Impaired pulmonary endothelial barrier function in sickle cell mice

Acute and chronic pulmonary complications leading to significant morbidity and mortality occur in persons with sickle cell disease (SCD). One of the leading causes of death is acute chest syndrome (ACS),[1] diagnosed by a new infiltrate on chest x-ray often triggered by infection.[2] The resulting low oxygen saturation leads to hemoglobin S polymerization, red blood cell sickling, vaso-occlusion and hypoxia,[3] the hallmark of ACS. Pulmonary endothelial cell (EC) barrier function is mainly regulated by the opening and closing of tight junctions in the intercellular space that controls the passage of macromolecules and cells across the vascular wall.[4] The association of tight junctions with the actin cytoskeleton is required for the dynamic regulation of junction opening and closure.[5] Under hypoxic and infection conditions, cell-cell junctions can be destabilized causing the passage of systemic inflammatory mediators into the lungs producing pulmonary edema. Extracellular nucleotides that function through cell surface purine receptors to enhance tight junctions are promising EC barrier protective agents.[6] A recent study delineated the role of P2Y receptors in EC barrier protective pathways involved in organizing the cytoskeleton and linking them to adherens and tight junctions.[7]

It was recently demonstrated in sickle cell mice that increased vascular permeability contributes to pulmonary edema and the pathophysiology of ACS.[8] Studies using Evans blue dye confirmed an increased permeability in the sickle cell mouse lung, however, EC barrier function was not investigated. To gain additional insights into barrier function, we performed studies with cultured EC from the lungs of the Townes knock-in sickle cell mouse (SS) and heterozygote (AS) littermates. Using endothelial-specific CD31 conjugated Dynabeads, we isolated mouse lung microvascular EC (MLMVEC) from SS (SS-MLMVEC) and AS (AS-MLMVEC) mice (Online Supplementary Methods). The EC were grown as a monolayer, and phase-contrast microscopy demonstrated the cobblestone structure (Figure 1A). They expressed endothelial nitric oxide synthase and platelet adhesion molecule and take up acetylated low density lipoprotein (*data not shown*), characteristics consistent with the EC phenotype. To determine the efficiency of the EC barrier function, trans-endothelial electrical resistance (TER) was performed using an electric cell-substrate impedance sensing instrument by methods which we have previously published.[9] At basal conditions, the TER in SS-MLMVEC gradually decreased over time compared to AS-MLMVEC (Figure 1B), where barrier function remained stable over time. The maintenance of EC barrier function is necessary for pulmonary health, but compromised cell-cell junction integrity contributes to the

