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Targeting Mac-1-mediated leukocyte-RBC interactions uncouples the benefits for acute vaso-occlusion and chronic organ damage

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Abstract

Vaso-occlusive crisis (VOC) is one of the most common complications of sickle cell disease (SCD). Recurrent episodes of VOC may cause irreversible organ damage and early mortality in patients with SCD. Emerging evidence suggests that VOC arises from a complex cascade that involves interactions among multiple blood and endothelial cells in the vasculature. Previous studies have identified $\alpha_M\beta_2$ integrin (Mac-1) as a critical molecule that mediates heterotypic interactions between red blood cells (RBCs) and adherent leukocytes, and promotes VOC in SCD mice. Here we show that RBC-leukocyte interactions are significantly diminished in Mac-1-deficient SCD mice, leading to an improvement of blood flow rates and prolonged survival time in a tumor necrosis factor α (TNF- α) and surgical trauma-induced VOC model. Mac-1-deletion, however, was not sufficient to reduce SCD-related chronic organ damage. Our results thus suggest uncoupled mechanisms between acute VOC benefits and the long-term complications of SCD which should be considered in future clinical trials.

Introduction

Sickle cell disease (SCD), an inherited blood disorder, results from a single nucleotide substitution in the gene encoding β -chain of hemoglobin (Hb β^s) [1]. Hb β^s polymerizes upon deoxygenation, which produces less deformable sickle red blood cells (sRBCs) with increased propensity to adhere to the endothelium. sRBCs can obstruct blood vessels and cause painful vaso-occlusive crisis (VOC) [2]. Recurrent episodes of VOC are thought to lead to irreversible organ damage and contribute to morbidity and mortality in patients with SCD [3].

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Emerging evidence demonstrated that VOC is a complex, multicellular process involving sRBC adhesion [4, 5], endothelial activation with upregulation of adhesion molecules [6], platelet activation and aggregation [7–9], and leukocyte activation and adhesion [10–13]. We have previously demonstrated that intravascular accumulation and activation of neutrophils is a key pathogenic event in the development of VOC in SCD [10–12]. Notably, adherent neutrophils can capture circulating RBCs in inflamed venules, leading to critical reduction in blood flow and vaso-occlusion [10]. Further, we identified that activated $\alpha_M\beta_2$ integrin (Mac-1) captured RBCs and mediated RBC-leukocyte interaction in SCD [12]. Blocking Mac-1 using M1/70 antibody was sufficient to significantly diminish RBC-leukocyte interaction, preventing VOC and prolonged survival in SCD mice challenged by tumor necrosis factor α (TNF- α) and surgical trauma [12]. These studies highlighted the role of Mac-1 in the pathophysiology of acute VOC in SCD.

Whether therapeutic strategies targeting acute VOC would benefit chronic organ damage remains unclear. SCD mice exhibit chronic organ damage reminiscent of human SCD characterized by multiorgan infarcts, progressive iron deposition in multiple organs, enlarged glomeruli in kidney, mononuclear-cell infiltration in kidney and liver, extramedullary hematopoiesis, and hepatic sinusoidal and pulmonary capillary congestion [14]. As Mac-1 blockade has been shown to improve acute VOC, we sought to evaluate whether the genetic deletion of Mac-1 would also lead to acute benefits and test whether such genetic deletion would translate into improved end-organ damage.

Materials and Methods

Mice

Berkeley sickle cell mice (Tg[Hu-miniLCR $\alpha_1^G\gamma^A\gamma\delta\beta^S$] *Hba*^{-/-} *Hbb*^{-/-}), referred to as SCD mice have been previously described [15, 16]. *Itgam*^{-/-} (*Itgam*^{tm1Myd}, Mac-1-deficient) mice and C57BL/6 mice were purchased from The Jackson Laboratory. SCD mice deficient (SCD.*Mac-1*^{-/-}) or heterozygous (SCD.*Mac-1*^{+/-}) in Mac-1 were generated in our laboratory by breeding SCD mice with *Itgam*^{-/-} mice. All experimental procedures in this study were approved by the Animal Care and Use Committee of Albert Einstein College of Medicine.

