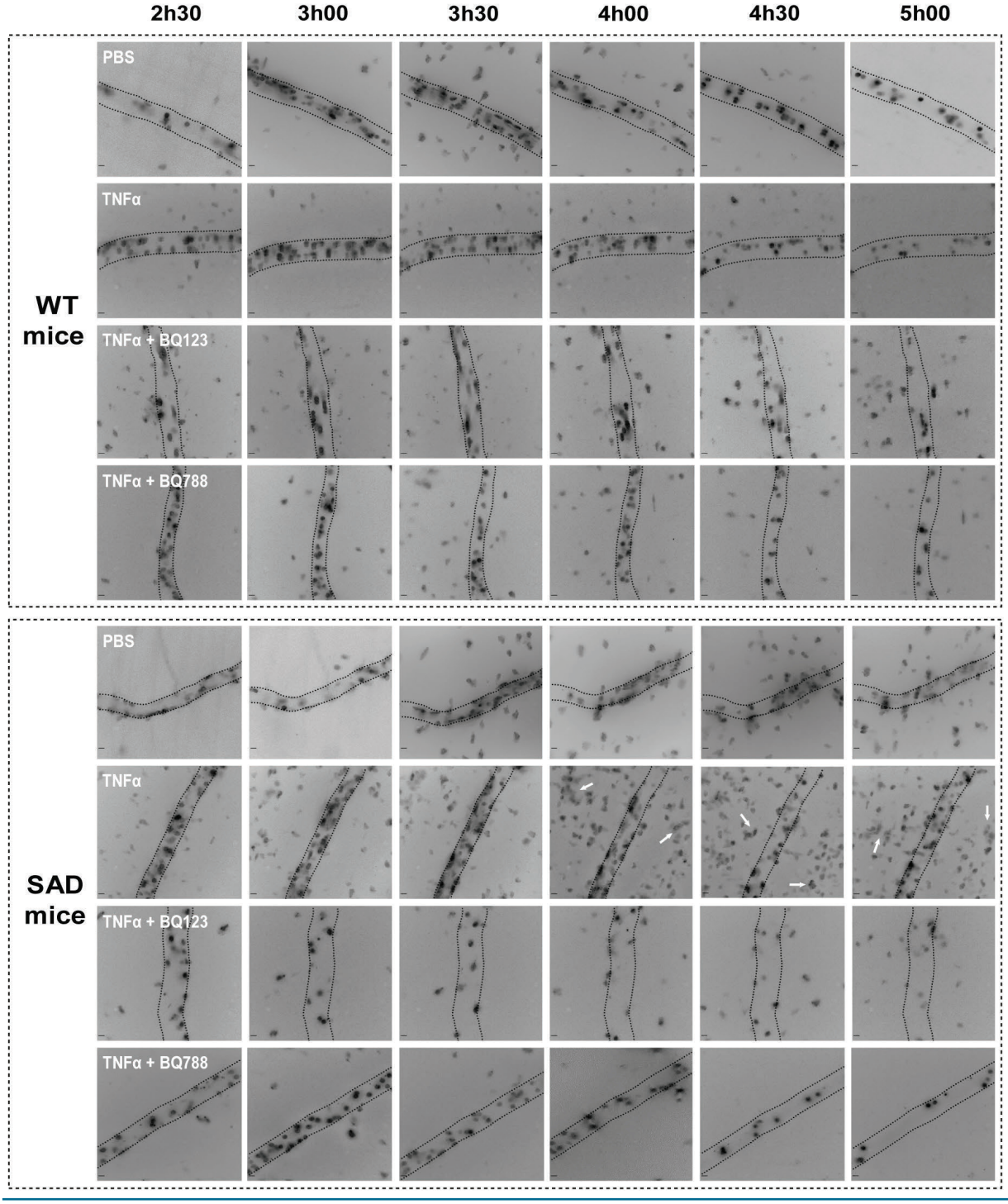

Figure 2. Kinetics of neutrophil recruitment. Representative images of cremasteric venules for each group witthout stimulation (administered phosphate - buffered saline, PBS) or after local TNFα stimulation associated or not with specific blocking of ET_A or ET_B receptors. White arrows indicate emigrated neutrophils. Scale bars: 10 μm.

neutrophils with high CD11b expression in neutrophils from TNFα-challenged SAD animals, whereas neither antagonist had any effect on CD11b expression in normal mice (*Online Supplementary Figure S2*). This suggests that involvement of the ET system in pro-inflammatory CD11b upregulation and neutrophil adhesion is specific to the SCD condition. No effect of either TNFα or the ET receptor antagonists was observed on the surface expression of CD11a and CD62L on neutrophils (*Online Supplementary Figure S2*) and no difference in cell viability (apoptosis or necrosis affected less than 3% of blood neutrophils) was observed between the different groups (*data not shown*).

