Epeleuton, a novel synthetic ω-3 fatty acid, reduces hypoxia/reperfusion stress in a mouse model of sickle cell disease

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Abstract

Inflammatory vasculopathy is critical in sickle cell disease (SCD)-associated organ damage. An imbalance between pro-inflammatory and pro-resolving mechanisms in response to different triggers such as hypoxia/reoxygenation or infections has been proposed to contribute to the progression of SCD. Administration of specialized pro-resolving lipid mediators may provide an effective therapeutic strategy to target inflammatory vasculopathy and to modulate inflammatory response. Epeleuton (15 hydroxy eicosapentaenoic acid ethyl ester) is a novel, orally administered, second-generation ω-3 fatty acid with a favorable clinical safety profile. In this study we show that epeleuton re-programs the lipidomic pattern of target organs for SCD towards a pro-resolving pattern. This protects against systemic and local inflammatory responses and improves red cell features, resulting in reduced hemolysis and sickling compared with that in vehicle-treated SCD mice. In addition, epeleuton prevents hypoxia/reoxygenation-induced activation of nuclear factor-κB with downregulation of the NLRP3 inflammasome in lung, kidney, and liver. This was associated with downregulation of markers of vascular activation in epeleuton-treated SCD mice when compared to vehicle-treated animals. Collectively our data support the potential therapeutic utility of epeleuton and provide the rationale for the design of clinical trials to evaluate the efficacy of epeleuton in patients with SCD.

Introduction

Sickle cell disease (SCD) is a genetic disease in which a point mutation of the globin gene at the β7 position leads to the production of hemoglobin S (HbS), resulting in chronic hemolysis and anemia.[1] SCD is also characterized by acute vaso-occlusive crises (VOC), a name based on the notion that occlusion of small vessels and/or capillaries by sickled cells is the triggering mechanism for the generation of inflammation and pain.[1] This inflammatory vasculopathy plays a key role in the pathogenesis of both acute and chronic sickle cell-related complications, interfacing the whole blood compartment and being involved in cell-cell pro-adhesion phenomena.[2] Regulatory-body approvals of new treatments such as voxelotor, an inhibitor of HbS polymerization, are important recent milestones.[3] However, the persistence of VOC, as well as chronic disease progression, still represent unmet therapeutic needs, which require additional approaches.

Studies in different models of inflammatory vasculopathy, such as atherosclerosis, diabetes or ischemic cardiomyopathy, have highlighted the yin-yang role of inflammatory response and pro-resolving mechanisms.[4,5] Evidence from this laboratory demonstrates that the pathophysiology of SCD goes beyond the well-known role of polymerized HbS and includes imbalances in pro-inflammatory *versus* pro-resolving mechanisms.[6] This imbalance in turn might promote disease progression and influence the severity of clinical manifestations.[4,5] Consistent with this notion, multiple independent clinical studies profiling plasma, red

cells, or platelet fatty acid composition in patients with SCD demonstrate that there is an underlying relative deficiency of ω-3 fatty acids and their pro-resolving metabolites in SCD under both basal and inflammatory conditions.[7-12] The observed decreases in fatty acids in SCD have also been shown to correlate with inflammation and hemoglobin in patients with SCD.[7,9] An attempt to target inflammatory vasculopathy and to modulate inflammatory response through pro-resolving mechanisms has been made in other diseases, such as cardiovascular diseases, by administering ω-3 fatty acids (ω-3 polyunsaturated fatty acids [PUFA]). Indeed, supplementation with ω-3 fatty acids might act as multimodal therapy by: (i) affecting cell membrane lipid composition; (ii) modulating soluble and cellular inflammatory responses and the coagulation cascade; and (iii) favoring nitric oxide production.[13,14] Previously, we reported that in the humanized mouse model for SCD, PUFA supplementation protected against acute sickle cell-related organ damage induced by hypoxia/reoxygenation (H/R) stress as a model for VOC.[15] These data were complemented by evidence from human studies that the administration of ω-3 PUFA to SCD subjects reduced VOC, pain episodes, and the need for blood transfusions.[16] However, the limited clinical and molecular data on PUFA administration for SCD patients still represents an obstacle to the routine use of PUFA as a therapeutic approach for patients with SCD. In addition, the specific formulation of PUFA supplements markedly affects their bioavailability and clinical outcomes, as demonstrated by the different effects of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) formulations on cardiovascular outcomes.[17]

Epeleuton, 15S-hydroxy eicosapentaenoic acid (15[S] HEPE) ethyl ester, is a second-generation synthetic ω-3 fatty acid derivative of C20:5 n-3 EPA.[18] 15(S)-HEPE, an oxylipin downstream of the 15-lipoxygenase metabolism of EPA, is endogenously produced by hypoxic vascular endothelial cells and can be further transformed to produce lipoxins A_5 and B_5.[19,20] At the time of submitting this article for publication, epeleuton has completed an extensive toxicological evaluation and been administered to more than 340 subjects in phase I and phase II clinical trials, with a favorable safety and tolerability profile. Studies in animal models of atherosclerosis and in patients with non-alcoholic fatty liver disease show that epeleuton has protective effects against inflammatory vasculopathy and markers of systemic inflammation, suggesting its possible transferability to SCD.[18] Notably, decreased concentrations of 15-HEPE were observed in humanized SCD mice under both normoxic and hypoxic conditions compared to those in healthy controls, a finding which is consistent with previously reported decreased fatty acid concentrations in patients with SCD.[6]

In this study, using a humanized mouse model of SCD, we found that epeleuton significantly increased organ concentrations of 15-HEPE, which has both anti-inflammatory and pro-resolving functions. We show the benefit of the oral administration of epeleuton in protecting against (H/R-induced stress in lung, liver, and kidney, known target organs for SCD. We also found that epeleuton reprograms the functional profile of spleen macrophages towards a pro-resolving signature and prevents H/R-induced vascular endothelial overactivation. Collectively our data support the potential therapeutic utility of epeleuton, elucidate further mechanisms for the beneficial effects of ω-3 fatty acids, and provide the rationale for the design of clinical trials to evaluate the efficacy of epeleuton in patients with SCD.

Methods

Animals and design of the study

Experiments were performed on 4- to 5-month-old and sex-matched (56.7% males and 43.3% females) healthy control mice (AA: $Hba^{tm1(HBA)Tow}$ $Hbb^{tm3(HBG1, HBB) Tow}$) and mice with SCD (SS: $Hba^{tm1(HBA)Tow}$ $Hbb^{tm2(HBG1, HBB*) Tow}$). We chose to use 4- to 5-month-old mice since we and others have previously reported that the sickle cell-related organ damage in animals of this age is not so severe as to generate confounding factors when the impact of H/R-induced stress is evaluated.[6,15,21-24] This is also corroborated by the observation of Kasztan *et al.*, who found that gender-related differences in kidney function of humanized SCD mice became significant after 24 weeks (6 months) of age.[22] The size of our mouse groups was established based on studies with the same mouse model for SCD generated by us and other laboratories.[6,15,21-24] The animal protocol was approved by the Animal Care and Use Committee of the University of Verona (CIRSAL) and the Italian Ministry of Health (protocol # 56DC9.64), following European directive 2010/63/EU and the Federation for Laboratory Animal Science Associations guidelines and recommendations. Mice were randomly assigned to the different treatment groups and analysis. Healthy and SCD mice were treated with either vehicle (hydroxypropyl methyl cellulose, 0.5%) alone or with epeleuton (1,000 mg/kg/day) in the vehicle, administered by gavage daily for 6 weeks. The dosage of epeleuton was based on previous pharmacokinetic studies in rodents, which yielded concentrations in plasma comparable to those observed in patients.[18] When indicated, mice underwent H/R stress: 10 hours of hypoxia (8% oxygen) followed by 3 hours of reoxygenation (21% oxygen) to mimic an acute VOC, as previously described.[6,15] Whole blood and organs were collected from each mouse under isoflurane anesthesia. Details on organ collection, as well as evaluation of the biochemical and hematologic parameters are reported in the *Online Supplementary Methods*.

Organ lipidomics

Details are reported in the *Online Supplementary Methods*.

Histological and immunoblot analyses

Histological analysis of lung, kidney and liver tissue and

immunoblot analyses were carried out as previously described.[6,15,21] Details are provided in the *Online Supplementary Methods*.

Analysis of circulating and spleen neutrophils and kidney leukocytes

Circulating and splenic total leukocytes and neutrophils were identified in freshly collected blood and spleen by flow cytometry as CD45$^+$ cells and CD45$^+$Ly6G$^+$ cells.[25] Details are provided in the *Online Supplementary Methods*.

Analysis of efferocytosis and macrophage receptors

Details are reported in the *Online Supplementary Methods*.[6]

Statistical analysis

A two-tailed unpaired Student *t* test or two-way analysis of variance with Tukey multiple comparisons test were used for data analyses. Whenever indicated we used an unpaired Student *t* test with Bonferroni correction. Normality was assessed with the Shapiro-Wilk test. Lipidomic data were analyzed using the non-parametric Kruskal Wallis test because normality was not met.

A *post-hoc* analysis was performed to determine the statistical power of the study (with computations carried out using G*Power 3.1.9.4). The achieved power for Figures 1A, 3A, 3B, and *Online Supplementary Figure S2* was greater than or equal to 88%.

Data show values from individual mice and are presented as the mean ± standard error of mean (SEM). Differences with $P<0.05$ were considered statistically significant. Whisker plots show data points and mean ± SEM.