Bone marrow transplantation (BMT)

We generated SCD.*Mac-1*^{+/-}, SCD.*Mac-1*^{-/-} and C57BL/6 mice by transplantation of BM nucleated cells from SCD.*Mac-1*^{+/-}, SCD.*Mac-1*^{-/-} and C57BL/6 mice into lethally irradiated C57BL/6 recipients as previously described [10, 17]. For intravital microscopy experiments, we used mice that were at least 3 months post BMT. Mice used in chronic tissue damage analysis were 6 months post BMT.

Flow cytometry

Blood was harvested through the retro-orbital sinus from SCD.*Mac-1*^{+/-} and SCD.*Mac-1*^{-/-} mice, collected in EDTA and RBC was lysed with lysis buffer (0.155 M NH₄Cl, 10mM KHCO₃, 0.1 mM EDTA). Single cell suspensions from blood were stained with fluorophore-conjugated anti-mouse Gr-1 and Mac-1 antibodies (eBioscience) and DAPI (sigma) and

analyzed by flow cytometry using an LSRII (BD Biosciences). Data were analyzed with FlowJo software.

Intravital experimental protocol

Mice were anesthetized by intraperitoneal (i.p.) injection of a mixture of 2% α -chloralose and 10% urethane in phosphate-buffered saline (PBS) and were treated with 0.5 μ g of TNF- α . A polyethylene tube was inserted into the trachea of anesthetized animals to facilitate spontaneous respiration. The cremaster muscle was gently exteriorized and then continuously superfused throughout the experiment with warmed (37°C) bicarbonate-buffered (pH 7.4) saline aerated with a mixture of 95% N₂ and 5% CO₂. 90 minutes after TNF- α administration, 10 venules of each mouse were videotaped over a period of 60 minutes, with each venule recorded continuously for 2 minutes. Venules were visualized with a custom-designed intravital microscope (MM-40; Nikon), using a 60X water immersion objective (Nikone). Images were recorded with a charge-coupled device video camera (Hamamatsu) and video recorder (Sony SVHS, SVO-9500). Venular diameter was measured with a video caliper. Centerline red cell velocity (V_{RBC}) was measured for each venule in real time with the use of an optical Doppler velocimeter (Texas A&M). Blood flow rate was calculated as $Q = V_{mean} \pi d^2 / 4$, where d is venule diameter and V_{mean} is estimated as $V_{RBC} / 1.6$ seconds.

Image analyses

All analyses were made with playback assessment of videotapes as previously described [10, 12, 17]. Briefly, adherent leukocytes were defined as those remaining stationary for at least 30 seconds over a 100- μ m venular segment. RBCs were identified by their size and shape (discoid and sickle-shaped cells). An interaction between RBCs and adherent leukocytes was defined as the arrest of an RBC on an adherent leukocyte for more than 2 video frames (> 0.07 second).

Hematologic parameters

Blood was collected in EDTA through the retro-orbital sinus. The RBC count, hemoglobin concentration (Hgb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin content (MCH), and mean corpuscular hemoglobin concentration (MCHC) were determined using an ADVIA 120 Hematology System (Siemens).

Measurement of urine osmolality

Mice were deprived of water for 16 hours before urine collection. Urine osmolality was measured with Model 3320 Osmometer according to the manufacturer's protocol (Advanced Instrument Inc.).

Histopathology

Organs were excised and fixed in 10% neutral buffered formalin for 36–72 hours and routinely processed for paraffin embedding. Samples for histopathology diagnostics were sectioned to a thickness of 5 μ m and stained with hematoxylin and eosin (H&E), Prussian blue (for iron) and Trichrome (for fibrosis). Samples collected from SCD.*Mac-1*^{+/-},

SCD.*Mac-1*^{-/-} and C57BL/6 mice were histologically evaluated blindly by board-certified veterinary pathologists with a qualitative grading scheme. The grading scale was on a 0–5 range: 0 (no lesion); 1 (minimal finding); 2 (mild finding); 3 (moderate finding); 4 (marked finding); and 5 (severe finding).

Statistics

Data are represented either as mean ± SEM or median with interquartile range. Comparisons between two samples were done using the unpaired Student's *t*-tests or Mann-Whitney test. Statistical analyses were performed with GraphPad Prism 6. * *p* < 0.05, ** *p* < 0.01.

