Subcutaneous injection of IHP-102 prevents lung vaso-occlusion in sickle cell disease mice

Sickle cell disease (SCD) is a monogenic disorder that affects ~100,000 African Americans and over 3 million people world-wide.[1,2] Acute systemic painful vaso-occlusive episode (VOE) is the primary reason for hospitalization of SCD patients and may serve as an antecedent to acute chest syndrome, a type of acute lung injury and one of the primary reasons for mortality among SCD patients.[3,4] Intravenous (IV) infusion of humanized anti-P-selectin antibody (Ab) has been shown to reduce frequency of VOE in SCD patients.[5] However, the IV route of administration often requires an out-patient visit to the clinic, thus highlighting the need for therapies that can cut down the health care cost by enabling self-administration by SCD patients at home during the prodromal phase of a VOE.[6] IHP-102, a novel glycan drug is the first subcutaneously (SQ) administrable therapeutic with dual blocking activity against P-selectin (half maximal inhibitory concentration [IC_{50}] =0.7 μg/mL) and complement pathway. Here, we show that IHP-102 is bioavailable in the blood circulation of rats within 30 minutes (min) following SQ administration and the P-selectin blocking activity persists up to 8 hours. Remarkably, intravital lung microscopy revealed that SQ administration of IHP-102 led to amelioration of IV oxy-hemoglobin (oxy-Hb) triggered lung vaso-occlusion in SCD mice by 80%. These findings highlight the therapeutic potential of IHP-102 as the first SQ administrable treatment for the prevention of VOE in SCD.

IV infusion of humanized anti-P-selectin Ab is now a Food and Drug Administration-approved therapy for the prevention of VOE in SCD patients.[5] Interestingly, VOE is often preceded by a 1- to 2-day long prodromal phase defined by fatigue and diffused body pain, which progresses to more severe and localized pain over the next few days.[6] Although this pathophysiology provides an opportunity for early intervention, this therapeutic window remains underutilized because the IV infusion requires visiting the hospital that many SCD patients wait to do until the onset of unbearable symptoms.[6,7] The annual medical care cost for SCD patients in the US is estimated to be over $3 billion and 80% of this cost accounts for inpatient hospitalization secondary to VOE.[8] VOE also result in reduced quality of life, uncompensated care and lost productivity, which indirectly further contributes to the economic burden of SCD.[8] SQ injection in the fatty tissue, just under the skin is widely used as a self-administrable mode of administering therapeutics like insulin, blood thinners, and fertility drugs. A disease modifying SQ therapeutic for VOE would have the potential to be self-administered by SCD patients at home, and if effective, could avoid hospitalization and the associated costs. Heparin or structurally similar semi-synthetic polysaccharides are widely used glycan drugs with both anticoagulant, anti-complement and anti-P-selectin activity.[9-11] IHP-102 is a novel glycan drug with dual activity against P-selectin-mediated cell adhesion and complement cascade, both of which contribute to the pathogenesis of VOE in SCD.[4,12,13] IHP-102 was developed by adding unique chemical modifications to heparin, resulting in relatively weak anti-thrombin activity (<10 U/mg), potent anti-P-selectin and anti-complement activity, and higher absorption following SQ administration. The goal of the current study was to determine whether SQ administered IHP-102 is effective in preventing vaso-occlusion in Townes knock-in humanized SCD mice.

We recently developed a model of vaso-occlusive crisis in Townes SCD mice triggered by IV administration of 10 μmol/kg oxy-Hb, which led to lung vaso-occlusion by large neutrophil-platelet aggregates.[14] Previously, we have also shown that IV administration of P-selectin blockers attenuate lung vaso-occlusion in SCD mice.[13,15] Therefore, we first assessed the efficacy of IHP-102 administered via the IV route, in preventing lung vaso-occlusion in SCD mice. SCD mice were IV administered 10 μmol/kg oxy-Hb without or with 30 mg/kg IHP-102 and lung vaso-occlusion was assessed using quantitative fluorescence intravital lung microscopy (qFILM) approach (Figure 1A) as described elsewhere.[13,14,16] Briefly, mice were anesthetized, ventilated with 95% oxygen containing 1-2% isoflurane, thoracic surgery was performed, and a small portion of the left lung was immobilized against a coverslip using a vacuum enabled micro-machined device as described elsewhere.[13,14,16] Next, fluorescein isothiocyanate (FITC)-conjugated dextran, Alexa Fluor 546-conjugated anti-Ly6g Ab and pacific-blue-conjugated anti-mouse CD49b Ab were IV administered for visualization of blood vessels and *in vivo* staining of neutrophils and platelets, respectively, and the lung microvasculature was visualized using a Nikon multi-photon-excitation fluorescence microscope. Although IV oxy-Hb triggered the occlusion of pulmonary arteriole “bottlenecks” (junction of pulmonary arterioles with pulmonary capillaries) with large neutrophil-platelet aggregates (marked with white ovals) in the lungs of SCD mice (Figure 1B), such aggregates were absent in the pulmonary arterioles of SCD mice IV administered both oxy-Hb and IHP-102 (Figure 1C). Time series of qFILM images were recorded in 10-15 field of view [FOV] size ~ 65,536 μm^2) in the lung of each mouse and analyzed using the strategy described elsewhere[13,14,16] to quantify pulmonary vaso-occlusion. The number of pulmonary vaso-occlusions per FOV (Figure 1D), number of large pulmonary

