ORIGINAL RESEARCH ARTICLE

MASP-2 and MASP-3 inhibitors block complement activation, inflammation, and microvascular stasis in a murine model of vaso-occlusion in sickle cell disease

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Patients with sickle cell disease (SCD) have ongoing hemolysis that promotes endothelial injury, complement activation, inflammation, vaso-occlusion, ischemia-reperfusion pathophysiology, and pain. Complement activation markers are increased in SCD in steady-state and further increased during vaso-occlusive crisis (VOC). However, the mechanisms driving complement activation in SCD have not been completely elucidated. Ischemia-reperfusion and heme released from hemoglobin during hemolysis, events that characterize SCD pathophysiology, can activate the lectin pathway (LP) and alternative pathway (AP), respectively. Here we evaluated the role of LP and AP in Townes sickle (SS) mice using inhibitory monoclonal antibodies (mAb) to mannose binding lectin (MBL)-associated serine protease (MASP)-2 or MASP-3, respectively. Townes SS mice were pretreated with MASP-2 mAb, MASP-3 mAb, isotype control mAb, or PBS before they were challenged with hypoxia-reoxygenation or hemoglobin. Pretreatment of SS mice with MASP-2 or MASP-3 mAb, markedly reduced Bb fragments, C4d and C5a in plasma and complement deposition in the liver, kidneys, and lungs collected 4 hours after challenge compared to control mAb-treated mice. Consistent with complement inhibition, hepatic inflammation markers NF-κB phosphorylated p65, VCAM-1, ICAM-1, and E-selectin were significantly reduced in SS mice pretreated with MASP-2 or MASP-3 mAb. Importantly, MASP-2 or MASP-3 mAb pretreatment significantly inhibited microvascular stasis (vaso-occlusion) induced by hypoxia-reoxygenation or hemoglobin. These studies suggest that the LP and the AP are both playing a role in promoting inflammation and vaso-occlusion in SCD. Inhibiting complement activation via the LP or the AP might inhibit inflammation and prevent VOC in SCD patients. (Translational Research 2022; 249:1–12)
Abbreviations: ANOVA = analysis of variance; AP = alternative pathway; C5aR = C5a receptor; CP = classical pathway; DAF = decay-accelerating factor; DHT = delayed hemolytic transfusion reactions; IF = immunofluorescence; I/R = ischemia-reperfusion; LP = lectin pathway; mAb = monoclonal antibody; MAC = membrane attack complex; MASP = MBL-associated serine protease; MBL = mannose binding lectin; OCT = optimal cutting temperature; PCSK6 = proprotein convertase subtilisin/kexin 6; PVDF = polyvinylidene fluoride; RBC = red blood cell; SCD = sickle cell disease; SS = sickle; TLR4 = toll-like receptor 4; VOC = vaso-occlusive crisis; VWF = von Willebrand factor; Z = zoom

At A Glance Commentary
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Background
Vaso-occlusive crises (VOC) in sickle cell disease patients are extremely painful and debilitating and a leading cause of emergency department visits and hospitalizations in this population. There is currently a lack of understanding the pathophysiological mechanisms underlying VOC. During VOC, sickle cell disease patients have been noted to have increased levels of complement activation and inflammation. However, the molecular pathway(s) leading to complement activation and their role in driving inflammation and VOC remains unexplored. We examined the effects of an anti-MASP-2 inhibitory monoclonal antibody that specifically blocks the lectin pathway and an anti-MASP-3 inhibitory monoclonal antibody that specifically inhibits the alternative pathway. Both MASP-2 and MASP-3 inhibited complement activation, hepatic inflammation, and microvascular stasis in a murine dorsal skinfold chamber model of VOC in sickle cell disease.

Translational Significance
These studies suggest that the LP and the AP are both playing a role in promoting inflammation and VOC in SCD patients. Inhibiting complement activation via the LP or the AP might inhibit inflammation and prevent VOC in SCD patients.