Results

Epeleuton reduces neutrophil counts and modulates inflammatory response in mice with sickle cell disease

Epeleuton was well tolerated and did not cause major

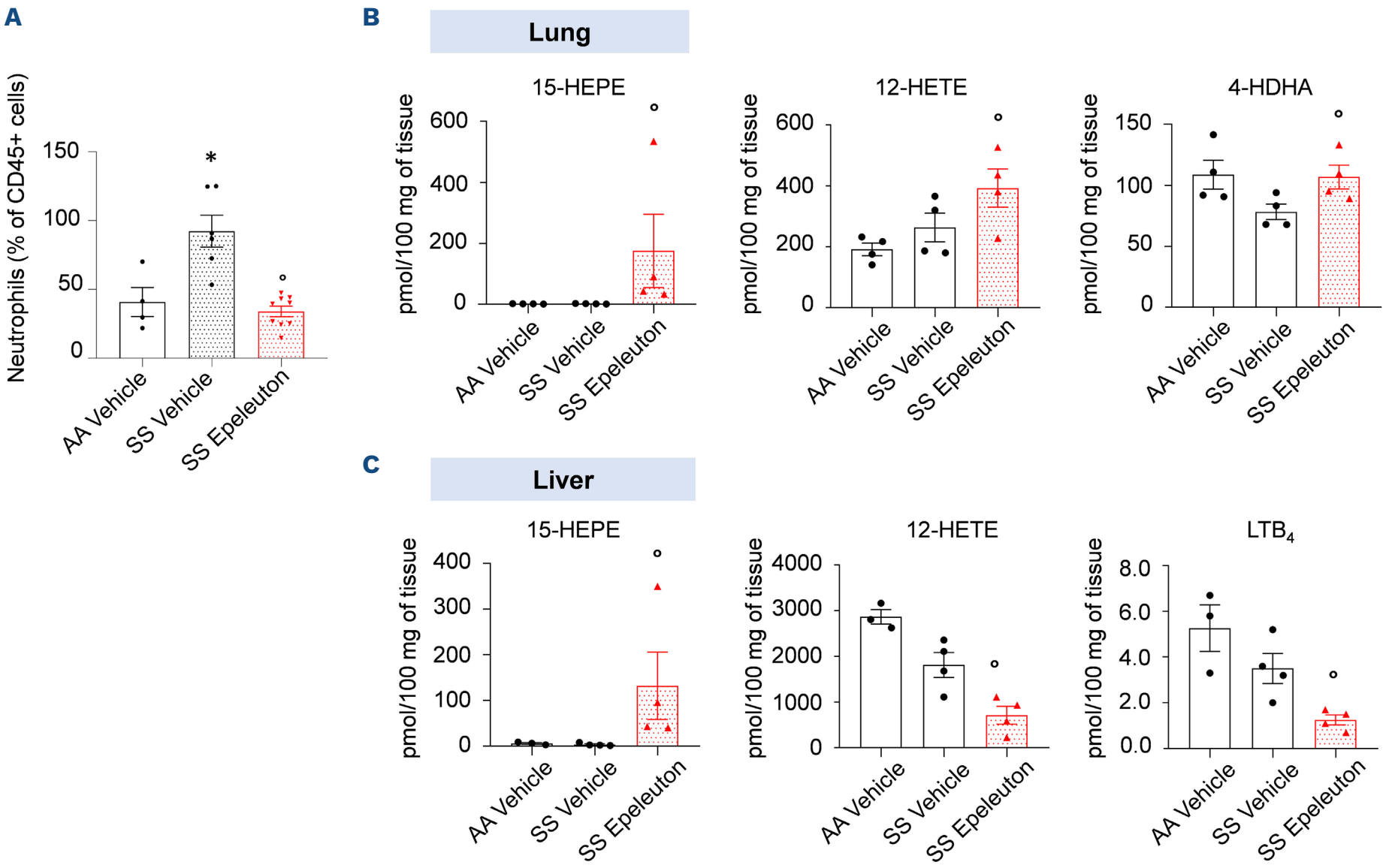

Figure 1. Epeleuton reduces circulating neutrophils and promotes a pro-resolving lipidomic profile in lung and liver of mice with sickle cell disease. (A) Circulating neutrophils identified by flow cytometric analysis as CD45$^+$Ly6G$^+$ cells in healthy mice (AA) and sickle cell disease (SCD) mice (SS) treated with either vehicle or epeleuton (1,000 mg/kg/day for 6 weeks). Data are presented as mean ± standard error of mean (SEM) (N=4-9). *$P<0.05$ compared to healthy mice; °$P<0.05$ compared to vehicle-treated SS animals by one-way analysis of variance. (B, C) Liquid chromatography tandem mass spectrometry-based lipidomics of oxylipins in lung (B) and liver (C) from AA, vehicle-treated SCD, or epeleuton-treated SCD mice. Results are mean ± SEM (N=3-4). °$P<0.05$ compared to vehicle-treated SS animals by the Kruskal Wallis test. 15-HEPE: 15-hydroxyeicosanopentaenoic acid; 12-HETE: 12-hydroxyeicosatetetraenoic acid; 4-HDHA: 4-hydroxydocosahexaenoic acid; LTB_4: leukotriene B4.

changes in body weight of the mice (*Online Supplementary Figure S1A*). We observed a trend toward a reduction of serum creatinine in epeleuton-treated SCD mice compared to vehicle-treated mice, with no change in blood urea nitrogen (*Online Supplementary Figure S1B*). A significant reduction in the level of alanine aminotransferase was observed in epeleuton-treated SCD mice compared to the level in either healthy or vehicle-treated SCD mice, with no change in aspartate aminotransferase (*Online Supplementary Figure S1C*). We found a significant reduction in C-reactive protein, and a trend toward a reduction of pentraxin-2, two markers of systemic inflammation (*Online Supplementary Figure S2A*), in epeleuton-treated SCD mice. These changes were accompanied by a lower plasma level of CCL2 monocyte chemoattractant protein 1, a mediator of neutrophil recruitment and function[26] (*Online Supplementary Figure S2B*). These data indicate a systemic anti-inflammatory effect of epeleuton in humanized SCD mice.

Under normoxia, epeleuton-treated SCD mice did not show significant changes in either hemoglobin concentration or reticulocyte counts (*Online Supplementary Figure S2C*), whereas we observed a significant reduction in circulating neutrophils (Figure 1A, *Online Supplementary Figure S3A*). Since the spleen might act as reservoir of inflammatory cells but also contribute to their clearance,[27] spleen-associated neutrophils were evaluated. No major changes in spleen weight/mouse weight ratio and or in spleen neutrophils were observed between epeleuton-treated SCD mice and vehicle-treated SCD animals (*Online Supplementary Figure S3B, C*).

Epeleuton promotes a pro-resolving profile in target organs and prevents the activation of the nuclear factor-κB pathway in mice with sickle cell disease

We carried out a lipidomic analysis of lung and liver from both mouse strains treated with either vehicle or epeleuton. In agreement with our previous study, we found abnormalities in the composition of both mono- and poly-unsaturated fatty acids in lung and liver from SCD mice compared to AA animals (*Online Supplementary Table S1*) despite the two mouse colonies having been fed the same diet.[15] These abnormalities consisted of a significantly lower ω-3 content, mainly α linolenic acid (C18:3 n-3), docosapentaenoic acid (DPA:5 -3), eicosatetraenoic acid (C20:4 n-3), and a lower ω-6/ω-3 ratio compared to that in healthy animals (*Online Supplementary Tables S1* and *S2*). As expected, there was a significant increase in lung and liver concentrations of 15-HEPE in epeleuton-treated SCD mice compared to vehicle-treated SCD or AA mice (Figure 1B, C). Total and differential amounts of saturated and unsaturated fatty acids in liver and spleen, as well as the ω-6/ω-3 ratio were not significantly different in epeleuton-treated SCD mice compared to vehicle-treated SCD or AA mice (*Online Supplementary Tables S1* and *S2*). In contrast, epeleuton caused striking changes in select oxylipins, increasing 12-HETE and 4-HDHA in lung from SCD when compared with the levels in vehicle-treated SCD mice (Figure 1B). This may have organo-protective effects, as observed in retinal tissue following H/R.[28] 12-HETE was significantly reduced by epeleuton in liver, which could reflect a blunted response of liver macrophages and/or platelets (which are 12-lipoxygenase-bearing cells)[29] and there was a trend towards an increase of liver 4-HDHA (Figure 1C). Furthermore, significantly lower concentrations of leukotriene B4, which is a main driver of neutrophil infiltration, and of the ω-3 docosapentaenoic acid (C22:5 n-3) were measured in liver from epeleuton-treated SCD mice than in vehicle-treated SCD mice (Figure 1C). These results indicate that the changes in SCD lipid profile produced by epeleuton are specific and not related to broad changes in lipid absorption or metabolism.

The transcriptional factor NF-κB is a key regulator of inflammation, and of the expression of genes involved in initiation of inflammation with cell-specific actions such as the modulation of vascular pro-adhesion molecules, including vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1).[6] We previously found that ω-3 fatty acid administration to SCD mice prevented the activation of NF-κB p65 in target organs for SCD, such as lung, kidney, and liver.[15] As shown in Figure 2A-C, the activation of NF-κB was significantly reduced in lungs, kidneys, and livers from humanized SCD mice treated with epeleuton compared to the activation in SCD mice treated with vehicle (*Online Supplementary Figure S4A*). Consistent with these observations, in lungs, kidneys, and livers from epeleuton-treated SCD mice we found significant reductions in the expression of VCAM-1 and ICAM-1, markers of inflammatory vasculopathy and endothelial activation, and in the expression of endothelin-1 (ET-1) a potent vaso- and broncho-constrictive cytokine involved in SCD-related organ damage[6] (Figure 2D, E; *Online Supplementary Figures S4B* and *S5A*). Livers from epeleuton-treated mice also displayed a marked reduction in liver protein oxidation, reaching values similar to those of healthy mice (Figure 2F, G; *Online Supplementary Figure S5B*).