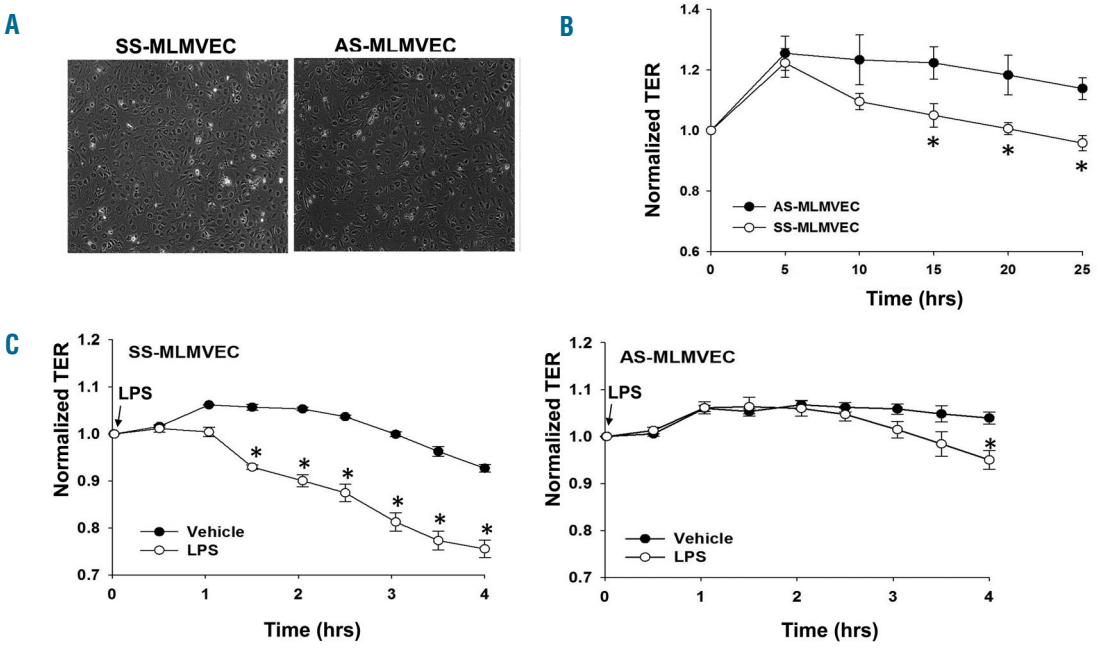

Figure 1. The SS-MLMVEC show decreased barrier function at baseline and in response to LPS toxin. (A) Shown are representative phase-contrast microscopy images of mouse lung microvascular endothelial cells (MLMVEC) from 4-6 weeks old Townes sickle cell mice (SS-MLMVEC) and heterozygote (AS-MLMVEC) littermates (Passage 3), isolated with platelet endothelial cell adhesion molecule-1 (PECAM-1) conjugated Dynabeads (20X magnification). (B) Equal numbers of SS-MLMVEC and AS-MLMVEC were seeded on electric cell-substrate impedance sensing (ECIS) arrays and grown to confluency. The growth medium was replaced with serum-free endothelial growth basal medium and the barrier function measured by trans-endothelial electrical resistance (TER) using the ECIS machine. Normalized resistance was calculated and plotted as a function of time. The data represents the mean ± standard deviation (n=3) *P<0.05. Vehicle control values were normalized to one. (C) Equal numbers of SS-MLMVEC and AS-MLMVEC were seeded on ECIS arrays and grown to confluency. When a constant resistance was attained, the cell monolayers were treated with vehicle (medium) or LPS (0.2 μg/ml) and the normalized resistance was calculated and plotted as a function of time. The data are represented as described in panel B. LPS: lipopolysaccharide; SS-MLMVEC: sickle cell mouse lung microvascular endothelial cells; AS-MLMVEC: heterozygote mouse lung microvascular endothelial cells.

leak response. Recent studies using siRNA approaches or transgenic mouse models demonstrated the requirement of endothelial junction and cytoskeleton proteins in the maintenance of proper barrier function. One study showed that the tight junction protein Z0-1 is a central regulator of the barrier function in EC.[10] Another study established the role of enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) in actin cytoskeleton remodeling and the maintenance of barrier function. Mice lacking functional Ena/VASP exhibited reduced EC barrier function and increased permeability.[11] Whether this protein plays a role in the mechanisms of dysregulated SS-MLMVEC barrier function remains to be investigated.

To understand the role of EC barrier dysfunction in the pathogenesis of ACS, an *in vitro* infection model was developed. The potent inflammatory molecule lipopolysaccharide (LPS) mimics the cellular response to bacterial infection in humans.[12] We and others have demonstrated that LPS mediates a decrease in TER of human lung EC. We and others have demonstrated that LPS mediates a concentration-dependent decrease in TER and reduces expression of the cell-cell junction protein VE-cadherin and formation of actin stress fibers.[13,14] Therefore, to gain insights into EC barrier dysfunction, monolayers of SS-MLMVEC and AS-MLMVEC were incubated with LPS (0.2 mg/ml) and TER was measured over 4 hrs (Figure 1C). For SS-MLMVEC treated with LPS, EC barrier dysfunction was observed for 60 min with a maximal 24% drop in TER at 4 hrs (P<0.05); by contrast for AS-MLMVEC the TER remained at basal level up to 2.5 hrs followed by a 10% decrease at 4 hrs (P<0.05). These results demonstrate the increased susceptibility of SS-MLMVEC to the effects of LPS, supporting the concept that EC in persons with SCD may be dysfunctional. Studies are ongoing to understand the cellular mechanisms involved in pulmonary EC barrier dysfunction in SS mice.