We next investigated the expression and the role of ET receptors on human neutrophils from SCD patients and healthy controls.

ET_A and ET_B mRNA expression in neutrophils from healthy volunteers and patients with sickle cell disease

As very few studies report on the expression of ET_B receptors in neutrophils from normal individuals[24-26] and none in SCD patients, we first investigated the expression of the *EDNRA* and *EDNRB* genes encoding ET_A and ET_B, respectively, in endothelial cells (HMEC-1) and in human neutrophils from SCD patients (SS) and healthy individuals (AA). ET_A and ET_B mRNA are expressed both in endothelial cells and in human neutrophils and at similar levels in neutrophils isolated from healthy controls and SCD patients (Figure 3).

To complete these results and confirm the presence of functional ET receptors on human neutrophils, we investigated the potential effect of neutrophil stimulation on the ET-1-mediated calcium response.

Endothelin-1 elicits an ET_B-mediated calcium response in neutrophils from healthy controls and individuals with sickle cell disease

Although ET_A and ET_B receptors have been shown to be expressed on many cell types, their presence on the surface of neutrophils and their signaling pathways are poorly understood. We investigated the activation of a calcium-dependent signaling pathway in response to stimulation with ET-1 (Figure 4A,B). In the presence of ET-1 in neutrophils from both SCD individuals and healthy volunteers, we found a rapid increase in intra-cytoplasmic calcium concentration, which progressively decreased to steady state in less than 100 s. The same experiment was performed with neutrophils pre-incubated for 15 min with BQ123 or BQ788. Neither antagonist altered baseline values (Figure 4C). ET_A antagonism did not prevent the ET-1-elicited calcium response (Figure 4A,C) whereas ET_B receptor antagonism provoked a significant reduction in the calcium response after the addition of ET-1 both in AA and SS neutrophils (Figure 4A,C). These results indicate the presence of a functional ET_B receptor on the surface of neutrophils.

We next investigated the potential secretion of ET-1 by human neutrophils.

Endothelin-1 secretion by neutrophils from healthy individuals and patients with sickle cell disease

We investigated the expression of preproET-1 mRNA in neutrophils from healthy controls and SCD patients, and compared this to expression levels in the endothelial HMEC-1 cell line. As shown in *Online Supplementary Figure S3*, preproET-1 mRNA is significantly expressed in neutrophils but at a much lower level than in endothelial cells.

To confirm the ability of neutrophils to secrete ET-1, we measured the concentration of ET-1 in the supernatant of isolated human neutrophils activated or not by TNFα (Figure 5). We found detectable concentrations of ET-1 in the supernatant of isolated neutrophils at baseline (*data not shown*) and after stimulation with TNFα. The level of extracellular ET-1 in the presence of TNFα was higher in supernatants of neutrophils from SS individuals than those from AA healthy controls (0.27 ± 0.15 pg/mL *versus* 1.64 ± 0.45 pg/mL respectively; P=0.033) and from SS patients treated with hydoxycarbamide (0.20 ± 0.08 pg/mL; P=0.04) (Figure 5).

These data highlight the ability of neutrophils to secrete ET-1, which may then activate endothelial cells and neutrophils themselves, with an autocrine and paracrine mechanism. Thus, we next investigated the effects of ET receptor antagonists on human neutrophil adhesion to endothelial cells.

ET_B receptor antagonism prevents neutrophil adhesion to endothelial cells in controls and patients with sickle cell disease

To mimic *in vivo* circulation accurately, we infused whole blood onto a layer of activated endothelial cells under conditions of laminar flow. Adhesion of neutrophils to TNFα-primed endothelium was similar in controls and SCD patients (Figure 6A). As shown in Figure 6B,C, ET_A receptor antagonism with BQ123 did not affect this. Conversely, adhesion of BQ788-treated neutrophils to endothelial cells was decreased by 42% (± 26%) compared to the adhesion of untreated neutrophils (P=0.0008) (Figure 6B,C). These data indicate an unexpected stimulatory role for the ET_B receptor in human neutrophil adhesion to endothelial cells, paralleling that observed *in vivo* in sickle mice.