Collectively our data indicate that epeleuton diminishes local sickle cell-related inflammatory responses and vascular endothelial activation in SCD-target organs.

Epeleuton protects against hypoxia/reoxygenation-induced hemolysis and re-programs spleen macrophages towards a pro-resolving pattern in mice with sickle cell disease

We tested epeleuton in sickle cell mice exposed to H/R stress, which is an experimental model of sickle cell-related VOC.[15,30] As shown in Figure 3A, the administration of epeleuton to SCD mice prevented the H/R-induced reduction in hematocrit and hemoglobin and decreased the amount of sickled red cells (*Online Supplementary Figure*

S6A). This suggests that in SCD mice epeleuton protects red cells against H/R-induced stress. The improvement of red cell membrane features was also supported by a reduction in the tyrosine phosphorylation state of band 3, an integral red cell membrane protein involved in membrane stability and release of erythroid microparticles[31] (Figure 3C, *Online Supplementary Figure S6B*), and the reduction in lactate dehydrogenase, a known marker of hemolysis (Figure 2D). In SCD mice epeleuton significantly reduced the H/R-induced increase in neutrophil counts (Figure 3E). This was associated with lower plasma C-reactive protein and pentraxin-2 levels (*Online Supplementary Figure S6C*) in epeleuton-treated SCD mice than in vehicle-treated mice. We also found a significant decrease of spleen neutrophils in epeleuton-treated SCD mice exposed to H/R compared to the counts in vehicle-treated SS animals (*Online Supplementary Figure S6D*). This was associated with a significant reduction in classic spleen macrophage inflammatory markers (CD80, CD68) (Figure 3F) and a trend towards a reduction in the clearance of damaged red blood cells in SCD mice treated with

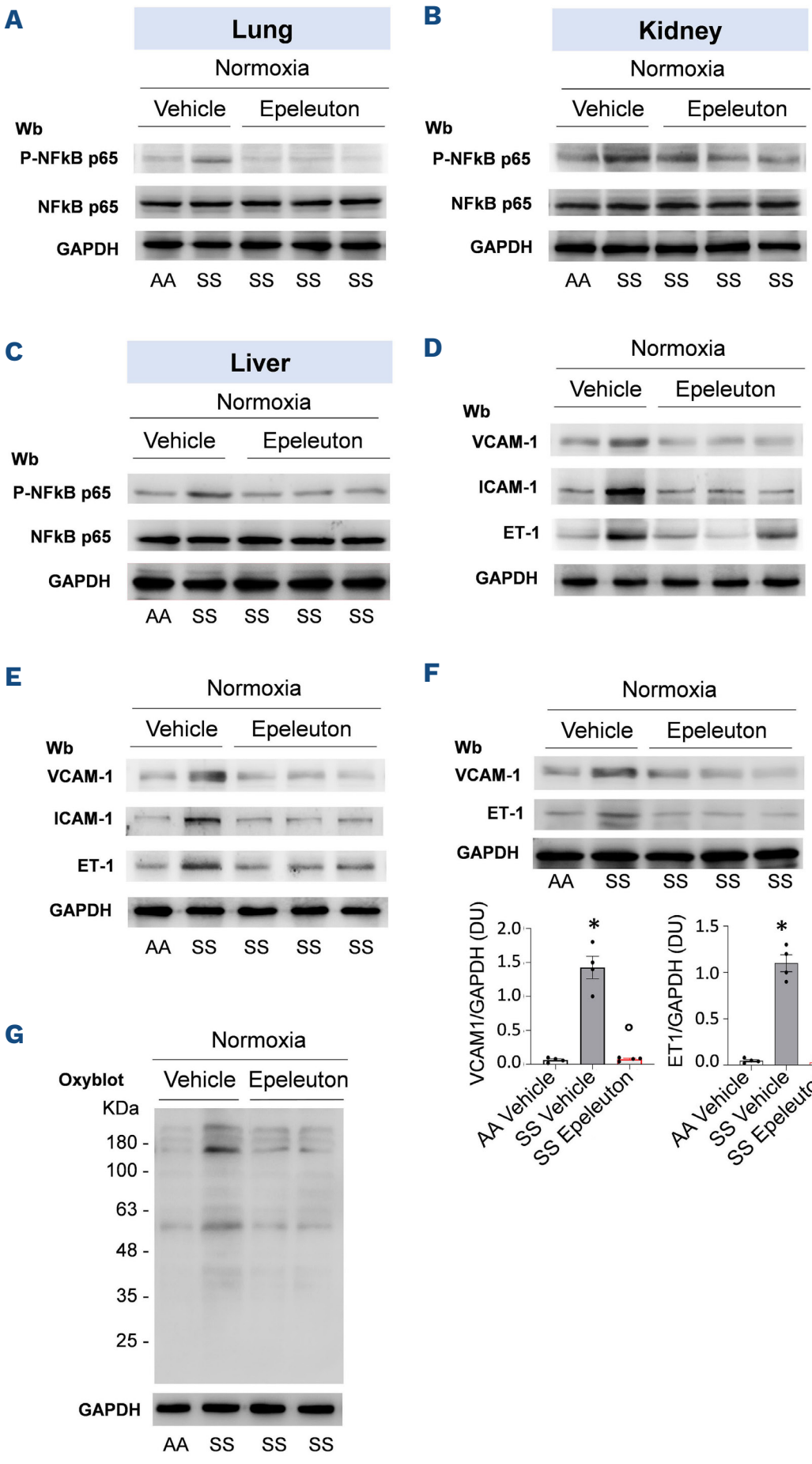

Figure 2. Epeleuton modulates inflammatory response with downregulation of markers of inflammatory vasculopathy. (A-C) Immunoblot analyses using specific antibodies against phosphorylated (p-) NF-κB p65 and NF-κB p65 in lung (A), kidney (B) and liver (C) from healthy mice (AA) and sickle cell disease (SCD) mice (SS) under normoxic conditions treated with either vehicle or epeleuton (1,000 mg/kg/day for 6 weeks). Lane 1: vehicle-treated AA mouse; lane 2: vehicle-treated SS mouse; lanes 3-5: epeleuton-treated SCD mice (results for 3 separate animals are shown). Protein (75 μg) was loaded on an 8% T, 2.5% C polyacrylamide gel. One representative gel from four with similar results is shown. Densitometric analysis of the immunoblots is shown in *Online Supplementary Figure S4A*. (D, E) Immunoblot analysis using specific antibodies against VCAM-1, ICAM-1, and ET-1 in lung (D) and kidney (E) from AA and SCD mice treated as in (A). Lane 1: vehicle-treated AA mouse; lane 2: vehicle-treated SS mouse; lanes 3-5: epeleuton-treated SCD mice (results for 3 separate animals are shown). Protein (75 μg) was loaded on an 11% T, 2.5% C polyacrylamide gel. One representative gel from four with similar results is shown. Densitometric analysis of immunoblots is shown in *Online Supplementary Figures S4B* and *S5A*. (F) Upper panel. Immunoblot analysis using specific antibodies against VCAM-1 and ET-1 in liver from AA and SCD mice treated as in (A). Lane 1: vehicle-treated AA mouse; lane 2: vehicle-treated SS mouse; lanes 3-5: epeleuton-treated SCD mice (results for 3 separate animals are shown). Protein (50 μg/μL) was loaded on an 8% T, 2.5% C polyacrylamide gel. One representative gel from four with similar results is shown. Lower panel. Densitometric analysis of the immunoblots. Data are presented as means ± standard error of mean (N=4); **P*<0.05 compared to AA mice; °*P*<0.05 compared to vehicle by one-way analysis of variance. (G) OxyBlot analysis of the soluble fractions of liver from AA and SCD mice treated as in (B). Lane 1: vehicle-treated AA mouse; lane 2: vehicle-treated SS mouse; lanes 3-5: epeleuton-treated SCD mice (results from 2 separate animals are shown). The carbonylated proteins (1 mg) were detected by treatment with 2,4-dinitrophenylhydrazine (DNP) and blotted with anti-DNP antibody. Quantification of band area is shown in *Online Supplementary Figure S5B*. GAPDH served as the protein loading control (A-G). Wb: western blot; NFkB: nuclear factor κB; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; VCAM-1: vascular cell adhesion molecule 1; ICAM-1: intercellular adhesion molecule 1; ET-1: endothelin-1; DU: densitometric units.