The complications of ACS contribute significant mortality in children and adults with SCD, however, the treatment remains supportive. Drug development efforts include two promising agents, L-glutamine, and the adenosine A2A receptor agonist, regadenoson. Recent studies indicate that extracellular nucleotides play an important role in anti-inflammatory mechanisms.[15] Signaling molecules interact with the cell-surface P2Y receptors which are activated by β-nicotinamide adenine dinucleotide (β-NAD), to enhance pulmonary artery EC barrier function *via* protein kinase A and Rac1-dependent actin cytoskeleton rearrangement.[13] Therefore, we expanded on these findings to determine whether β-NAD could improve EC barrier function. Monolayers of SS-MLMVEC and AS-MLMVEC were incubated with β-NAD (100 μM) for 4 hrs, which enhanced EC barrier function (P<0.05) for both cell types, peaking at 2 hrs (Figure 2A). However, maximal enhancement was 40% for SS-MLMVEC and 70% for AS-MLMVEC compared to vehicle control (Figure 2B). The lower response of SS-MLMVEC to β-NAD may be due to higher basal barrier dysfunction as shown in Figure 1B. A recent study verified increased vascular permeability in the lungs of Berkley sickle mice treated with red blood cell lysates, supporting abnormal EC barrier function.

Based on the function of β-NAD in the autocrine/paracrine protective signaling mechanism activated by extracellular nucleotides,[16] we speculated that an improvement in EC barrier function might involve the reorganization of the actin cytoskeleton. Therefore, we performed immunochemical staining and observed the