Results

Impact of Mac-1 deletion on acute VOC in SCD mice

Previously, our laboratory has demonstrated that administration of an antibody to Mac-1 (M1/70) in SCD mice significantly decreased interactions between RBC and adherent leukocytes and prolonged survival of SCD mice compared to mice infused with isotype control antibody [12]. To confirm and extend studies on the role of Mac-1 integrin in SCD pathophysiology, we generated SCD.*Mac-1*^{-/-} mice (Fig. 1A). F1 hybrids were generated by breeding SCD males with Mac-1-deficient females since SCD females exhibit difficulties in feeding their pups [16]. Because the genes encoding murine β-globin (*Hbb*) and Mac-1 (*Itgam*) are closely linked on mouse chromosome 7 and mapped in close proximity (~15 centimorgan apart; MGI database for the laboratory mouse), the F1 hybrids would carry *Hbb*(-) on paternal chromosome and *Itgam*(-) on maternal chromosome (*trans* configuration) (Fig. 1A). We screened for F2 hybrids with *Hbb*(-) and *Itgam*(-) in *cis* configuration (termed *cis* mice) from the crossing between F1 hybrids and wild-type mice (Fig. 1A) and finally generated SCD.*Mac-1*^{-/-} mice from intercrossing the F2 *cis* mice.

We confirmed the absence of Mac-1 expression on Gr-1⁺ blood leukocytes of SCD.*Mac-1*^{-/-} mice (Fig. 1B). Mac-1-deficiency did not significantly change systemic leukocyte counts in SCD mice (Fig. 1C). We next evaluated leukocyte behavior by intravital microscopy of cremasteric postcapillary venules. We found that the number of leukocytes recruited in venules of TNF-α-treated SCD.*Mac-1*^{+/-} and SCD.*Mac-1*^{-/-} mice was not affected by Mac-1 deficiency (Fig. 1D). However, Mac-1-deficiency significantly diminished heterotypic interaction between RBCs and adherent leukocytes in SCD mice (Fig. 1E). Interaction between RBCs and adherent leukocytes can trigger VOC which impedes the blood flow and leads to early death in SCD mice [10]. In parallel with the reduced RBC-leukocyte interactions, the microcirculatory blood flow rate of SCD.*Mac-1*^{-/-} mice was significantly higher than that of SCD.*Mac-1*^{+/-} mice (Fig. 1F). In addition, Mac-1-deficiency significantly prolonged the survival of SCD mice during or following the experimental procedure (Fig. 1G). These data indicate that Mac-1 integrin mediates interaction between RBC and adherent leukocytes, and promotes VOC in SCD.

Impact of Mac-1 deletion on chronic organ damage in SCD mice

To investigate whether there is a beneficial effect of Mac-1-deficiency on chronic organ damage of SCD mice, we harvested blood, spleen, kidney, lung, and liver from

SCD.*Mac-I^{+/-}* and SCD.*Mac-I^{-/-}* mice at 6 months post bone marrow transplantation. RBC counts and red blood cell indexes were comparable between SCD.*Mac-I^{+/-}* and SCD.*Mac-I^{-/-}* mice (Table 1). Both groups of mice exhibited severe anemia with reduced RBC count, decreased concentration of hemoglobin (Hgb) in the blood, and reduced hematocrit (Hct), mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC), similar to previously published data on SCD mice [15, 16].

Histological examination of the kidneys revealed membranous glomerulopathy characterized by thickened capillary basement membranes (Fig. 2A, upper panels). Histopathological changes, however, were similar between SCD.*Mac-I^{+/-}* and SCD.*Mac-I^{-/-}* mice. Similar amount of iron was present in the proximal tubular epithelial cells in SCD.*Mac-I^{+/-}* and SCD.*Mac-I^{-/-}* kidneys (Fig. 2A, middle panels, arrows), but not present within the C57BL/6 kidneys (Fig. 2A, middle right panel). The trichrome staining confirmed increased collagen in glomerular tufts and demonstrated minimally increased interstitial collagen in both groups of mice (Fig. 2A, lower panels). Notably, the interstitial and glomerular trichrome staining was slightly greater in SCD.*Mac-I^{-/-}* mice than SCD.*Mac-I^{+/-}* mice (Fig. 2A, lower panels). To evaluate kidney function, we determined the ability to concentrate urine after 16 hours of water deprivation. We found that urine osmolality was significantly reduced in SCD, SCD.*Mac-I^{+/-}* and SCD.*Mac-I^{-/-}* mice, as compared to healthy C57BL/6 mice (Fig. 2B). Consistent with the similar alterations in renal histopathology, Mac-1-deficiency did not ameliorate the severity of renal dysfunction in SCD mice (Fig. 2B).