INTRODUCTION
Sickle cell disease (SCD) is a painful disorder that afflicts millions of people worldwide. SCD is an autosomal recessive disorder caused by an amino acid substitution in the beta-globin gene that causes hemoglobin S polymerization and red blood cell (RBC) sickling when deoxygenated. This alters RBC physiology, resulting in hemolysis, anemia, endothelial injury, and recurrent episodes of painful vaso-occlusion.1

In SCD patients and mice, complement has been shown to be abnormally activated in asymptomatic disease, during crisis, and in patients with delayed hemolytic transfusion reactions. The first report of complement activation in SCD was published in 1967,2 and studies3-7 have since reported increased levels of complement-derived fragments in the blood of SCD patients, particularly during crisis, demonstrating that complement is activated in SCD and suggesting that complement may play an important role in the pathophysiology of the disease. Lombardi et al4 found increased serum levels of complement anaphylatoxin C5a in SCD patients, deposition of C5b-9 in small vessels of skin biopsies, and C3b on sickle (SS) RBC membranes. These SS RBCs were more adhesive to endothelium with adhesion inhibited by complement factor H, a soluble plasma regulator of the AP. Roumenina et al showed that treatment of SCD patients with hydroxyurea reduces plasma C5b-9 and C3d deposition on SS RBCs.6 Yoo et al reported that Bb, a fragment of activated factor B and an enzymatic component of the AP C3 convertase, and anaphylatoxins C3a and C5a are elevated in the plasma of pediatric patients during vaso-occlusive pain crises compared to steady-state.7 In SS mice, infusion of C5a quickly promotes vaso-occlusion and tissue inflammation and these effects are abrogated by infusing an anti-C5a receptor (C5aR) mAb that blocks C5a/C5aR pro-inflammatory signaling.8

Heme has been implicated in AP activation in SCD. Heme released during intravascular hemolysis in SCD activates the AP on endothelial surfaces and this complement activation is attenuated in vivo and in vitro by the heme scavenger hemopexin.5 The AP C3 and C5 convertases can assemble on the A3 domain of ultra-large von Willebrand factor9 and by noncovalent anchoring of C3(H2O) and C3 activation fragments to P-selectin expressed on endothelial cells in a toll-like receptor 4 (TLR4)-dependent manner.10

In addition to AP activation by heme, other complement pathways may be activated in SCD. Ischemia-reperfusion (I/R) and endothelial damage are central to the pathophysiology of SCD.11,12 Endothelial injury can occur as a direct response to hemolysis, I/R, oxidative stress, and nitric oxide depletion.13-16 Endothelial injury can trigger activation of the LP, promoting inflammation and further endothelial injury.17,20 Activation of the LP may also trigger coagulation via MASP-2
cleavage of prothrombin to thrombin.\textsuperscript{21,22} In experimental I/R, inhibition of the LP blocks subsequent inflammatory responses and organ damage.\textsuperscript{23-30} Experimental I/R in SS mice can be achieved using hypoxia-reoxygenation, which further activates complement and induces vascular inflammation and vaso-occlusion that are inhibited by infusing anti-C5aR or anti-C5 mAb.\textsuperscript{8}

Complement activation by I/R is initiated through carbohydrate pattern recognition molecules of the LP including MBLs, ficolins, and collectins. Juxtaposition of these pattern recognition molecules in close proximity to each other leads to the proteolytic activation of associated MASP-1 and 2 proenzymes,\textsuperscript{31} resulting in cleavage of C2 and C4, and formation of the LP C3 convertase (C4b2a). Both MASP-1 and MASP-2 can cleave C2, but only MASP-2 can cleave C4.\textsuperscript{30} LP C3 convertase activity results in C3b deposition, which can associate with factor B, and in combination with factor D and properdin drive the formation of the AP C3 convertase C3bBb to augment C3b deposition. LP-generated C3b can also bind to either AP or LP/classical pathway (CP) convertase to produce C5 convertases (C3bBbC3b and C4bC2aC3b) that cleave C5 to produce C5a and C5b. Complement activation by the AP MASP-3 requires cleavage of profactor D to mature factor D, which in turn cleaves factor B, to drive the formation of the C3 convertase (C3bBb) of the AP.\textsuperscript{32-35} MASP-3 is the sole activator of profactor D and indispensable for AP function.\textsuperscript{33,34}