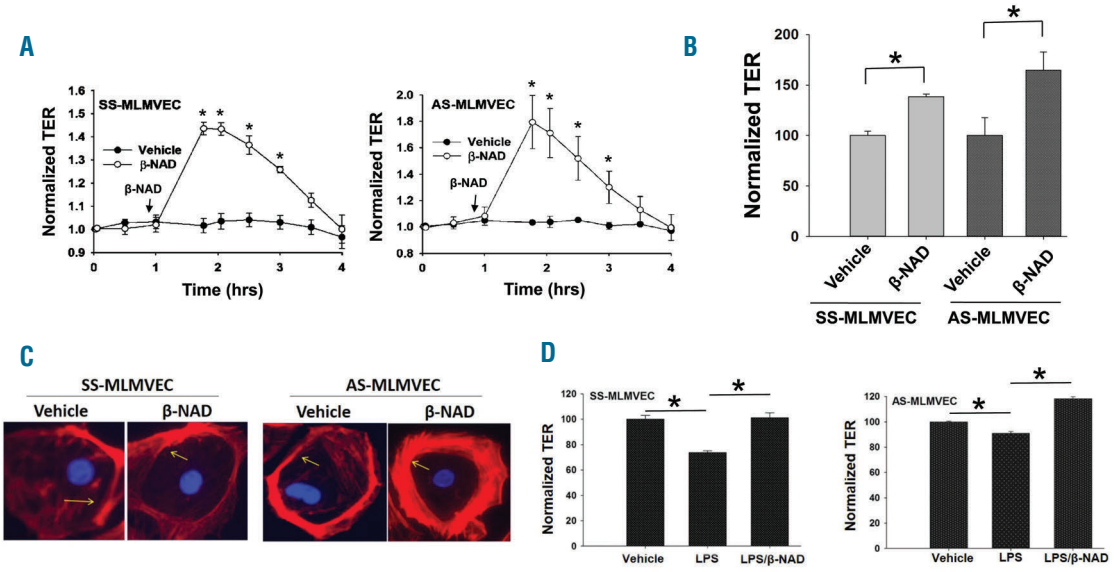

Figure 2. β-NAD enhances the basal barrier function of SS-MLMVEC and AS-MLMVEC littermates and protects against LPS toxin. (A) The SS-MLMVEC and AS-MLMVEC were seeded on electric cell-substrate impedance sensing (ECIS) arrays and grown to confluency; the data were collected and analyzed as described in Figure 1B. (B) The 2 hr values from the data in Panel A were plotted in the bar graphs. Representative data from two independent isolations are shown. (C) After passage 3, endothelial cells (ECs) were seeded on microscope slides with coverslips, allowed to form monolayers for 48 hrs and then were treated with vehicle (medium) or β–NAD (100 μM) for 30 min. The ECs were washed with phosphate buffered saline, fixed with formaldehyde (4%) in phosphate buffered saline and permeabilized with Triton X-100 (0.25%). The ECs were stained with the Alexa Fluor 488 Phalloidin (F-actin) probe and images captured using a confocal microscope (ZEISS) (160X magnification). (D) The SS-MLMVEC and AS-MLMVEC were seeded on ECIS arrays and grown to confluency by the same methods described in Figure 1B. EC monolayers were treated with vehicle (medium), LPS (0.2 μg/ml), or LPS/β-NAD (0.2 μg/ml and 100 μM, respectively) and TER measured for 4 hrs. Representative data from two independent isolations are shown. *P<0.05 represent a comparison of data obtained for LPS compared to vehicle or data analysis between LPS and LPS/β-NAD. LPS: lipopolysaccharide; SS-MLMVEC: sickle cell mouse lung microvascular endothelial cells; AS-MLMVEC: heterozygote mouse lung microvascular endothelial cells; TER: trans-endothelial electrical resistance; β-NAD: β-nicotinamide adenine dinucleotide.

disorganization of the actin ring at baseline in SS-MLMVEC compared to AS-MLMVEC (Figure 2C). Interestingly, β-NAD treatment enhanced actin ring formation in both types of EC, in agreement with the improvement in TER measurements (Figure 2B). We next investigated whether β-NAD could protect against LPS toxicity. We incubated SS-MLMVEC and AS-MLMVEC with LPS (0.2 μg/ml) or LPS (0.2 μg/ml) combined with β-NAD (100 μM), and then TER was measured; β-NAD significantly attenuated LPS-induced EC barrier dysfunction in both cell types (Figure 2D). Moreover, basal TER levels were further improved for AS-MLMVEC, similar to that reported in humans.[13] Thus, P2Y receptor activation by β-NAD enhanced basal EC barrier function and protected against the disruptive effects of the bacterial toxin LPS.

To determine if the effects of β-NAD would provide protection against LPS toxicity and lung injury *in vivo*, we performed studies in SS mice. Initial dose-finding studies were conducted with LPS (0.5, 0.1, 0.08, and 0.05 mg/kg) by intraperitoneal injections (200 μl). At LPS doses >0.08 mg/kg all SS mice died within 4-6 hrs, however, at the 0.05 mg/kg dose mice survived for up to 10 hrs, therefore this dose was used for *in vivo* studies. We evaluated SS mice in three groups, which received 200 μl of either vehicle (normal saline), LPS (0.05 mg/kg), or LPS/β-NAD (0.05 mg/kg and 11mg/kg, respectively) by intraperitoneal injections. Kaplan-Meier survival curves showed that mice in the LPS group died within 8-10 hrs (Figure 3A). By contrast, mice treated with LPS/β-NAD survived for 21-24 hrs, whereas survival was unchanged for mice treated with normal saline. To determine whether ACS develops after LPS, the study was repeated and lung tissue harvested at the time of death for the LPS group (8-10 hrs) and at 24 hrs for LPS/β-NAD treated and control mice. The Hematoxylin and Eosin (H&E) stained lung showed alveolar thickening, indicative of pulmonary edema after LPS treatment (Figure 3B), which was reduced in the lung tissues of LPS/β-NAD treated mice. To gain further evidence of ACS, the number of neutrophils were counted by light microscopy from the H&E stained sections (6-8 fields from each group). We observed a 6-fold increase in neutrophils after LPS treatment, which was decreased by 52% by combined LPS/β-NAD treatment (Figure 3C). As further evidence of the positive effects of β-NAD, neutrophil myeloperoxidase staining was completed. The level of myeloperoxidase positive cells decreased in LPS/β-NAD treated mice compared to LPS alone (Figure 3D), suggesting less neutrophil extravasation. Moreover, myeloperoxidase enzyme activity showed a significant 2.73-fold increase in activity (P<0.05) after LPS toxin, which was attenuated by 50.2% (P<0.05) by combined LPS/β-NAD treatment (Figure 3E). With combined β-NAD treatment, myeloper-