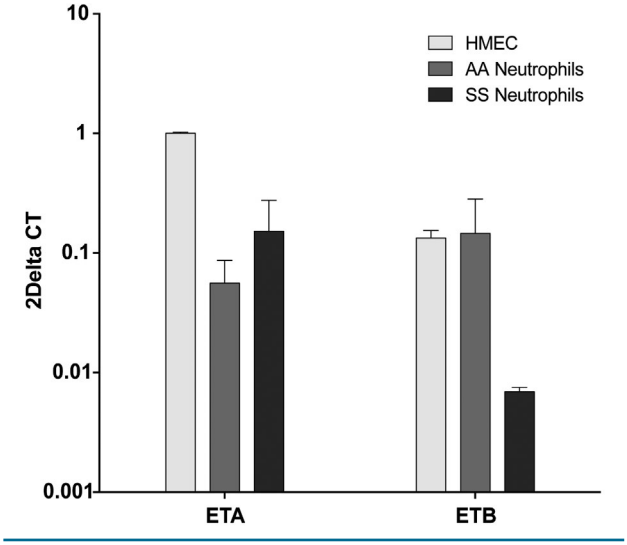

Figure 3. Endothelial cells and neutrophils from both healthy controls (AA), and SCD patients (SS) express ET_A and ET_B transcripts. Results of real time quantitative reverse transcriptase polymerase chain reaction are expressed as a comparison with $ET_{A/B}$ mRNA transcripts in endothelial cells (HMEC-1) with the ΔΔCT method. The reference gene used was the β_2 microglobulin gene. Results are means (± SEM) of eight independent samples (4 controls, 4 patients) in duplicate.

ET_B receptor antagonism prevents neutrophil adhesion to endothelial cells through effects on both endothelial cells and neutrophils

During the adhesion experiments described above, ET receptor antagonists were added to whole blood, then infused directly onto endothelial cells. We were not, therefore, able to exclude any potential effect of the pharmacological antagonists on endothelial cells. To discriminate between the effects of the antagonists on endothelial and neutrophil ET receptors, we performed the same experiments by incubating only endothelial cells with the two selective antagonists. We found a 28% (±33%) significant decrease in the adhesion of neutrophils following treatment of endothelial cells with BQ788 (but not with BQ123) compared to the untreated condition (P=0.02) (Figure 6D).

To study the specific effects of ET_A and ET_B antagonists on leukocytes, we used a different protocol since it was not possible to rinse the whole blood and totally exclude contact between the antagonists and the endothelial cells. We therefore infused whole blood directly onto channels coated with purified adhesion proteins (P-selectin, VCAM-1 and ICAM-1). The results illustrated in Figure 6E show that neutrophil adhesion after incubation with BQ123 was not different to that in the untreated condition. In contrast, there was a 16% (±18%) decrease in the adhesion of neutrophils on recombinant adhesion molecules after incubation with BQ788 compared to the untreated sample (P=0.03) (Figure 6E). We could also rule