Vaso-occlusive pain crisis (VOC) has an inflammatory etiology and is the leading cause of emergency department visits and hospitalizations in the SCD population.\textsuperscript{36} Prevention and treatment of VOC is a major unmet medical need. To further understand the role of the AP and LP in VOC, we examined the effects of a MASP-2 mAb that specifically inhibits the LP and a MASP-3 mAb that specifically inhibits the AP, on complement activation,
inflammation, and vaso-occlusion in SS mice. A diagram of the complement activation pathways and their inhibition by MASP-2 or MASP-3 mAb is shown in Fig 1. We hypothesize that both the LP and AP are involved in VOC pathophysiology.

MATERIALS AND METHODS

Mice. All experiments in sickle (SS) mice were approved by the University of Minnesota’s Institutional Animal Care and Use Committee. These studies used equal numbers of male and female Townes-SS mice on a 129/B6 mixed genetic background with knockout of murine α- and β-globin sites and knock in of human α- and βS-globins into the same sites. All animals were housed in specific pathogen-free rooms to limit infections, and kept on a 12-hour light/dark cycle at 21°C. All animals were monitored daily for health problems, food and water levels, and cage conditions. All animals were included in each endpoint analysis and there were no unexpected adverse events that required modification of the protocol. The initial studies were performed in SS mice 24–35 weeks of age challenged with H/R and subsequent studies were performed in SS mice 12–16 weeks of age challenged with hemoglobin (Hb). Each study used 4 SS mice per group (2 male and 2 female). Studies for ex vivo assessment of MASP antibodies were performed using C57BL/6 mice by Omeros Inc. (MASP-3 mAb) and MPI Research, Inc. (MASP-2 mAb) using protocols approved by their Institutional Animal Care and Use Committees.

Measurement of microvascular stasis (vaso-occlusion). Townes SS mice were anesthetized with a mixture of ketamine (106 mg/kg) and xylazine (7.2 mg/kg) and implanted with dorsal skinfold chambers. After implantation, mice were placed in an intravital microscopy stage and 20–23 flowing subcutaneous venules in the chamber window were selected and mapped as previously described. MASP-3 mAb (10 mg/kg body weight, Omeros, Inc) was injected subcutaneously 4 days prior to study, whereas MASP-2 or isotype control mAb (10 mg/kg body weight, Omeros, Inc) was infused intravenously via the tail vein 30 minutes prior to challenge with hypoxia-reoxygenation or hemoglobin. Mice challenged with hypoxia-reoxygenation were exposed to hypoxia (7% O2 , 93% N2) for 1 hour followed by normoxia for 4 hours. Mice challenged with hemoglobin were infused via the tail vein with human hemoglobin (1 μmol/kg body weight). Each of the same venules selected and mapped at baseline, were visually re-examined for stasis (no flow) at 1 hour after hypoxia-reoxygenation or hemoglobin infusion. The static venules in each mouse were counted and percent stasis at 1 hour was calculated by dividing the number of static venules by the total (static + flowing) number of venules.

Tissue collection. Mice with dorsal skinfold chambers were euthanized in CO2 at room temperature 4 hours after hypoxia-reoxygenation or infusion of hemoglobin. Whole blood was collected by cardiac puncture into EDTA anticoagulant. Blood was centrifuged at 3000 g at 4°C and plasma was collected, flash frozen in liquid N2, and stored in a freezer at −85°C until use. After blood collection, the lungs, livers, and kidneys were placed in optimal cutting temperature (OCT) compound and/or aluminum foil, flash frozen in liquid N2, and stored at −85°C until further processing.