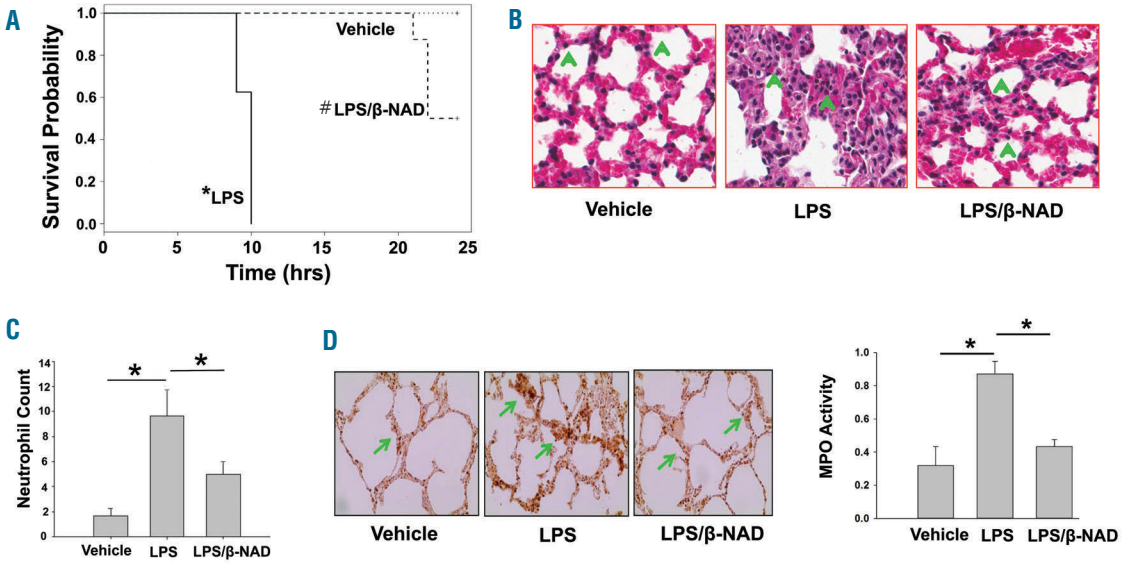

Figure 3. The β-NAD treatment protects against LPS mortality and acute lung injury. (A) Three groups of mice were injected with either vehicle (normal saline; 7 mice), LPS (0.05 mg/kg; 8 mice) or LPS/β-NAD (0.05 mg/kg-11 mg/kg; 8 mice) and were monitored until death. All LPS treated mice died within 8-10 hrs but the LPS/β-NAD treated mice survived for 21-24 hrs; survival was unchanged for the normal saline group. The data represent the mean ± standard deviation and a student's t-test was used to compare between groups. The survival of mice treated with LPS was compared to vehicle control using a log-rank test and the second analysis compared the LPS *versus* LPS/B-NAD treatment groups; *P<0.05 represent a comparison of data between LPS and vehicle and #P<0.05 a comparison between LPS and LPS/β-NAD. (B) The three treatment conditions were repeated and the mice were sacrificed at 8-10 hrs for LPS treated mice and 24 hrs for the LPS/β-NAD and vehicle normal saline groups. The lung sections were stained with Hematoxylin and Eosin (H&E) to determine morphological changes. The arrows point to the thickened alveoli and pulmonary fluid infiltration (vehicle *versus* LPS section). (C) The neutrophils were manually counted (6-8 fields from each group) from the H&E stained lung tissue. *P<0.05 represent a comparison between LPS versus vehicle or LPS versus LPS/β-NAD. (D) MPO staining was performed to confirm neutrophil infiltration into the lung under the different treatment conditions. The arrows point to representative MPO staining. (E) The assay to measure MPO enzyme activity was performed with lung homogenates according to the manufacturer protocol (Cayman Chemical, Ann Arbor, MI, USA). Lung tissue was harvested and homogenized in cold phosphate buffered saline; the supernatant obtained after centrifugation was used to measure MPO activity; *P<0.05 represent a comparison of LPS *versus* vehicle or LPS *versus* LPS/β-NAD. MPO: myeloperoxidase; LPS: lipopolysaccharide; β-NAD: β-nicotinamide adenine dinucleotide.

oxidase levels were comparable to normal saline treated mice, however, whether this improvement was due to a decrease in neutrophil extravasation *versus* altered myeloperoxidase activity requires additional studies. Nevertheless, these results suggest β-NAD attenuated LPS-induced acute lung injury in SCD mice.