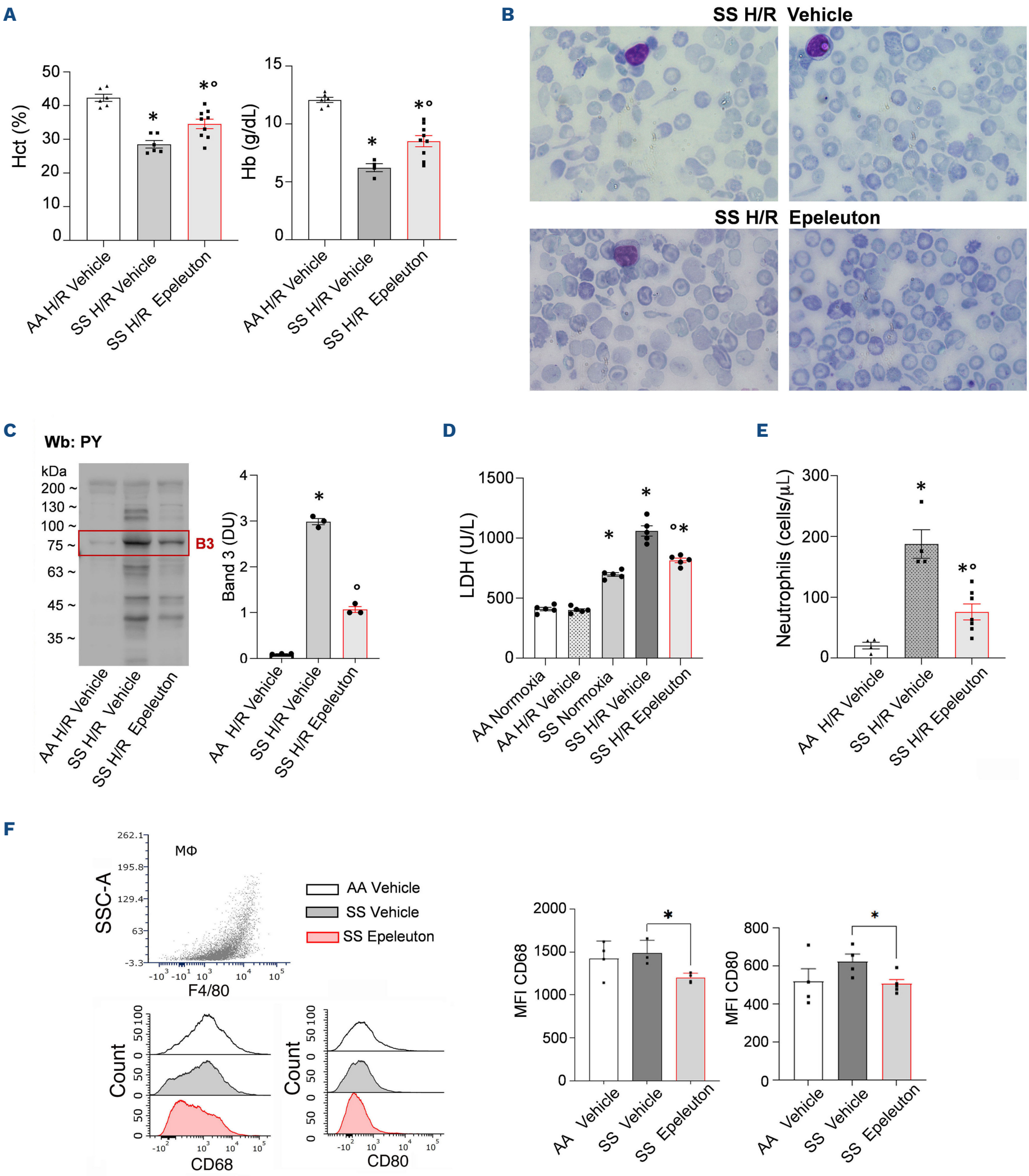

Figure 3. In sickle cell disease mice exposed to hypoxia/reoxygenation stress, epeleuton reduces the stress-induced hemolysis, modulates the inflammatory response, and reprograms spleen macrophages towards a pro-resolving pattern. (A) Hematocrit (left panel) and hemoglobin (right panel) in healthy mice (AA) and sickle cell disease (SCD) mice (SS) exposed to hypoxia/reoxygenation (H/R): stress, hypoxia (8% oxygen; 10 hours) followed by reoxygenation (21% oxygen; 3 hours), treated with either vehicle or epeleuton (1,000 mg/kg/day for 6 weeks). Data are presented as means ± standard error of mean (SEM) (N=4-9). *P<0.05 compared to AA mice; °P<0.05 compared to vehicle-treated mice by an unpaired t test with Bonferroni correction. (B) Erythrocyte morphology in a blood smear of SCD mice treated as in (A). One representative image is shown. Orig-

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inal magnification 100×. Quantification is shown in *Online Supplementary Figure S6A*. (C) Immunoblot analysis using specific anti-phospho-tyrosine antibody of red cell membrane proteins from mice treated as in (A). Lane 1: vehicle-treated AA mouse exposed to H/R; lane 2: vehicle-treated SCD mouse exposed to H/R; lane 3: epeleuton-treated SS mouse exposed to H/R. Proteins (75 μg) were loaded on an 8% T, 2.5% C polyacrylamide gel (see *Online Supplementary Figure S6B* for Coomassie staining, used as the loading control). Densitometric analysis of the phosphorylation of band-3 is shown on the right. Data are presented as means ± standard error of mean (SEM) (N=3). **P*<0.05 compared to AA mice; °*P*<0.05 compared to vehicle-treated mice by an unpaired *t* test with the Bonferroni correction. (D) Plasma concentrations of lactate dehydrogenase in AA and SCD mice under conditions of normoxia or exposed to H/R and treated as in (A). Data are mean ± SEM (N=5). **P*<0.05 compared to AA mice; °*P*<0.05 compared to vehicle-treated mice by one-way analysis of variance (ANOVA). (E) Circulating neutrophils identified by flow cytometric analysis as $CD45^+Ly6G^+$ cells in mice treated as in (A). Data are mean ± SEM (N=4-7), **P*<0.05 compared to AA mice; °*P*<0.05 compared to vehicle-treated mice by one-way ANOVA. (F) Flow cytometry gating strategy and representative plots of spleen macrophages from AA or SCD mice treated with vehicle or epeleuton. M1 marker expression on spleen macrophages and red blood cell clearance from AA or SS mice fed with epeleuton, as determined by flow cytometry in $F4/80^+$ cells. Results are means ± standard deviation (N=4 mice/group); **P*<0.05 (one-way ANOVA). Hct: hematocrit; Hb: hemoglobin; Wb: western blot; PY: phosphotyrosine; B3: band 3; DU: densitometric units; LDH: lactate dehydrogenase; SSC: side scatter; MFI: mean fluorescence intensity.

epeleuton compared with that in mice treated with vehicle (*Online Supplementary Figure S6E*). It is noteworthy that the effects of epeleuton on neutrophils and macrophage re-programing in SCD mice are consistent with the pro-resolving actions we previously established for 17R-RvD1 in SCD mice.[6]

Taken together, these data indicate that epeleuton protects against H/R-induced stress, attenuating inflammation, and leading to early initiation of resolution events.

Epeleuton reduces lung injury and prevents the overactivation of nuclear factor κB and hypoxia-induced lung inflammatory vasculopathy in mice with sickle cell disease

In SCD mice treated with epeleuton, we observed a trend towards a reduction in lung inflammatory cell infiltrates compared to those in SCD mice treated with the vehicle (Figure 4A, *Online Supplementary Table S3*). Consistent with these observations, we found a significant decrease in the active forms of NF-κB p65 induced by H/R in SCD mice treated with epeleuton (Figure 4B). To assess whether epeleuton was protective against H/R-induced inflammation and vascular dysfunction, we evaluated lung expression of: (i) the NLRP3 inflammasome, a key player in sterile inflammation; (ii) vascular endothelial activation; and (iii) neutrophil vascular recruitment.[6] As shown in Figure 4C, in lung tissue from epeleuton-treated SCD mice exposed to H/R, we found downregulation of the NLRP3 inflammasome, which has been reported to participate in inflammation in models of acute lung injury such as acute respiratory disease syndrome and asthma.[32] In addition, epeleuton reduced the expression of VCAM-1, E-selectin, and thromboxane synthase 1 (TXAS) (Figure 4C). TXAS is controlled by the NF-κB p65 signaling pathway, and it has been linked to activation of both vascular endothelial cells and platelets in other models of ischemia-reperfusion damage.[6,33,34]

These data provide evidence that epeleuton protects SCD mice against H/R-induced lung injury by modulation of NF-κB and key mediators of vascular damage.

Epeleuton diminishes hypoxia/reoxygenation-induced kidney damage and markers of vascular dysfunction in mice with sickle cell disease

Histopathological analysis of kidney tissue from SCD mice exposed to H/R revealed no major effect of epeleuton on kidney inflammatory cell infiltrates in hematoxylin and eosin-stained preparations (Figure 5A, *Online Supplementary Table S3*), whereas we found a significant reduction in total leukocyte infiltrates, as determined by flow-cytometric analysis, in kidney from epeleuton-treated SCD mice compared to vehicle-treated animals (Figure 5B). We also observed a reduction of H/R-induced increases in plasma creatinine and blood urea nitrogen levels in the epeleuton-treated SCD mice compared to the levels in the vehicle-treated SCD animals (Figure 5C). This is consistent with the decreased H/R-induced activation of NF-κB p65 in kidneys from epeleuton-treated SCD mice compared to activation in vehicle-treated animals (Figure 5D). The beneficial effects of epeleuton in SCD mice were further supported by the reduction in H/R-induced increase in expression of NLRP3, VCAM-1, ET-1, and TXAS-1, which are involved in vascular activation, reduction of kidney vascular tone, and inflammation (Figure 5E, *Online Supplementary Figure S7*).

Taken together our data indicate that epeleuton mitigates H/R-induced acute kidney damage and modulates the related amplified inflammation and vascular dysfunction.

In sickle cell disease mice exposed to hypoxia/reoxygenation stress, epeleuton reduces liver injury and prevents the overactivation of inflammatory and redox-related pathways

Compared to vehicle-treated mice, SCD mice treated with epeleuton and exposed to H/R stress had fewer inflammatory cell infiltrates and a significant reduction in thrombi formation (Figure 6A). In addition, we found a significant reduction in liver iron accumulation, mainly characterized by decreased iron accumulation in hepatocytes of SCD mice treated with epeleuton and exposed to H/R stress (Figure 6A, lower panel; *Online Supplementary Figure S8A*).