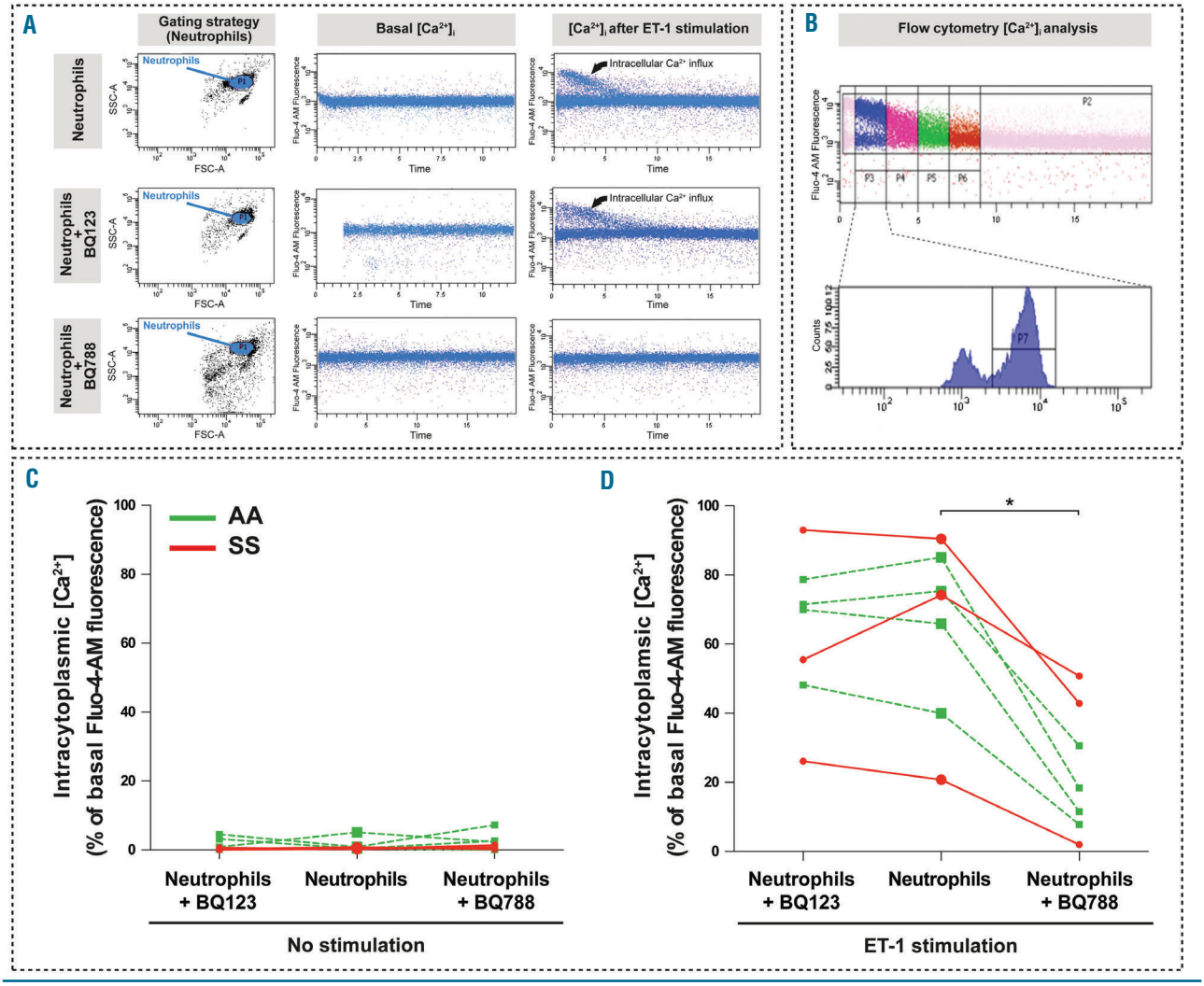

Figure 4. Endothelin-1 elicits an ET_B-mediated calcium response in neutrophils from healthy controls and individuals with sickle cell disease. (A) Representative images of the intracellular calcium measurements in isolated neutrophils: rapid increase of calcium concentration after addition of ET-1, similar effect on neutrophils previously incubated with BQ123, significant reduction of the ET-1-elicited calcium response in neutrophils previously incubated with BQ788. (B) Gating strategy for the comparison of the fluorescence intensity before and after addition of ET-1. (C) Comparison between the percentages of the Fluo4 AM-associated highly fluorescent sub-population (population "P7") from seven independent samples (neutrophils from 4 healthy controls and 3 SCD patients) in the different conditions: (C) Percentages of the Fluo4 AM-associated highly fluorescent neutrophil sub-population (population "P7") from AA individuals (green squares) and from SS patients (red circles) previously incubated with phosphate-buffered saline (unlabeled), BQ123, or BQ788. (D) Percentages of the Fluo4 AM-associated highly fluorescent neutrophil sub-population measured within the first 30 s after addition of ET-1 (period P3), after phosphate-buffered saline, BQ123 or BQ788. Paired comparisons were made. *P<0.05 *vs.* ET-1 + PBS.

out that ET-1 altered human and mouse neutrophil viability as neither exogenous ET-1 nor ET_A or ET_B antagonists altered the proportion of apoptotic and necrotic neutrophils *in vitro* and *in vivo*, respectively (*data not shown*).

Overall these results indicate that activation of the ET_B receptor promotes neutrophil adhesion through action on two compartments: endothelial cells and neutrophils.

Discussion

Polymorphonuclear neutrophils play a major role in the pathogenesis of endothelial injury characteristic of conditions such as ischemia-reperfusion and SCD.[9] Sequestration of neutrophils into the microvasculature is the essential initiating step in neutrophil recruitment in inflammation and this exacerbates vaso-occlusion in SCD.[2] Neutrophils from SCD patients and mice are activated, and show increased adhesion.[2,11] The identification of specific mediators of neutrophil recruitment in SCD is important given that the broad spectrum and non-specific inhibition of neutrophil-endothelium interactions may lead to an increased risk of bacterial infections and trigger VOC and death.