In summary, we demonstrated that SS-MLMVEC exhibit basal barrier dysfunction and increased susceptibility to LPS toxicity when compared to AS-MLMVEC. Furthermore, β-NAD treatment enhanced basal EC barrier function and provided protection against LPS toxicity in SCD mice.

The disorganization of the cortical actin ring in SS-MLMVEC at steady-state implicates this process as part of the mechanism of barrier dysfunction in SCD mice. Our results provide insights into EC barrier function, which warrant mechanistic studies to support the development of novel therapies for the pulmonary complications of SCD.

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References

1. Gladwin MT, Vichinsky E. Pulmonary complications of sickle cell disease. N Engl J Med. 2008;359(21):2254-2265.
2. Miller ST, Hammerschlag MR, Chirgwin K, et al. Role of Chlamydia pneumoniae in acute chest syndrome of sickle cell disease. J Pediatr. 1991;118(1):30-33.
3. Elshazly SA, Heiba NM, Abdelmageed WM. Plasma PTX3 levels in sickle cell disease patients, during vaso occlusion and acute chest syndrome (data from Saudi population). Hematology. 2014;19(1):52-59.
4. Vandenbroucke E, Mehta D, Minshall R, Malik AB. Regulation of endothelial junctional permeability. Ann N Y Acad Sci. 2008; 1123:134-145.
5. Prasain N, Stevens T. The actin cytoskeleton in endothelial cell phenotypes. Microvasc Res. 2009;77(1):53-63.
6. Idzko M, Ferrari D, Riegel AK, Eltzschig HK. Extracellular nucleotide and nucleoside signaling in vascular and blood disease. Blood. 2014; 124(7):1029-1037.
7. Zemskov E, Lucas R, Verin AD, Umapathy NS. P2Y receptors as regulators of lung endothelial barrier integrity. J Cardiovasc Dis Res. 2011;2(1):14-22.
8. Ghosh S, Tan F, Ofori-Acquah SF. Spatiotemporal dysfunction of the vascular permeability barrier in transgenic mice with sickle cell disease. Anemia. 2012;2012:582018.
9. Umapathy SN, Kaczmarek E, Fatteh N, et al. Adenosine A1 receptors promote vasa vasorum endothelial cell barrier integrity via Gi and Akt-dependent actin cytoskeleton remodeling. PloS One. 2013; 8(4):e59733.
10. Tornavaca O, Chia M, Dufton N, et al. ZO-1 controls endothelial adherens junctions, cell-cell tension, angiogenesis, and barrier formation. J Cell Biol. 2015;208(6):821-838.
11. Furman C, Sieminski AL, Kwiatkowski AV, et al. Ena/VASP is required for endothelial barrier function in vivo. J Cell Biol. 2007; 179(4):761-775.
12. Sukriti S, Tauseef M, Yazbeck P, Mehta D. Mechanisms regulating endothelial permeability. Pulm Circ. 2014;4(4):535-551.
13. Umapathy NS, Zemskov EA, Gonzales J, et al. Extracellular beta-nicotinamide adenine dinucleotide (beta-NAD) promotes the endothelial cell barrier integrity via PKA- and EPAC1/Rac1-dependent actin cytoskeleton rearrangement. J Cell Physiol. 2010; 223(1):215-223.
14. Kolosova IA, Mirzapoiazova T, Moreno-Vinasco L, Sammani S, Garcia JG, Verin AD. Protective effect of purinergic agonist ATPgammaS against acute lung injury. Am J Physiol Lung Cell Mol Physiol. 2008;294(2):L319-324.
15. Idzko M, Ferrari D, Eltzschig HK. Nucleotide signalling during inflammation. Nature. 2014;509(7500):310-317.
16. Jacobson JR, Dudek SM, Singleton PA, Kolosova IA, Verin AD, Garcia JG. Endothelial cell barrier enhancement by ATP is mediated by the small GTPase Rac and cortactin. Am J Physiol Lung Cell Mol Physiol. 2006;291(2):L289-295.