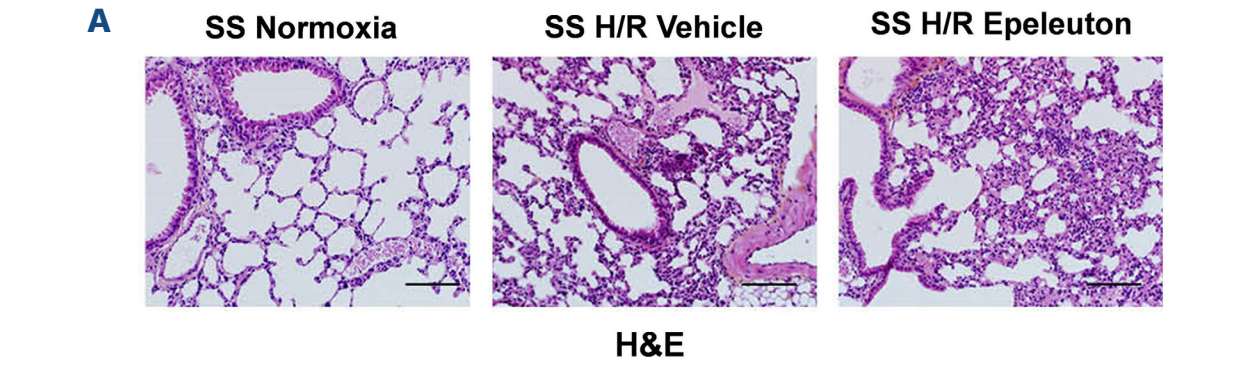

Figure 4. Epeleuton reduces lung injury, preventing the overactivation of nuclear factor κB and hypoxia-induced lung inflammatory vasculopathy in mice with sickle cell disease. (A) Representative micro-picture of hematoxylin and eosin-stained sections of lung at 200x magnification from sickle cell disease (SCD) mice (SS) in conditions of normoxia and exposed to hypoxia/reoxygenation (H/R),hypoxia (8% oxygen; 10 hours) followed by reoxygenation (21% oxygen; 3 hours), treated with either vehicle or epeleuton (1,000 mg/kg/day for 6 weeks) (scale bar: 50 µm) (see also *Online Supplementary Table S3*). (B) Left panel. Immunoblot

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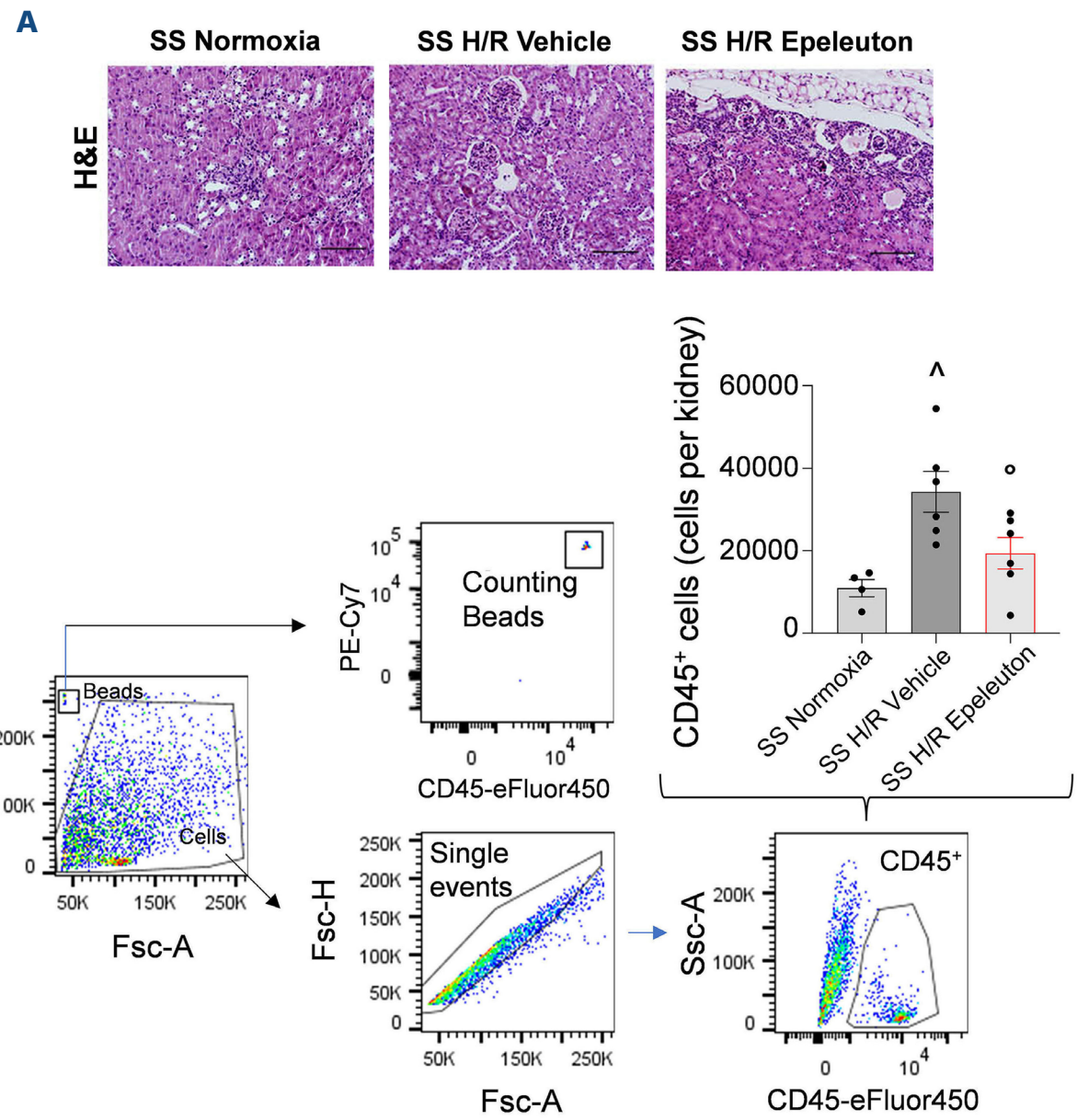
analysis using specific antibodies against phosphorylated (p-)NF-κB p65 and NF-κB p65 in lung from AA and SS mice treated as (A). Lane 1: AA mouse under normoxia; lane 2: SS mouse under normoxia; lane 3: vehicle-treated AA mouse exposed to H/R; lane 4; vehicle-treated SS mouse exposed to H/R; lanes 5 and 6: epeleuton-treated SS mice exposed to H/R (results from 2 separate animals are shown). Protein (75 μg) was loaded on an 8% T, 2.5% C polyacrylamide gel. GAPDH served as the protein loading control. One representative gel from four with similar results is shown. Right panel. Densitometric analysis of the immunoblots. Data are presented as means ± standard error of mean (SEM) (N=4). *P<0.05 compared to AA mice; ^P<0.05 compared to normoxia; °P<0.05 compared to vehicle by one-way analysis of variance (ANOVA). (C) Left panel. Immunoblot analysis using specific antibodies against NLRP3, VCAM-1, E-selectin and TXAS in lung from AA and SS mice treated as in (A). Lane 1: AA mouse under normoxia; lane 2: SS mouse under normoxia; lane 3: vehicle-treated AA mouse exposed to H/R; lane 4: vehicle-treated SS mouse exposed to H/R; lanes 5 and 6: epeleuton-treated SS mice exposed to H/R (results for 2 separate animals are shown). Protein (75 μg) was loaded on an 8% T, 2.5% C polyacrylamide gel. GAPDH served as the protein loading control. One representative gel from four with similar results is shown. Right panels. Densitometric analyses of the immunoblot. Data are presented as means ± SEM (N=4). *P<0.05 compared to AA mice; ^P<0.05 compared to normoxia; °P<0.05 compared to vehicle by one-way ANOVA. H&E: hematoxylin and eosin; Wb: western blot; NFkB: nuclear factor κB; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; DU: densitometric units; NLRP3: nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3; VCAM-1: vascular cell adhesion molecule 1; TXAS: thromboxane synthase.



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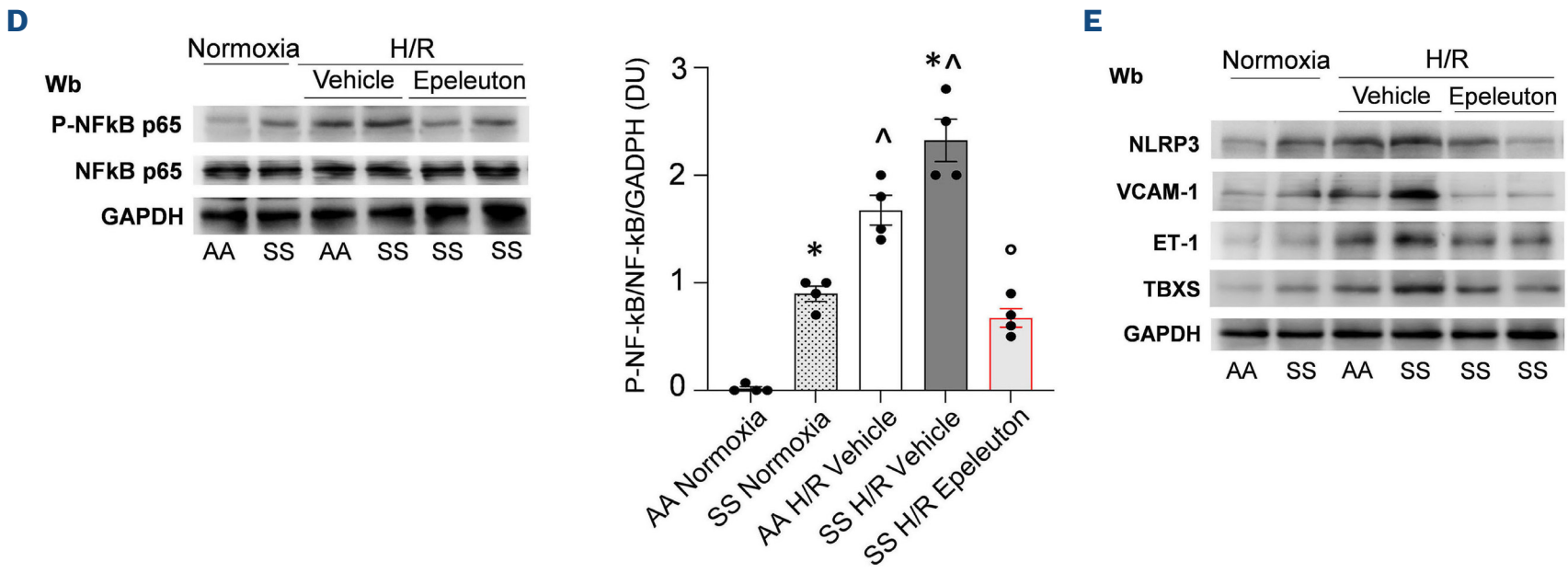