Following studies indicating that the ET system is activated in SCD individuals,[27-32] our group previously showed that ET receptor antagonism not only blunts experimental VOC-associated vasoconstriction but also substantially inhibits acute VOC-induced increases of the numbers of peripheral blood total leukocytes (and specifically neutrophils), neutrophil infiltration of the bronchoalveolar space, renal and pulmonary myeloperoxidase activity and organ damage.[19] Furthermore, early studies suggested that ET-1 acts on neutrophils by increasing intracellular free calcium mobilization,[33] N-formyl-L-methionyl- L-leucyl-L-phenylalanine-mediated superoxide anion production,[34] or aggregation.[35]

We, therefore, hypothesized that the ET system may play a direct role in neutrophil-endothelial interactions in SCD. We found that ET receptors are involved in several steps of neutrophil microvascular recruitment in SCD mice. Whereas rolling adhesion in sickle SAD mice involves the ET_B receptor alone, firm adhesion and post-adhesive dynamic behavior with transmigration involve both the ET_A and ET_B receptors. We also demonstrated an unexpected stimulatory role for the ET_B receptor in adhesion of human neutrophils to endothelial cells under laminar flow conditions. Our findings suggest a differential role for ET receptors between mice and humans given that we found no pro-adhesive role for the ET_A receptor in human neutrophil adhesion, in contrast to the situation in sickle mice.

Another feature was the more intense and prolonged neutrophil adhesion to endothelial cells *in vivo* in sickle mice compared to control mice, whereas no differences were found in human neutrophil adhesion *in vitro*. Our study with human samples may have lacked statistical power, given the greater variability in neutrophil adhesion compared to that measured in mice. This might be due to species specificities and greater genetic heterogeneity in humans than in mice, a factor that should be taken into account when translating animal studies into the clinical setting. One limitation to the *in vitro* experiments was the lack of significant matching of AA with SS individuals providing blood, due to ethical constraints. Differences in models caused by *in vitro* manipulation of human blood with phenotypic heterogeneity of cultured endothelial cells should also be considered. Moreover, chronic *in vivo* exposure of the endothelium to cytokines, products of hemolysis and blood cell micro-vesicles may alter endothelial phenotype and trigger expression of different patterns of adhesion molecules.[36] Nevertheless, both murine and human models provide consistent evidence for a powerful anti-adhesive role for ET_B receptor antagonism.

Our work also challenges the common opinion that adhesion of neutrophils to endothelial cells is always increased in SCD subjects. In fact, Fadlon *et al.*, using a different approach (counting the radioactivity associated with adherent extensively centrifuged, washed and radiolabeled isolated neutrophils bound to layers of human umbilical vein endothelial cells in a static condition), could not show any difference in adhesion of neutrophils from 25 control subjects and 25 patients out of crisis to untreated and TNF-treated endothelial cells.[12] Thus, our measurements of in-flow adhesion of neutrophils in whole blood from six healthy control subjects and 12 patients are consistent with the seminal results of Fadlon *et al*. In contrast, peripheral blood neutrophils from the majority of SCD patients in crisis were more adhesive to cultured endothelial monolayers than neutrophils from patients out of crisis or from healthy control subjects.[162] Likewise, TNFα–injected SCD mice exhibited significant increases in neutrophil adhesion compared to AS mice, as measured by multi-channel fluorescence intravital microscopy analyses.[2,37] In retrospect it is interesting to note that despite the absence of anemia in young animals, the SAD mouse model still presents remarkable neutrophil features in common with other SCD mouse models such as the βS "BERK" and SS knock-in sickle strains, with high counts of circulating leukocytes and neutrophils at steady state and during experimental VOC, thus mimicking what is observed in humans,[19] and exquisite sensitivity to TNFα-induced leukocyte adhesion.[2,37] One of the advantages of the SAD strain is its robustness with a fully controlled

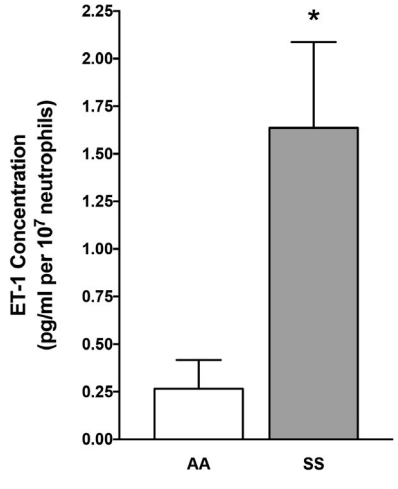

Figure 5. Endothelin-1 secretion by neutrophils from healthy controls and sickle cell disease patients at steady state. ET-1 concentration in supernatants of human neutrophils. Results represent means of 22 independent samples [9 healthy controls (AA), 9 SCD patients without hydroxycarbamide therapy (SS)]. Concentration is normalized for 10^7 neutrophils per condition. *P<0.05 *vs.* AA.

congenic C57Bl6/J background and easy breeding thus enabling experiments without the use of fetal liver or bone marrow transplant. It has also proven its relevance as a model of vaso-occlusion[20,38-40] and of typical chronic degenerative organ injury.[20,38-41] Thus, the SAD mouse model displays a characteristic inflammatory microvascular disease. At least at the level of neutrophil adhesion to the endothelium, our study indicates that this model shares common pathways relevant to the human condition.