Immunoblots. Microsomes and nuclear extracts were isolated from the livers as previously described.39 Immunoblots of cellular subfractions (30 μg protein/lane) or EDTA plasma (5 μL/lane) were prepared using SDS PAGE 5–12% gels (BioRad), transferred to polyvinylidene fluoride (PVDF) membranes. PVDF membranes were immunostained with primary antibodies to NF-κB phospho-p65 (Ser536, Cell Signaling #3031), total p65 (Cell Signaling #3034), VCAM-1 (Abcam #174279), ICAM-1 (Abcam #ab124759), E-selectin (BioVision #3631) complement activation fragment Bb (GeneTex #GTX86947), C4d (Bios Antibodies #bs-10453R), C5a (MyBioSource #MBS2007023), GAPDH (Sigma-Aldrich #G9545), or IgG (Bio-Rad #170-6518). Primary antibodies were detected with appropriate secondary antibodies conjugated to alkaline phosphatase and visualized with ECF substrate (GE Healthcare) and a Typhoon FLA 9500 imager (GE Healthcare).

Immunofluorescence. Frozen kidneys (n = 2), livers (n = 2), and lungs (n = 3) in OCT compound were sectioned in a microtome-cryostat into 6-μm sections. Tissues were stained with primary antibodies to C3 activation fragments C3b/C3c/C3e (Hycent #HM1065), MAC (C5b-9, Abcam #ab55811), and endothelial cell marker VE cadherin (R&D Systems #AF1002). Primary antibodies in tissues were identified with the appropriate fluorescent-labeled secondary antibodies (Jackson ImmunoResearch). Slides were mounted using DPX mounting medium (Electron Microscope Sciences #13514), visualized, and images were acquired using a Fluoview FV1000 BX2 upright confocal microscope (Olympus, Center Valley, Pa) with a UPLXAP060XO/1.42 objective (Olympus) with zoom (Z) 2. Images were processed with FluoView (Olympus) and Adobe Photoshop software (San Jose, Calif). C3 activation fragments and MAC (C5b-9) were quantitated by counting the number of positive pixels and dividing that by the total number of tissue pixels in the image (Adobe Photoshop) to generate percent positive pixels for each protein of interest. Results in the livers, kidneys, and lungs were counted from 4
image fields collected from each organ for a total of 8–12 independent fields used for each condition.

**Statistics.** Analyses were performed with GraphPad Prism 9.3. Data were analyzed for statistical differences using one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test. Data that failed the normality test were re-evaluated with the Kruskal-Wallis test with Dunn's multiple comparison test. Statistical significance is indicated on the figures with asterisks as *P < 0.05, **P < 0.01, and ***P < 0.001.

**RESULTS**

**Ex vivo inhibition of the LP and AP by MASP mAbs.** MASP-2 mAb HG4 selectively inhibits LP-induced complement activation, whereas MASP-3 mAb 13B1 inhibits conversion of pro-factor D to active factor D, thereby inhibiting the activity of the AP. We examined the effects of our MASP-2 and MASP-3 mAbs on LP and AP inhibition ex vivo using serum samples from mice pretreated with MASP-2 or MASP-3 mAb. To examine MASP-2 mAb inhibition of the LP, C57BL/6 mice received a single subcutaneous injection of MASP-2 mAb (1, 5, or 20 mg/kg). Serum was collected at the indicated times and assayed for LP-dependent C3 deposition on mannan-coated plates and C3 deposition was detected using anti-C3c IgG. LP functional activity was normalized to LP activity in pooled sera from naive C57BL/6 mice (Supplemental Fig 1, A). LP activity in serum, as measured by lectin-induced C4 cleavage, was inhibited in a dose-dependent fashion. While 1 mg/kg of the MASP-2 mAb did not appreciably inhibit LP activity in mice, 5 mg/kg maximally inhibited LP activity for at least 48 hour, whereas 20 mg/kg maintained maximal inhibition of LP activity for at least 7 days after MASP-2 mAb treatment. To examine MASP-3 mAb inhibition of the AP, C57BL/6 mice received a single subcutaneous injection of MASP-3 (10 mg/kg) mAb or vehicle. Serum was collected at the indicated times and assayed for C3 deposition on zymosan coated plates using anti-C3c IgG in AP-specific buffer containing 5 mM MgCl2 and 8 mM EGTA (Supplemental Fig 1, B). AP activity in serum, as measured by C3c deposition on zymosan, was inhibited in a time-dependent fashion, reaching maximal inhibition 4 days after injection and remained effective for at least 7 days after treatment.