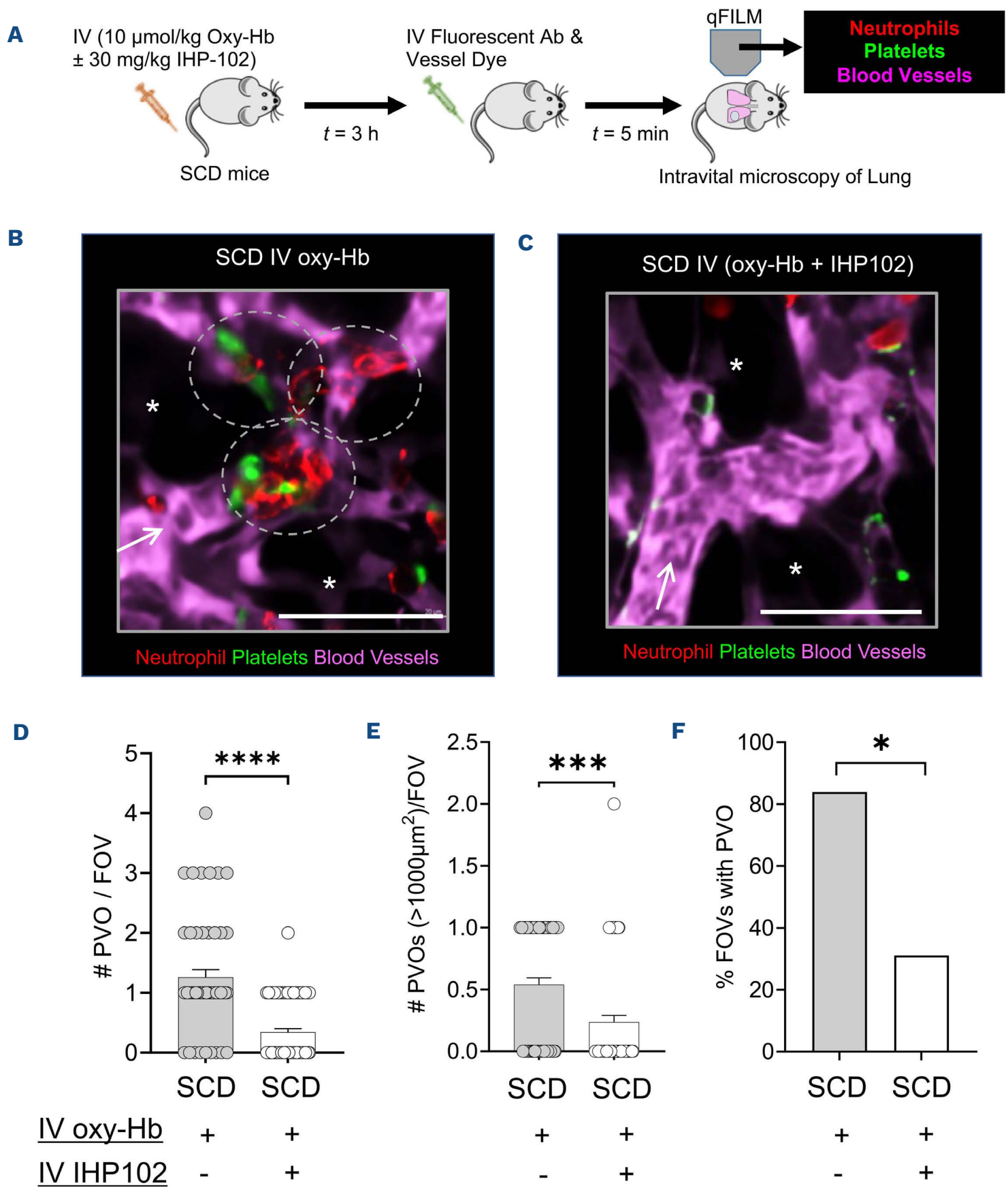

Figure 1. Intravenously administered IHP-102 ameliorates lung vaso-occlusion in SCD mice. (A) Experimental scheme - SCD mice were intravenously (IV) administered 10 µmol/kg oxy-hemoglobin (oxy-Hb) without or with 30 mg/kg IHP-102 and quantitative fluorescence intravital lung microscopy (qFILM) was used to assess the absence or presence of vaso-occlusion in the lung. Pulmonary microcirculation (pseudo-colored purple), neutrophils (red), and platelets (pseudo-colored green) were labeled *in vivo* by IV administration of fluorescein isothiocyanate (FITC) dextran, AF546-anti-Ly6G antibody (Ab), and pacific blue-anti-CD49b Ab, respectively. (B) Representative qFILM image showing occlusion of arteriolar bottlenecks by large neutrophil-platelet aggregates (marked by dotted white ovals) in the lung of an SCD mouse IV administered 10 µmol/kg oxy-Hb. (C) Representative qFILM image showing absence of neutrophil-platelet aggregates in the pulmonary arteriole of an SCD mouse IV administered 10 µmol/kg oxy-Hb + 30 mg/kg IHP-102. Scale bars 50 µm. Arrows denote blood flow direction in the pulmonary arteriole. Alveolar air spaces marked by *. (D) Number of pulmonary vaso-occlusions per field of view (#PVO/FOV), (E) number of large PVO (with area >1,000 µm^2) per FOV, and (F) FOV and PVO were significantly reduced by greater than 50% in SCD mice administered IV oxy-Hb + IHP-102 compared to SCD mice administered IV oxy-Hb. Each data point (circle) in (D) and (E) denotes #PVO observed in a single qFILM FOV (size ~65,536 µm^2). IV oxy-Hb: 56 FOV in 4 mice. IV oxy-Hb + IHP-102: 73 FOV in 4 mice. Bars in (D) and (E) represents mean ± standard error and compared using Student's *t* test. Bars in (F) represent percentage and compared using 4-fold table analysis with χ^2 statistics. *P<0.05, ***P<0.001; ****P<0.0001.