Contrasting with the findings of previous studies, which used pharmacological blockade or genetic deficiency models[42-45] to unravel physiological functions of the ET_B receptor, our current data suggest that neutrophil endothelial recruitment is primarily dependent upon an unblocked ET_B receptor. However, previous studies used both non-SCD animal models and human subjects at steady state, in which the primary role of endothelial ET_B is to contribute to ET-1 clearance[42] and stimulation of nitric oxide production which is essential for termination of ET-1 signaling.[45,46] Our novel findings demonstrate an important contribution of the ET_B receptor to vascular inflammation in SCD.

Here, we provide the first series of systematic investigations aimed at unravelling the role of ET receptors in the different phases of the neutrophil-endothelial interaction. They complement earlier work showing that endothelins are chemoattractants for neutrophils[47] *in vitro*. The first *in vivo* studies used perfusion of exogenous ET-1; this led to a time- and dose-dependent sequential entrapment of platelets and neutrophils in the pulmonary circulation.[48] Similarly, the multi-step recruitment of rabbit peritoneal neutrophils was stimulated by ET-1 and inhibited by a specific antagonist of the ET_A receptor.[49] These findings were recently confirmed and expanded, as treatment with a mixed $ET_{A/B}$ receptor antagonist, bosentan, and selective

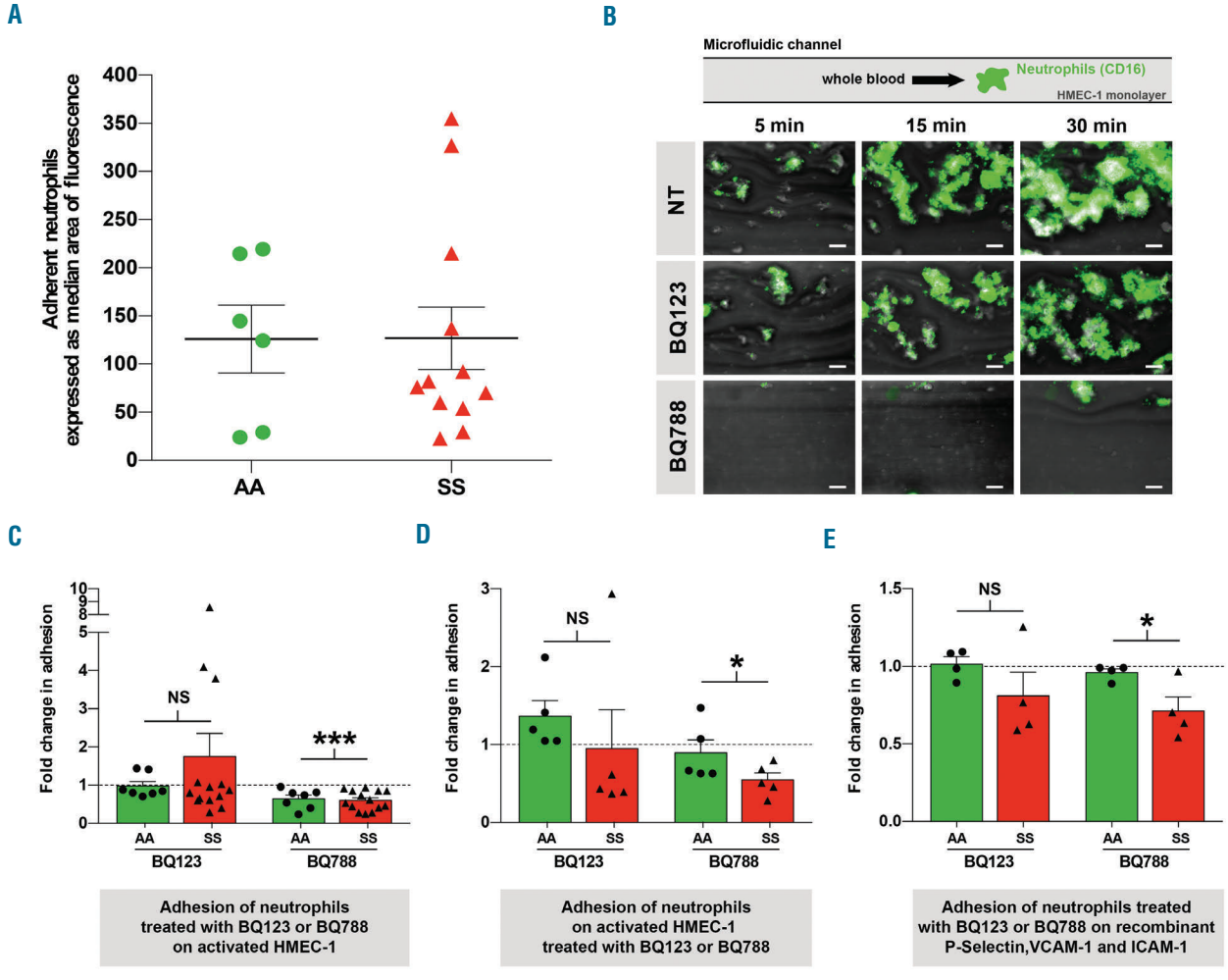

Figure 6. ET_B but nor ET_A blockade prevents adhesion of neutrophils from healthy individuals and patients with sickle cell disease on activated endothelial cells and recombinant adhesion proteins. (A) Adhesion of neutrophils to endothelial cells (HMEC-1) over a 30 min period in a microfluidic system at a shear stress of 1 dyn/cm^2, expressed as median area of neutrophil-associated fluorescence per field comparing six healthy controls (AA) to 12 SCD patients (SS). (B) Representative photomicrographs of anti-CD16 Alexa 488 (in green)-labeled neutrophils that have gradually adhered to the endothelial cells in microfluidic channels. Three channels were infused with the same batch of whole blood. Conditions were: NT: untreated blood; BQ123: blood preincubated with 1 μM BQ123; BQ788: blood previously treated with 1 μM BQ788. (C) Relative change in neutrophil adhesion on activated HMEC-1 after 30 min of infusion of whole blood from seven healthy AA controls (green circles) and 14 SS SCD patients (red triangles) previously incubated with 1 μM BQ123 or 1 μM BQ788 compared with non-treated blood. (D) Relative change in neutrophil adhesion 30 min after infusion of whole blood from five healthy donors (green circles) and five SCD patients (red triangles) on activated HMEC previously incubated with BQ123 or BQ788 compared with untreated HMEC. (E) Neutrophil adhesion on recombinant P-selectin, VCAM-1 and ICAM-1 proteins 30 min after infusion of whole blood from four healthy donors (green circles) and four SCD subjects (red triangles) previously incubated with BQ123 or BQ788 compared with non-treated blood. *P<0,05, ***P<0,001 with paired, non-parametric t-test (Wilcoxon test) vs. untreated blood or HMEC.