**In vivo inhibition of the LP and AP by MASP mAbs.** We examined the inhibitory activities of MASP-2 and MASP-3 mAbs on complement activation in sickle (SS) mice (Fig 2). PBS, isotype control mAb or MASP-2 mAb was infused intravenously via the tail vein into Townes-SS mice 30 minutes prior to a challenge with hypoxia-reoxygenation or hemoglobin infusion. MASP-3 mAb was injected subcutaneously 4 days prior to challenge to allow sufficient time for depletion of pre-existing circulating active factor D and thus achieve effective AP blockade. EDTA plasma was collected 4 hours after hypoxia-reoxygenation or hemoglobin challenge and analyzed for circulating complement activation fragments Bb, C4d, and C5a on immunoblots. Compared to PBS or control mAb, pretreatment with either MASP-2 mAb (LP inhibitor), or MASP-3 mAb, (AP inhibitor), markedly decreased plasma Bb levels after a challenge with hypoxia-reoxygenation (Fig 2, A) or hemoglobin (Fig 2, B) and plasma C4d (Fig 2, C) and C5a (Fig 2, D) after challenge with hemoglobin. Quantifications of Bb, C4d, and C5a immunoblots are presented in Supplemental Fig 2. Plasma Bb, C4d, and C5a were significantly lower in SS mice pretreated with MASP-2 or MASP-3 mAb compared to PBS or control mAb.

**Inhibition of complement deposition in organs by MASP mAbs.** The livers, kidneys, and lungs from SS mice pretreated with isotype control, MASP-2, or MASP-3 mAb were examined by immunofluorescence (IF) for deposition of C3 fragments and MAC (C5b-9) 4 hours after hemoglobin challenge. Representative images and quantitation of IF images are shown in Fig 3. Organs collected from SS mice pretreated with MASP-2 or MASP-3 mAb had significantly reduced deposition of C3 activation fragments and MAC compared to SS mice pretreated with isotype control mAb. While qualitatively reduced in all organs, the mean reduction in C3 fragment levels in the livers of mice pretreated with MASP-2 mAb and the mean reduction in MAC levels in the lungs of mice pretreated with MASP-3 mAb did not reach statistical significance compared to isotype control mAb. Most of the complement deposition in tissues was seen in animals pretreated with control mAb and was located near blood vessels, which were immunostained with the endothelial cell marker VE cadherin, especially in the liver and lungs. MAC staining in the kidney appeared to include other cell types.