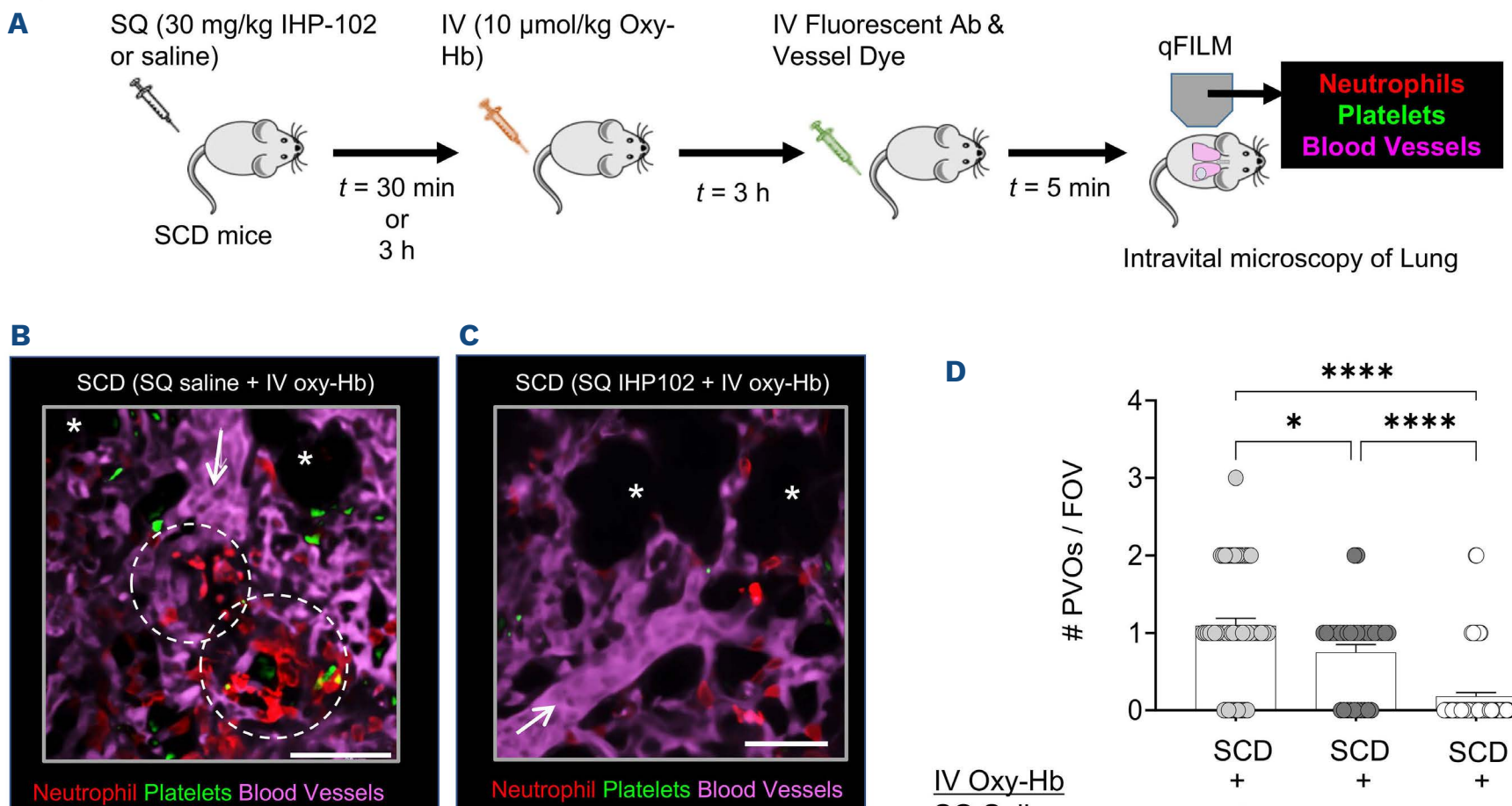

Figure 2. Subcutaneously administered IHP-102 attenuates lung vaso-occlusion in SCD mice. (A) Experimental scheme - SCD mice were subcutaneously (SQ) administered saline or 30 mg/kg IHP-102. After 30 minutes (min) or 3 hours (h), 10 µmol/kg oxy-hemoglobin (oxy-Hb) was intravenously (IV) administered to trigger vaso-occlusive crisis, and quantitative fluorescence intravital lung microscopy (qFILM) was used to assess the absence or presence of vaso-occlusion in the lung. Pulmonary microcirculation (pseudo-colored purple), neutrophils (red), and platelets (pseudo-colored green) were labeled *in vivo* by IV administration of fluorescein isothiocyanate (FITC) dextran, AF546-anti-Ly6G antibody (Ab), and pacific blue-anti-CD49b Ab, respectively. (B) Representative qFILM image showing two large neutrophil-platelet aggregates (marked by dotted white ovals) occluding the arteriolar bottlenecks in the lung of an SCD mouse administered SQ saline + IV oxy-Hb. (C) Representative qFILM image showing absence of neutrophil-platelet aggregates in the pulmonary arteriole of an SCD mouse administered SQ IHP-102 + IV oxy-Hb. Scale bars 50 µm. Arrows denote blood flow direction in the pulmonary arteriole. Alveolar air spaces marked by *. Complete time series for (B) and (C) are shown in *Online Supplementary Movie S1* and *S2*, respectively. (D) Number of pulmonary vaso-occlusions per field of view (#PVO/FOV) were significantly less in SCD mice SQ administered 30 mg/kg IHP-102 than SCD mice SQ administered saline prior to IV administration of 10 µmol/kg oxy-Hb (30% and 80% reduction with IHP-102 administration 30 min and 3 h, respectively prior to IV oxy-Hb). Each data point (circle) in D denotes #PVO observed in a single qFILM FOV (size ~ 65,536 μm^2). SQ saline + IV oxy-Hb: 55 FOV in 4 mice. SQ IHP-102 (30 min) + IV oxy-Hb: 32 FOV in 3 mice. SQ IHP-102 (3 h) + IV oxy-Hb: 78 FOV in 5 mice. Bars in (D) show mean ± standard error and compared using Student's *t* test. *P<0.05; ****P< 0.0001.