ET_A and ET_B receptor antagonists (BQ-123 and BQ-788, respectively) inhibited ET-1 and ovalbumin-induced neutrophil migration to the peritoneal cavity, suggesting that ET-1, acting through both ET_A and ET_B, is an important mediator of neutrophil recruitment in adaptive inflammation.[50] Further studies will be needed to explore these chemotactic actions of ET-1.

It is intriguing that ET receptor blockers had a significantly more potent effect on neutrophil adhesion in sickle SAD mice than in their WT counterparts. This may be due to a potentially stronger chemotactic ET-1 concentration gradient emanating from the pathological endothelium, creating higher ET-1 concentrations in the SCD circulation. We hypothesize that SCD-specific vascular inflammation may prime neutrophils and cause increased neutrophil adhesion and trans-endothelial migration of neutrophils to tissues through increased local ET-1 availability from endothelial cells, neutrophils and, potentially, other, yet to be identified blood cells. Another complementary hypothesis could be that neutrophils from SCD individuals may differ in their ability to degrade local endothelial-derived and autocrine-derived ET-1, as reported for human neutrophils *in vitro*.[51] Our findings may also foster further studies to investigate the role of ET-3, a selective ET_B agonist that may be induced in SCD.[52]

Increased potency of ET receptor blockers on neutrophil adhesion in SCD conditions may also be related to distinct patterns of ET receptor expression, although we did not observe this, at least in human neutrophils. Excess of ligand usually promotes desensitization of G-protein-coupled receptors such as ET receptors, a counter-regulatory mechanism that may be defective in SCD neutrophils or endothelial cells. We did not perform any comparative dose-response studies to evaluate neutrophil sensitivity to ET-1 in SCD and control subjects. Defective desensitization or SCD-specific bias in post-receptor signaling could cause increased ET receptor-dependent cell adhesion. This would be consistent with the more intense and prolonged adhesion of neutrophils to post-capillary venules induced by TNFα in sickle mice compared to that in their WT congenic controls. Thus, the SAD mouse model exhibits exquisite sensitivity to TNFα, reminiscent of TNF-induced leukocyte stasis in the cremasteric venules, which was fatal in a high percentage of mice transplanted with bone marrow from the severe βS "BERK" mouse model.[2,37] The reason for this magnified response to TNFα is still unclear. Such induced blood stasis may trigger VOC in the context of infections, as classically reported in septic patients.