**Inhibition of hepatic inflammation by MASP mAbs.** Hepatic inflammation in SCD can induce acute hepatic sequestration or VOC, which has been associated with significant mortality. Since complement activation releases anaphylatoxins C3a and C5a, which are potent inducers of inflammation, we examined NF-κB and adhesion molecule expression using immunoblots of cellular subfractions isolated from the livers of SS mice pretreated with MASP mAbs and challenged with hypoxia-reoxygenation or hemoglobin. NF-κB is a major pro-inflammatory transcription factor. Nuclear expression of NF-κB phospho-p65 is a measure of NF-κB activation and pro-inflammatory gene transcription. Therefore, we examined the effects of MASP-2 or MASP-3 mAb on NF-κB expression in liver nuclear
extracts; total nuclear p65 expression was similar in all treatment groups. VCAM-1, ICAM-1, and E-selectin adhesion molecule expression in liver microsomes was lower in SS mice pretreated with MASP-2 or MASP-3 mAb compared to PBS or isotype control mAb after a challenge with hypoxia-reoxygenation or hemoglobin (Fig 4). Quantification of hepatic NF-κB phospho-p65/total p65 ratios, and VCAM-1, ICAM-1 and E-selectin/GAPDH ratios in each treatment group are presented in Supplemental Fig 3. All pro-inflammatory ratios were significantly lower in SS mice pretreated with MASP-2 or MASP-3 mAb compared to control mAb.

**Inhibition of stasis (vaso-occlusion) by MASP mAbs.** We have previously shown that C5a induces microvascular...
stasis (vaso-occlusion) in SS mice. Therefore, we tested if inhibition of complement activation with MASP-2 or MASP-3 mAb would decrease microvascular stasis in SS mice with dorsal skinfold chambers. Pretreatment with either MASP-2 or MASP-3 mAb markedly decreased microvascular stasis in SS mice 1 hour after challenge with hypoxia-reoxygenation, or hemoglobin, compared to SS mice pretreated with PBS or isotype control mAb (Fig 5).

DISCUSSION

SCD is a hemolytic condition with underlying endothelial dysfunction. It has been demonstrated that heme when added to normal human serum activates complement, generating C3a, C5a, and soluble C5b-9. In addition, heme can decrease membrane cofactor protein and decay-accelerating factor expression on endothelial cells. Heme-induced complement activation can also occur on the surface of endothelial cells in TLR4-dependent manner with exocytosed Weibel-Palade body von Willebrand factor (VWF) and P-selectin promoting the binding, assembly, and activation of C3 convertase (C3bBb) and C5 convertase (C3bBbC3b) of the AP. This has implications for inflammation and vaso-occlusion in SCD. We have previously shown that infusion of C5a into SS mice induces hepatic inflammation including P-selectin and VWF on endothelial cells, and microvascular stasis in the dorsal skinfold chamber model that can

Fig 3. Pretreatment of SS mice with MASP-2 or MASP-3 mAb decreased complement deposition in the tissues of SS mice after a challenge with hemoglobin. SS mice, 12–16 weeks of age, were pretreated with isotype control, MASP-2, or MASP-3 mAb. The liver, kidneys, and lungs were removed 4 hours after hemoglobin infusion and frozen in OCT compound. Frozen tissues were sectioned in a microtome-cryostat into 6 μm sections and stained with primary antibodies to C3 activation fragments C3b/iC3b/C3c (red), MAC (C5b-9; green), and endothelial cell marker VE cadherin (blue). Four image fields were collected from each organ (n = 2–3). Representative liver, kidney, and lung images are 60X magnification. White bar in each image represents 30 μm. C3 and MAC IF pixels are expressed as a percent of the total pixels. Results in the livers, kidneys, and lungs were counted from 4 image fields collected from each organ for a total of 8–12 independent fields used for each condition. Quantification graphs are presented on the right. Mean values for each field are shown (open circles) and horizontal lines are means ± SEM. *P < 0.05, **P < 0.001, and ***P < 0.001 using one-way ANOVA with Dunnett’s multiple comparisons test and the Kruskal-Wallis test with Dunn’s multiple comparison test.
**Fig 4.** Pretreatment with anti-MASP mAbs decreased inflammation in the livers of SS mice after a challenge with hypoxia-reoxygenation or hemoglobin. MASP-3 mAb (10 mg/kg body weight) was injected subcutaneously into Townes SS mice (n = 4/group) 4 days prior to challenge, whereas MASP-2 or isotype control mAb (10 mg/kg body weight) was infused via the tail vein 30 minutes prior to challenge with hypoxia-reoxygenation (1 h hypoxia at 7% O₂ followed by normoxia) or hemoglobin (1 μmol/kg body weight). Livers were collected 4 hours after hypoxia-reoxygenation or hemoglobin challenge and analyzed for protein expression on immunoblots. NF-κB phospho- and total p65 expression were measured in hepatic nuclear extracts. VCAM-1, ICAM-1, E-selectin, and GAPDH were measured in hepatic microsomes. SS mice 24–35 weeks of age were challenged with hypoxia-reoxygenation and SS mice 12–16 weeks of age were challenged with hemoglobin.