Figure 5. Epeleuton diminishes hypoxia/reoxygenation-induced kidney damage and markers of vascular dysfunction in mice with sickle cell disease. (A) Representative micro-pictures of hematoxylin and eosin-stained sections of kidney at 200x magnification from sickle cell disease (SCD) mice (SS) exposed to hypoxia/reoxygenation (H/R): hypoxia (8% oxygen; 10 hours), followed by re-oxygenation (21% oxygen; 3 hours) treated with either vehicle or epeleuton (1,000 mg/kg/day for 6 weeks) (scale bar: 50 μm) (see also *Online Supplementary Table S3*). (B) Kidney leukocyte infiltrates determined by flow cytometry gating analysis (the gating strategy is shown in *Online Supplementary Figure S8*). Data are presented as means ± standard error of mean (SEM) (N=4-6). ^*P*<0.05 compared to normoxia; °*P*<0.05 compared to vehicle by an unpaired *t* test with Bonferroni correction. (C) Plasma blood urea nitrogen (left panel) and creatinine (right panel) in healthy (AA) and SCD (SS) mice under conditions of normoxia or exposed to H/R as in (A). Data are means ± SEM (N=6). **P*<0.05 compared to AA mice; ^*P*<0.05 compared to normoxia; °*P*<0.05 compared to vehicle by one-way analysis of variance (ANOVA). (D) Left panel. Immunoblot analysis using specific antibodies against phosphorylated (p-) NF-κB p65 and NF-κB p65 in kidney from AA and SS mice treated as in (A). Lane 1: AA mouse under normoxia; lane 2: SS mouse under normoxia; lane 3: vehicle-treated AA mouse exposed to H/R; lane 4: vehicle-treated SS mouse exposed to H/R; lanes 5 and 6: epeleuton-treated SS mice exposed to H/R (results from 2 separate animals are shown). Protein (75 μg) was loaded on an 8% T, 2.5% C polyacrylamide gel. One representative gel from four with similar results is shown. Right panel. Densitometric analysis of the immunoblots. Data are presented as means ± SEM (N=4). **P*<0.05 compared to AA mice; ^*P*<0.05 compared to normoxia; °*P*<0.05 compared to vehicle by one-way ANOVA. (E) Immunoblot analysis, using specific antibodies against NLRP3, VCAM-1, ET-1 and TBXS in kidney from AA and SS mice treated as in (A). Lane 1: AA mouse under normoxia; lane 2: SS mouse under normoxia; lane 3: vehicle-treated AA mouse exposed to H/R; lane 4: vehicle-treated SS mouse exposed to H/R; lanes 5 and 6: epeleuton-treated SS mice exposed to H/R (results for 2 separate animals are shown). Protein (75 μg) was loaded on an 11% T, 2.5% C polyacrylamide gel. One representative gel from four with similar results is shown. Densitometric analysis of immunoblots is shown on the right. Data are presented as means ± SEM (N=4). **P*<0.05 compared to AA mice; ^*P*<0.05 compared to normoxia; °*P*<0.05 compared to vehicle by one-way ANOVA. GAPDH served as the protein loading control (D, E). H&E: hematoxylin and eosin; BUN: blood urea nitrogen; Wb: western blot; NFkB: nuclear factor κB; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; DU: densitometric units; Ssc: side scatter; Fsc: forward scatter; PE-Cy7: phycoerythrin cyanine 7; NLRP3: nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3; VCAM-1: vascular cell adhesion molecule 1; ET-1: endothelin 1; TBXS: thromboxane synthase.

In agreement with modulation of local inflammatory response and oxidation, we found reduced activation of both acute phase inflammatory and redox-related transcriptional factors NF-κB p65 and Nrf2 in epeleuton-treated SCD mice exposed to H/R (Figure 6B). This was associated with a marked reduction in liver protein oxidation (*Online Supplementary Figure S8B*) and a consistent downregulation of the antioxidants NQO1 and HO-1, which are regulated by Nfr2[35] (*Online Supplementary Figure S8C*). We then explored the effects of epeleuton on markers of inflammatory response and vascular activation. As shown in Figure 6C, we found lower expression of NLRP3 in livers from epeleuton-treated SCD mice exposed to H/R. Similar NLRP3 inflammasome activation has been reported in models of liver diseases, such as chronic hepatitis B or C and non-alcoholic steatohepatitis.[36] In addition, epeleuton prevented the H/R-induced upregulation of VCAM-1 in livers from SCD mice exposed to H/R stress, while we found a trend towards a reduction of ET-1 in the same group of mice when compared to vehicle-treated animals (Figure 6C).

Epeleuton protects against progression of inflammatory vasculopathy related to acute hypoxia/reoxygenation stress in mice with sickle cell disease

Given the beneficial effects of epeleuton on vascular dysfunction in SCD target organs and of fatty acid supplementation on different models of vascular dysfunction and inflammatory vasculopathy,[6,15,16] we evaluated the impact of epeleuton treatment on sickle cell-related inflammatory vasculopathy.

In isolated aorta from SCD mice under normoxia, epeleuton significantly downregulated the expression of ICAM-1 and ET- 1 but not of VCAM-1 compared to the expression in vehicle-treated SCD mice (*Online Supplementary Figure S9*)