The current studies set the stage for investigation of the ET system in neutrophil recruitment in other conditions beyond SCD. Indeed, early studies suggested a particular role for neutrophil recruitment in models of ischemia-reperfusion injury[53,54] a condition that is particularly relevant to SCD-associated VOC. Conflicting reports regarding the respective roles of the ET_A and ET_B receptors suggest that pathophysiological context matters. Overall, both receptor subtypes have been shown to be involved in undefined steps of neutrophil recruitment[55,56] and our findings further demonstrate the potent effect of ET receptor antagonists on neutrophil adhesion and transmigration in SCD with a particular emphasis on the role of the ET_B receptor.

Our results with human neutrophils and cultured endothelial cells in microfluidic flow chambers indicate that the ET_B receptor promotes neutrophil adhesion through activation at both the neutrophil and the endothelial cell surface.

In endothelial cells, the exact mechanisms whereby recruitment of neutrophils is dependent on an unblocked ET_B receptor remain to be clarified. As discussed above, more efficient coupling of the ET_B receptor to adhesion molecules may be at play.

On the other hand, ET-1 was shown to promote neutrophil aggregation (homotypic adhesion)[35,57] as well as β_2-integrin-dependent attachment of neutrophils to cultured human and bovine vascular endothelial cells (heterotypic cell adhesion). We provide here the first direct evidence of functional ET_B receptors coupled to calcium signaling in neutrophils from control and sickle cell subjects. Together with our results showing that selective ET_B blockade alleviated adhesion of human neutrophils to adhesion molecules, this suggests that ET_B signaling may trigger calcium-induced activation of integrins. Interestingly, increased adhesion has been linked to the higher membrane expression of CD11b/CD18 on neutrophils from SCD patients than on those from controls.[13] Furthermore, blocking CD11b/Mac-1 using M1/70 antibody was sufficient to diminish the erythrocyte–leukocyte interaction significantly, preventing VOC and prolonging survival in SCD mice challenged by TNFα and surgical trauma.[23] These studies highlighted the role of CD11b in the pathophysiology of acute VOC in SCD. It is worth noting that expression of CD18 and CD11b on the surface of neutrophils was also increased by ET-1 *in vitro*[53,58,59] although it was not investigated which ET receptor was involved. In line with these results, administration of TNFα induced very significant upregulation of CD11b expression in neutrophils from both WT and SAD animals but this effect was markedly asymmetric with a further 50% increase in the sickle cell condition. Surprisingly, ET receptor antagonists limited CD11b-associated mean fluorescence surface intensity and the proportion of neutrophils with high CD11b expression in neutrophils from TNFα–challenged SAD animals. Moreover, ET_A and ET_B antagonists had no effect on CD11b expression in normal mice. This suggests that involvement of the ET system in pro-inflammatory CD11b upregulation and neutrophil adhesion is specific to the SCD condition. Moreover, the expression of the integrin is probably not the only regulatory mechanism involved, given that rapid alterations in β_2 integrin conformation, and consequently ligand affinity,[60] could also play a very important role in neutrophil adhesion.

Surprisingly, and contrary to previous reports, we found no difference in CD62L expression in neutrophils from sickle mice compared to normal ones.[9,11] An explanation for this phenomenon might be that, *in vivo*, strongly activated neutrophils may have left the circulation and are therefore underrepresented in the blood samples used for flow cytometry analysis.

In summary, our current findings suggest that human neutrophils display functional ET_B receptors with calcium signaling capability, leading to accentuated adhesion to the endothelium. Using our high-speed imaging platform, we observed that the ET_B response promoted rolling and firm adhesion of neutrophils, resulting in significant subsequent migration of these cells through venular walls to tissue, specifically in sickle cell mice. Furthermore, we confirmed that human neutrophils synthesize ET-1, which may be involved in autocrine and paracrine pathophysiological actions, and may contribute to the higher

plasma levels of ET-1 reported in SCD individuals. Thus, the ET-1-ET_B axis should be considered as a cytokine-like potent pro-inflammatory pathway in SCD. If ET receptor antagonists are proven to be safe and effective in the prevention or treatment of acute VOC in the clinical setting, they should include anti-ET_B potency. Such antagonists may provide major clinical benefits, improve quality of life, and prolong survival of SCD patients.

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