|                     | Control mAb | MASP-2 mAb | MASP-3 mAb |
|---------------------|-------------|------------|------------|
| NF-κB Phospho-P65   |             |            |            |
| NF-κB Total P65     |             |            |            |
| VCAM-1              |             |            |            |
| ICAM-1              |             |            |            |
| E-Selectin          |             |            |            |
| GAPDH               |             |            |            |

**Fig 5.** Pretreatment with anti-MASP mAbs decreased microvascular stasis in SS mice after a challenge with hypoxia-reoxygenation or hemoglobin. Townes SS mice (n = 4/group) were anesthetized with a mixture of ketamine (106 mg/kg) and xylazine (7.2 mg/kg) and implanted with dorsal skinfold chambers. After implantation, mice were placed on an intravital microscopy stage and 20–23 flowing subcutaneous venules in the chamber window were selected and mapped. After baseline selection of flowing venules, mice were challenged with hypoxia-reoxygenation (1 h hypoxia at 7% O₂ followed by normoxia) or hemoglobin (1 μmol/kg body weight). MASP-3 mAb (10 mg/kg body weight) was injected subcutaneously 4 days prior to challenge, whereas MASP-
be prevented by blocking C5a signaling with a mAb to C5aR. In addition, hepatic inflammation and stasis in SS mice induced by hypoxia-reoxygenation can be blocked by an anti-C5 mAb that blocks murine C5 cleavage. Thus, inflammation and vaso-occlusion in SS mice induced by hypoxia-reoxygenation require complement activation suggesting that inhibition of C5a generation or signaling may be beneficial in treating VOC in SCD patients.

In addition to hemolysis activating complement, vaso-occlusion in SCD produces ischemia-reperfusion (I/R) pathophysiology. I/R is a well-known trigger of the LP of complement activation that leads to amplification of complement activation by the AP and generation of additional C5a and vaso-occlusion in a positive feedback loop. Stasis peaks at 30–60 minutes and subsequently the vessels gradually reopen over several hours even without anti-inflammatory treatment. This effect creates I/R physiology that is likely promoting complement activation by the LP. Thus, complement activation and vaso-occlusion are the proverbial “chicken or the egg” conundrum. Which comes first, complement activation or vaso-occlusion? Since the β5 mutation is the ultimate cause of SCD pathophysiology, perhaps hemolysis and TLR4 signaling initiates complement activation with subsequent I/R pathophysiology and the LP triggering addition complement activation, inflammation, and vaso-occlusion that is amplified by the AP. This might explain the beneficial effects of inhibiting vaso-occlusion with either the heme scavenger hemopexin, inhibiting the AP with MASP-3 mAb, the terminal pathway with C5 or C5aR mAb, or the LP with MASP-2 mAb. Alternatively, damaged endothelium may initiate complement activation via the LP, which may be further amplified by the AP and ultimately lead to vaso-occlusion. The observation that LP inhibition by MASP-2 mAb and AP inhibition by MASP-3 mAb were similarly effective at inhibiting complement activation, inflammation, and microvascular stasis suggests that the LP and AP work in tandem, most likely in a positive feedback loop, to promote SCD pathophysiology.