vaso-occlusions (size > 1,000 μm^2) per FOV (Figure 1E), and percent FOV with at least one pulmonary vaso-occlusion (Figure 1F) were significantly reduced by greater than 50% in SCD mice IV administered oxy-Hb + IHP-102 compared to SCD mice administered IV oxy-Hb only. Recently, we have shown that lung vaso-occlusion is reduced by ~50% in P-selectin-deficient SCD mice, suggesting that the remaining ~50% vaso-occlusive pathophysiology is P-selectin independent.[14] Therefore, greater than 50% reduction in lung vaso-occlusion caused by IV IHP-102 is suggestive of additional therapeutic effects beyond P-selectin-inhibition. As shown in *Online Supplementary Figure S1A*, *in vitro* pretreatment with IHP-102 led to significant inhibition of complement pathway in human serum samples. Interestingly, complement activation is known to contribute to vaso-occlusion in SCD.[12] Although IHP-102 also manifests potent anti-complement activity (*Online Supplementary Figure S1A*), more elaborate studies would be needed in the future to assess the role of complement inhibition in the P-selectin-independent therapeutic effects of IHP-102. Next, we assessed the pharmacokinetics of IHP-102 bioavailability in the blood following SQ administration. Importantly, such studies require blood draws at multiple time points, which is hard to achieve in mice with relatively small total blood volume (~1-1.5 mL). Therefore, Sprague Dawley rats were SQ administered IHP-102 (30 mg/kg) and serum samples collected at different time points were analyzed for P-selectin blocking activity using an *in vitro* P-selectin

cell adhesion assay (*Online Supplementary Figure S1B*). Remarkably, P-selectin blocking activity was detectable in the serum in less than 30 min and persisted for greater than 8 hours post SQ administration of IHP-102 (*Online Supplementary Figure S1B*). Based on this, SCD mice were SQ administered IHP-102 (30 mg/kg) or saline ~30 min or 3 h prior to IV administration of 10 μmol/kg oxy-Hb and lung vaso-occlusion was assessed using qFILM (experimental scheme shown in Figure 2A). Large neutrophil-platelet aggregates were observed to occlude the pulmonary arterioles in the lung of SCD mice administered SQ saline + IV oxy-Hb (representative FOV shown in Figure 2B and *Online Supplementary Movie S1*), however, such aggregates were fewer in the lung of SCD mice administered SQ IHP-102 + IV oxy-Hb. A representative FOV (Figure 2C; *Online Supplementary Movie S2*) shows unobstructed blood flow through the pulmonary arteriole and into the pulmonary capillary network, suggestive of the absence of lung vaso-occlusion in an SCD mouse administered SQ IHP-102 + IV oxy-Hb. Indeed, the quantitative analysis of qFILM data revealed that the number of pulmonary vaso-occlusions per FOV were significantly reduced by ~30% and 80% in SCD mice administered IHP-102 SQ 30 min and 3 h, respectively prior to IV oxy-Hb compared to SCD mice administered SQ saline + IV oxy-Hb (Figure 2D).

Taken together, our findings suggest that IHP-102 prevents lung vaso-occlusion in SCD mice possibly through potent anti-P-selectin and anti-complement mechanisms. Most importantly, IHP-102 is effective in preventing vaso-occlusion in the lung of SCD mice following administration *via* the SQ route. The interpretation of our findings is associated with few limitations, justifying the need for further investigation in more elaborate future studies. First, the efficacy of IHP-102 was assessed in the current study by only using IV oxy-Hb to trigger vaso-occlusive crisis in SCD mice, therefore, it would be beneficial to validate these findings in future studies using inflammatory triggers other than oxy-Hb as well. Second, the efficacy of SQ IHP-102 in preventing vaso-occlusion needs to be compared with the efficacy of SQ heparin in future studies. Third, the efficacy of IHP-102 also needs to be assessed in P-selectin deficient SCD mice to validate the P-selectin independent benefits of IHP-102. Fourth, our current study only assesses the efficacy of IHP-102 as an acute SQ therapy in preventing vaso-occlusion in SCD mice, therefore, the potential of IHP-102 as a prophylactic chronic therapy remains to be determined in future studies. Fifth, it would be useful to assess the efficacy of SQ IHP-102 administered post IV oxy-Hb challenge in SCD mice. Notwithstanding these limitations, our current findings inform the need for clinical studies to evaluate the safety and efficacy of IHP-102 as a potential self-administrable SQ therapy for early treatment of VOE and prevention of VOE-associated hospitalization among SCD patients.

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Contributions

RKD and RV performed the qFILM studies with SCD mice. TWK and TB contributed to qFILM studies in SCD mice. OK was involved in breeding and genotyping SCD mice. JT prepared oxy-hemoglobin used in *in vivo* mice studies. GN and JP were involved in formulation of IHP-102, complement testing, and conducting pharmacokinetic studies in rats. JP contributed to study design. PS was responsible for experimental design and project supervision. PS and RKD wrote the manuscript in consultation with all the co-authors.

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Data-sharing statement

Data will be made available upon reasonable request to the corresponding author.

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