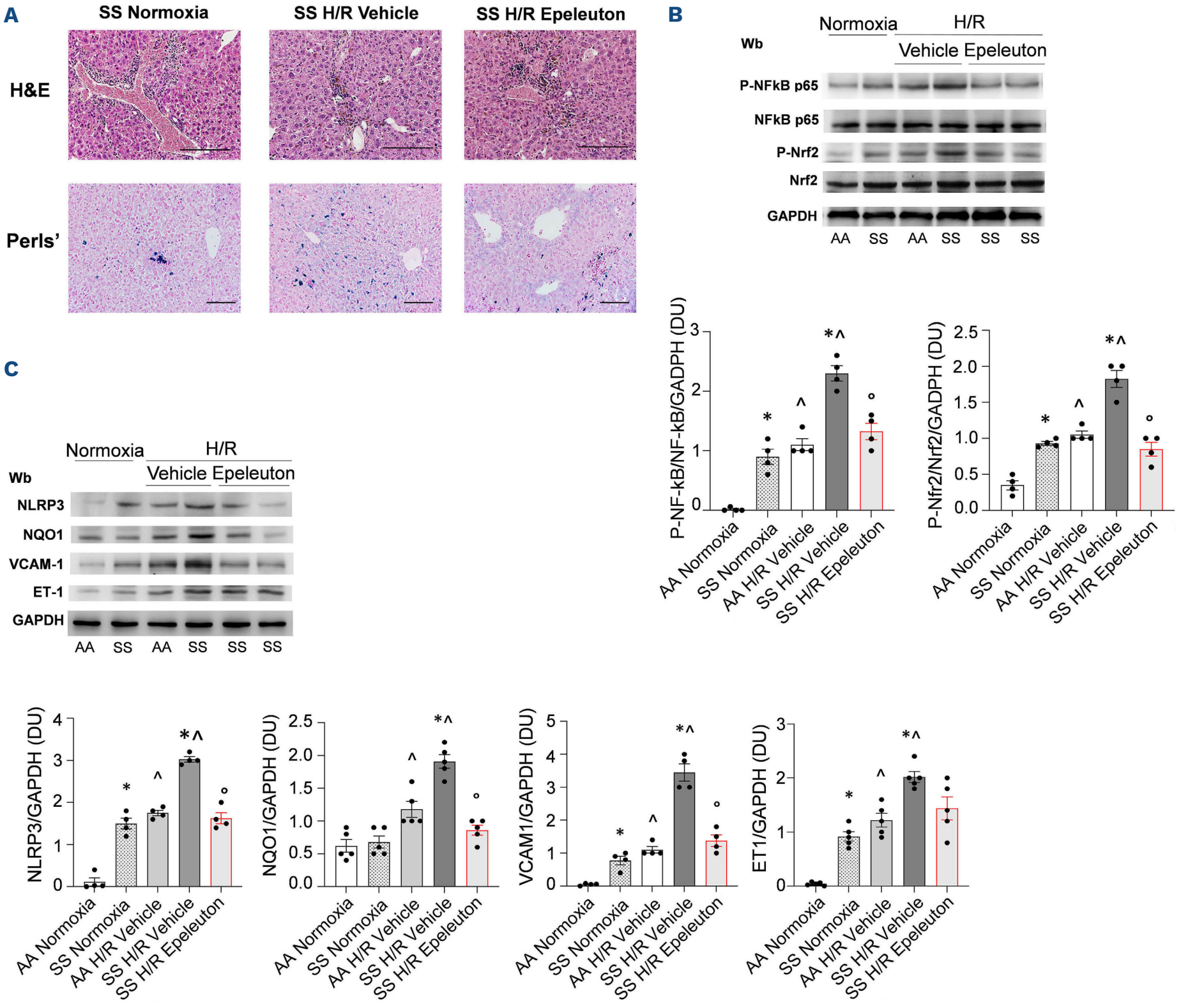

Figure 6. In sickle cell disease mice exposed to hypoxia/reoxygenation stress, epeleuton reduces liver injury and prevents the overactivation of inflammatory and redox-related pathways. (A) Representative micro-picture of hematoxylin and eosin-stained and Perls-stained sections of liver at 200x magnification from sickle cell disease (SCD) mice (SS) exposed to hypoxia/reoxygenation (H/R), hypoxia (8% oxygen; 10 hours) followed by reoxygenation (21% oxygen; 3 hours), treated with either vehicle or epeleuton (1,000 mg/kg/day for 6 weeks) (scale bar: 50 μm) (see also *Online Supplementary Table S3*). (B) Immunoblot analysis using specific antibodies against phosphorylated (p-)NF-κB p65, NF-κB p65, p-Nrf2, and Nrf2 in liver from normal (AA) and SS mice as in (A). Lane 1: AA mouse under normoxia; lane 2: SS mouse under normoxia; lane 3: vehicle-treated AA mouse exposed to H/R; lane 4: vehicle-treated SS mouse exposed to H/R; lanes 5 and 6: epeleuton-treated SS mice exposed to H/R (results from 2 separate animals are shown). Protein (75 μg) was loaded on an 8% T, 2.5% C polyacrylamide gel. One representative gel from four with similar results is shown. Densitometric analyses of the immunoblots are shown in the panels below. Data are presented as means ± standard error of mean (SEM) (N=4).*P<0.05 compared to AA mice; ^P<0.05 compared to normoxia; °P<0.05 compared to vehicle by one-way analysis of variance (ANOVA). (C) Immunoblot analysis, using specific antibodies against NLRP3, NQO1, VCAM-1 and ET-1 in liver from AA and SS mice treated as in (A). Lane 1: AA mouse under normoxia; lane 2: SS mouse under normoxia; lane 3: vehicle-treated AA mouse exposed to H/R; lane 4: vehicle-treated SS mouse exposed to H/R; lanes 5 and 6: epeleuton-treated SS mice exposed to H/R (results from 2 separate animals are shown). Protein (75 μg) was loaded on an 11% T, 2.5% C polyacrylamide gel. One representative gel from four or five with similar results is shown. Densitometric analyses of the immunoblots are shown in the panels below. Data are presented as means ± SEM (N=4/5). *P<0.05 compared to AA mice; ^P<0.05 compared to normoxia; °P<0.05 compared to vehicle by one-way ANOVA. GAPDH served as the protein loading control (B, C). H&E: hematoxylin and eosin; Wb: western blot; NFkB: nuclear factor κB; Nrf2: nuclear factor erythroid 2-related factor 2; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; DU: densitometric units; Ssc: side scatter; Fsc: forward scatter; PE-Cy7: phycoerythrin cyanine 7; NLRP3: nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3; NQO1: NAD(P)H quinone dehydrogenase 1; VCAM1: vascular cell adhesion molecule 1; ET1: endothelin 1.

As shown in Figure 7A, we found a significant reduction in H/R-induced expression of VCAM-1 in epeluton-treated SCD mice compared with vehicle-treated SCD mice. These data indicate that treatment with epeleuton may prevent the worsening of vascular dysfunction induced by H/R stress, with a potential to delay disease progression.

Discussion

In this study, we show that epeleuton, a novel synthetic ω-3 fatty acid derivative, protects against cellular stress associated with SCD and supports early initiation of the resolution phase of inflammation. The observed effects may limit disease progression and the initiation of acute VOC, which have a negative impact on patients' quality of life.[37] Previous studies have shown the beneficial effects of dietary ω-3 fatty acid supplementation on SCD phenotype.[16,38,39] However, the formulation, the route of administration and the bioavailability of ω-3 fatty acids influence the use of such supplementation in clinical practice for patients with SCD. Epeleuton has an advantageous functional profile compared to that of other formulations of ω-3 fatty acids tested in SCD such as purified ω-3 formulations including Lovaza™ (ω-3 acid ethyl esters) or SC411 and fish oils.[15,39,40] Lovaza™ is a prescription esterified ω-3-acid mixture (~55.1% EPA, ~44.9% DHA), while SC411 is a DHA ethyl ester formulation with minimal food-drug interaction. As a second-generation ω-3 enzymatic derivative that is metabolically downstream of EPA, epeleuton has advantages as a therapeutic for SCD. From biochemical and pharmacological perspectives, therapeutics on downstream purified ω-3 derivatives are likely to be more rapidly and potently bioactive *per se* or upon further enzymatic reactions. The advantage conferred in SCD from bypassing the initial stages of metabolism of fatty acids such as EPA and DHA is highlighted by the previous

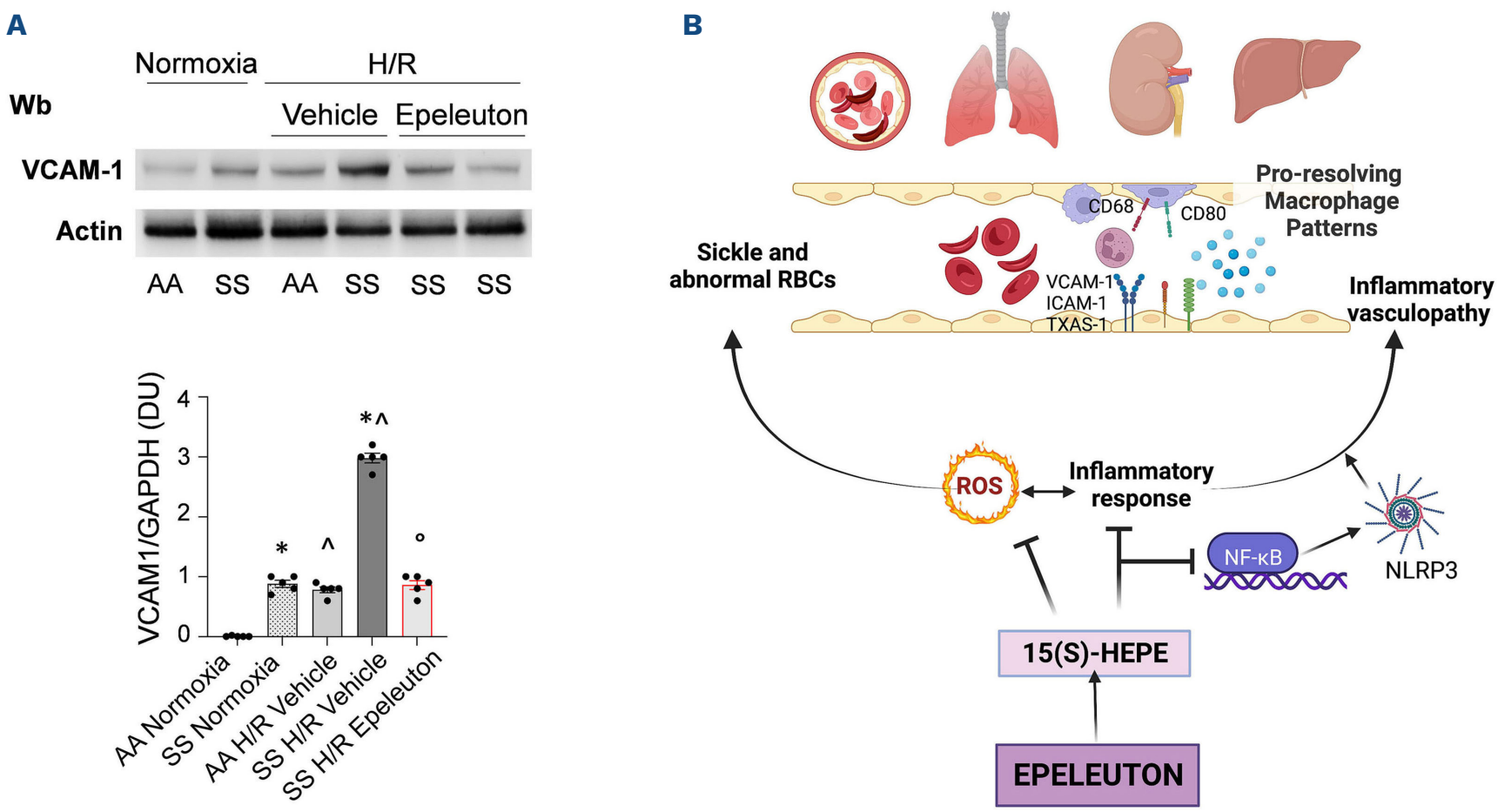

Figure 7. Epeleuton protects against progression of inflammatory vasculopathy related to acute hypoxia/reoxygenation stress in mice with sickle cell disease. (A) Immunoblot analysis, using specific antibodies against VCAM-1 (40 µg of protein loaded on an 8% T, 2.5% C polyacrylamide gel) in isolated aorta from healthy mice (AA) and sickle cell disease (SCD) mice (SS) under normoxia or exposed to hypoxia/reoxygenation (H/R) treated with either vehicle or epeleuton (1,000 mg/kg/day for 6 weeks). Lane 1: AA mouse under normoxia; lane 2: SS mouse under normoxia; lane 3: vehicle-treated AA mouse exposed to H/R; lane 4: vehicle-treated SS mouse exposed to H/R; lanes 5 and 6: epeleuton-treated SS mice exposed to H/R (results from 2 separate animals are shown). Actin served as the protein loading control. One representative gel from five with similar results is shown. Densitometric analysis of immunoblots is shown in the lower panel. Data are presented as means ± standard error of mean (N=5). *P<0.05 compared to AA mice; ^P<0.05 compared to normoxia; °P<0.05 compared to vehicle by one-way analysis of variance. (B) Schematic diagram of the dual anti-inflammatory and pro-resolution effects of epeleuton in the humanized mouse model of SCD. Epeleuton and its active moiety 15(S)-HEPE favor pro-resolving mechanisms targeting inflammation, the reactive oxygen species burst, NF-κB activation and NLRP3 inflammasome expression. This results in prevention of red blood cell sickling, inflammatory vasculopathy reduction and macrophages pro-resolving reprogramming (CD68 and CD80) in target organs for SCD. Wb: western blot; VCAM-1: vascular cell adhesion molecule 1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; DU: densitometric units; RBC: red blood cells; ICAM-1: intercellular adhesion molecule 1; TXAS-1: thromboxane synthase 1; ROS: reactive oxygen species; NF-kB: nuclear factor κB; NLRP3: nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3; 15(S)-HEPE: 15(S) hydroxy eicosapentaenoic acid.