MASP-3 and factor D circulate predominantly in an active form in normal plasma, which requires the administration of anti-MASP-3 mAb 4 days prior to challenge. MASP-3 can be activated by MASP-1, but recent studies suggest that the circulating proprotein convertase subtilisin/kexin 6 (PCSK6) might be the primary activator of MASP-3 in circulation. MASP-1 and MASP-3 are secreted primarily by the liver by alternative splicing from the common Masp1 gene, with the result that they have a common heavy chain and distinct light chains. The light chains consist of the serine protease domain, transcribed from either MASP-1—specific exons or a MASP-3—specific exon.

MASP-2 deposition has been found in the pulmonary microvasculature in severe COVID-19 infection-associated vascular injury and thrombosis. MASP-2 and 3 have been therapeutically targeted in several clinical and preclinical studies, including transplantation-associated thrombotic microangiopathy, severe COVID-19, IgA nephropathy, traumatic brain injury, myocardial infarction, and post-ischemic brain injury. This preclinical study adds SCD to that list.

We used plasma Bb, C4d, C5a, and tissue deposition of C3 fragments and MAC as read-outs for complement activation after challenge with hypoxia-reoxygenation or hemoglobin. Bb is produced by AP activation, but was inhibited by either MASP-3 or MASP-2 mAb. Similarly, plasma C4d is produced by LP or CP activation, but was inhibited by either MASP-2 (LP) or MASP-3 (AP) mAb. Finally, either MASP-2 or MASP-3 mAb inhibited downstream C5a generation, complement deposition, and microvascular stasis. These observations indicate that LP and AP activation work together in a positive-feedback loop to drive SCD; perhaps through vaso-occlusion (stasis) and I/R. The AP serves as an amplification loop for the LP. Inhibition of AP with MASP-3 mAb would blunt LP amplification by the AP, and inhibit C5a generation, stasis, I/R, and I/R-mediated activation of the LP. Inhibition of the LP with MASP-2 mAb would inhibit formation of the C3 convertase (C2C4b), C3b generation, formation of the C5 convertases, and C5a generation. Thus, blockade of either the LP or AP may be beneficial in preventing or treating VOC in SCD.

One limitation of this study is that although we measured microvascular stasis in the skin, we did not measure inflammation in the skin. However, we have previously demonstrated that inflammation in SS mice is occurring in vascular beds throughout the body including the lungs, liver, kidneys, and skin.

Another limitation is we only evaluated MASP...
inhibitors in a model to prevent VOC; we did not evaluate MASP inhibitors in a treatment modality.

Several questions remain, but perhaps the most important question is whether complement activation is playing a role in VOC and other SCD complications in patients. Anti-C5 therapy eculizumab has been used in SCD patients with delayed hemolytic transfusion reactions (DHTTR) with hyperhemolysis.62–66 There is a case report of a pediatric SCD patient with VOC where rapid improvement in hemolysis and clinical status was observed within 48 hours of a single eculizumab dose.64 Guidelines by the American Society of Hematology recommend complement inhibition in patients with DHTTR and ongoing hyperhemolysis.67 It is important to note that the classical pathway may be activating complement in DHTTR.68 Should complement blockade be used to prevent or treat VOC in SCD? Fortunately, we may have an answer to this question soon, as the CROSSWALK clinical trial will assess the benefits of a C5 inhibitor on VOC prevention and treatment.69,70 In conclusion, these preclinical data suggest that MASP-2 or MASP-3 inhibition may be beneficial in preventing VOC and other SCD complications in patients.

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Conflicts of interest: JDB and GMV receive research funding from CSL-Behring and Mitobridge/Astellas and have applied for funding from Omeros. In addition, JDB and GMV are consultants to Mitobridge/Astellas. TD and JC are employees of Omeros. JDB, GMV, TD, and JC have a pending patent application for the use of anti-MASP monoclonal antibodies in sickle cell disease.

SUPPLEMENTARY MATERIALS
Supplementary material associated with this article can be found in the online version at doi:10.1016/j.trsl.2022.06.018.

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