finding that humanized SCD mice were not as readily able to synthesize downstream fatty acid metabolites compared to healthy control mice.[6] Importantly, 15(S)-HEPE, which has also been identified in resolving exudates from mice treated with ω-3 fatty acids, directly reduces polymorphonuclear leukocyte trans-endothelial migration, as does its metabolite lipoxin A_5 at a higher potency.[41] In SCD mice, epeleuton might attenuate the inflammatory response by favoring pro-resolving mechanisms mediated by 15(S)-HEPE. In agreement with this notion, we found increased organ content of 15-HEPE and reduction of systemic inflammation, modulation of the CCL2 chemotactic cytokine and a decrease in neutrophil counts in SCD mice treated with epeleuton. This is of particular interest since epeleuton reduces splenic neutrophils and reprograms spleen macrophages towards a pro-resolution pattern, supporting the peculiarity of epeleuton as a source of pro-resolving lipid mediators. The peculiar effect of epeleuton on lung leukotriene B4 content in SCD mice is concordant with a pro-resolving effect of epeleuton, similar to the effects described in the same model treated with exogenous 17R-RvD1.[6]

The dual anti-inflammatory and pro-resolution effects of epeleuton in SCD mice are highlighted by changes in lipidomic profile and a reduction in NF-κB p65 activation in target organs for SCD such as lung, liver, and kidney (Figures 1, 3, 4, and 5). This is further corroborated by decreased expression of the NLRP3 inflammasome, which activates inflammatory reactions in response to disrupted cell homeostasis in lung, kidney, and liver of SCD mice exposed to H/R stress. Growing evidence in both patients with SCD and cell/animal-based models of SCD support the importance of NLRP3 inflammasome activation in the severity of the clinical manifestations of SCD as well as in monocyte and platelet function/activation.[42-44] Although the mechanism of activation of NLRP3 in SCD is still under investigation, oxidation and free heme/hemolysis have been suggested to trigger the NLRP3 inflammasome in SCD.[45,46] In this model, epeleuton reduces both oxidation and hemolysis when SCD mice are exposed to H/R stress and prevents the worsening of liver iron overload and the activation of the redox-related transcription factor Nrf2. It is worth noting that previous studies have shown that the NLRP3 inflammasome might favor Nrf2 degradation, affecting total cell antioxidant power, supporting the importance of limiting reactive-oxygen species induced by activation of the NRLP3 inflammasome.[47,48] Here, we found that epeleuton protects against NLRP3 inflammasome activation by: (i) preventing the activation of NF-κB p65; (ii) decreasing oxidation and the related activation of Nrf2; and (iii) reducing hemolysis. Indeed, studies in different models of acute or acute-on-chronic disease of lung, kidney or liver have previously shown that inhibition of the NLRP3 inflammasome ameliorated disease outcomes.[32,36,49] In addition, the observations that ω-3 fatty acids and specialized pro-resolving mediators prevent NLRP3 inflammasome activation in different models of inflammatory and degenerative disorders further support the beneficial effects of epeleuton on sickle cell-related organ damage.[50] In agreement, epeleuton prevented the expression of markers of inflammatory vasculopathy in target organs for SCD, including isolated aorta, providing a rationale for considering epeleuton as a potential, new therapeutic tool for SCD. Finally, the improvement of hemolysis in epeleuton-treated SCD mice exposed to H/R suggests a possible beneficial effect also on red cell deformability, which will be explored in future studies.

The present results support the designation of epeleuton as an orphan drug, as determined by the USA Food and Drug Administration (*https://www.accessdata.fda.gov/scripts/opdlisting/oopd/detailedIndex.cfm?cfgridkey=880022*) and the European Medicines Agency (*https://www.ema.europa.eu/en/medicines/human/orphan-designations/eu-3-22-2695*).[18]

In conclusion, we show here in humanized SCD mice that epeleuton acts as a multimodal agent targeting hemolysis, balancing inflammatory response and pro-resolving mechanisms, and vascular dysfunction. Our data support the potential therapeutic utility of epeleuton and provide a rationale for the design of a clinical trial (NCT05861453) to evaluate the efficacy of epeleuton in patients with SCD.

Disclosures

LDF received research funding from Afimmune. MH and DC are Afimmune employees. JC is a shareholder of Afimmune. DLB has sat on advisory boards for Angiowave, Bayer, Boehringer Ingelheim, Cardax, CellProthera, Cereno Scientific, Elsevier Practice Update Cardiology, High Enroll, Janssen, Level Ex, McKinsey, Medscape Cardiology, Merck, MyoKardia, NirvaMed, Novo Nordisk, PhaseBio, PLx Pharma, and Stasys; is a member of the Board of Directors of the American Heart Association New York City; holds stock or stock options with Angiowave (stock options), Bristol Myers Squibb (stock), DRS.LINQ (stock options), and High Enroll (stock); is a consultant for Broadview Ventures and Hims; has participated in data monitoring committees for Acesion Pharma, Assistance Publique-Hôpitaux de Paris, Baim Institute for Clinical Research (formerly Harvard Clinical Research Institute, for the PORTICO trial, funded by St. Jude Medical, now Abbott), Boston Scientific (Chair, PEITHO trial), Cleveland Clinic, Contego Medical (Chair, PERFORMANCE 2), Duke Clinical Research Institute, Mayo Clinic, Mount Sinai School of Medicine (for the ENVISAGE trial, funded by Daiichi Sankyo; for the ABILITY-DM trial, funded by Concept Medical), Novartis, Population Health Research Institute, Rutgers University (for the NIH-funded MINT trial); has received honoraria from the American College of Cardiology (Senior Associate Editor, Clinical Trials and News, ACC.org; Chair, ACC Accreditation Oversight Committee), Arnold and Porter law firm (work related to Sanofi/Bristol-Myers Squibb clopidogrel litigation), Baim Institute for Clinical Research (formerly Harvard Clinical Research Institute; RE-DUAL PCI

clinical trial steering committee funded by Boehringer Ingelheim; AEGIS-II executive committee funded by CSL Behring), Belvoir Publications (Editor-in-Chief, Harvard Heart Letter), Canadian Medical and Surgical Knowledge Translation Research Group (clinical trial steering committees), CSL Behring (AHA lecture), Cowen and Company, Duke Clinical Research Institute (clinical trial steering committees, including for the PRONOUNCE trial, funded by Ferring Pharmaceuticals), HMP Global (Editor-in-Chief, Journal of Invasive Cardiology), Journal of the American College of Cardiology (Guest Editor; Associate Editor), K2P (Co-Chair, interdisciplinary curriculum), Level Ex, Medtelligence/ReachMD (CME steering committees), MJH Life Sciences, Oakstone CME (Course Director, Comprehensive Review of Interventional Cardiology), Piper Sandler, Population Health Research Institute (for the COMPASS operations committee, publications committee, steering committee, and USA national co-leader, funded by Bayer), WebMD (CME steering committees), and Wiley (steering committee); Other: Clinical Cardiology (Deputy Editor); is named on a patent for sotagliflozin assigned to Brigham and Women's Hospital who assigned to Lexicon (neither DLB nor Brigham and Women's Hospital receives any income from this patent); has received research funding from Abbott, Acesion Pharma, Afimmune, Aker Biomarine, Alnylam, Amarin, Amgen, AstraZeneca, Bayer, Beren, Boehringer Ingelheim, Boston Scientific, Bristol-Myers Squibb, Cardax, CellProthera, Cereno Scientific, Chiesi, CinCor, Cleerly, CSL Behring, Eisai, Ethicon, Faraday Pharmaceuticals, Ferring Pharmaceuticals, Forest Laboratories, Fractyl, Garmin, HLS Therapeutics, Idorsia, Ironwood, Ischemix, Janssen, Javelin, Lexicon, Lilly, Medtronic, Merck, Moderna, MyoKardia, NirvaMed, Novartis, Novo Nordisk, Otsuka, Owkin, Pfizer, PhaseBio, PLx Pharma, Recardio, Regeneron, Reid Hoffman Foundation, Roche, Sanofi, Stasys, Synaptic, The Medicines Company, Youngene, and 89Bio; has received royalties from Elsevier (Editor, Braunwald's Heart Disease); is or has been a site co-investigator for Abbott, Biotronik, Boston Scientific, CSI, Endotronix, St. Jude Medical (now Abbott), Philips, SpectraWAVE, Svelte, and Vascular Solutions; is a trustee for the American College of Cardiology; and has performed unfunded research for FlowCo. CB has provided consultancy services for Afimmune. The other authors have no conflicts of interest to disclose.

Contributions

AM, EF contributed to the experimental design, carried out experiments, and analyzed data. AS, VR and AP carried out immunoblot analyses. JC, FM, EG, JC and DLB critically reviewed the paper. DC and MH discussed the experimental design and data and critically reviewed the data. AR and GF analyzed lipidomic data. CB and LDF designed the experiments, analyzed data and wrote the paper.

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Data-sharing statement

All the data and protocols are stored in the Nas Synology DS216se Hard Disk, located at the University of Verona, Italy and are available on request.

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