Both SCD.*Mac-I^{+/-}* and SCD.*Mac-I^{-/-}* mice had similar splenomegaly (680 ± 41 mg and 658 ± 31 mg, respectively) when compared to that of C57BL/6 mice (64 ± 3 mg). Histologically, spleens from both SCD.*Mac-I^{+/-}* and SCD.*Mac-I^{-/-}* mice had moderate lymphoid depletion in the white pulp and increased extramedullary hematopoiesis in the red pulp, with no statistically significant difference detected (Fig. 2C).

Livers in both genotypes exhibited degenerative and inflammatory histological changes with increased hepatocellular and macrophage accumulations of iron and increased sinusoidal and perivascular collagen as compared to wild-type mice. Similar histological findings were identified in SCD.*Mac-I^{+/-}* and SCD.*Mac-I^{-/-}* livers including sinusoidal congestion (Fig. 2D, upper panels, black arrows), leukocyte infiltration (Fig. 2D, upper panels, white arrow heads), siderosis (Fig. 2D, middle panels, black arrows), and extensive areas of chronic hepatocellular degeneration, necrosis, fibrosis and mixed inflammation. Increased collagen, aside from that associated with areas of visible fibrosis, was evident along sinusoids and around vessels in both SCD.*Mac-I^{+/-}* and SCD.*Mac-I^{-/-}* mouse livers (Fig. 2D, lower panels) but extent of fibrosis was similar between the two groups.

In the lungs, both groups of mice had evidence of patchy alveolar leukocyte accumulation (Fig. 2E, upper panels, yellow arrow heads) and intravascular sickle cell congestion (Fig. 2E, upper panels, black arrows). In addition, both groups of mice had minimally increased trichrome staining present in the alveolar septa with no difference observed between groups (Fig. 2E, lower panels).

Collectively, both SCD.*Mac-1*^{+/-} and SCD.*Mac-1*^{-/-} mice revealed end-organ damage characteristic of SCD that includes loss of urine concentrating ability, splenomegaly, liver damage characterized by chronic inflammation and iron deposition, and pulmonary congestion. Our data thus indicate that Mac-1-deficiency does not alleviate the chronic organ damage induced by SCD.

Discussion

Leukocytes play an important role in promoting sickle cell vaso-occlusion. Early clinical studies have revealed a positive correlation between high leukocyte counts and poor disease outcome in patients with SCD [3, 18–21]. Intravital microscopy studies using SCD mouse models have identified adherent neutrophils as the major population that interacts with sickle RBCs and promotes vaso-occlusion [10, 12, 22]. Blockade of leukocyte adhesion or RBC-neutrophil interaction effectively improves the blood flow rates and prolongs survival during VOC in SCD mouse models [10, 12]. These studies have led to exciting clinical trials of anti-adhesion therapies, including reagents that target selectins (e.g. Rivipansel, SelG1) or neutrophil activation (e.g. intravenous immunoglobulin (IVIG), PF04447943) [23]. However, whether anti-adhesion therapies can benefit chronic tissue damage in SCD remains unclear.

In this manuscript, we show that while RBC-leukocyte interactions are significantly diminished in SCD.*Mac-1*^{-/-} mice, leading to an improvement of blood flow rate and prolonged survival of these mice in acute VOC, Mac-1-deletion is insufficient to alleviate chronic organ damage as evidenced by the lack of improvement in multi-organ histopathology of SCD.*Mac-1*^{-/-} mice compared to those of SCD.*Mac-1*^{+/-} mice. These results therefore suggest distinct mechanisms for acute vaso-occlusion and chronic tissue damage in SCD, and argue that Mac-1 can be a useful therapeutic target for managing acute VOC, but not for prevention of chronic organ damage in SCD.

The development of end-organ damage in SCD is likely multifactorial. Chronic hemolysis, oxidative stress and recurrent vaso-occlusive episodes may emerge as major determinants of SCD-related organ damage [24, 25]. Intravascular hemolysis releases cell-free hemoglobin into the circulation, which can deplete nitric oxide, generate oxidative stress and increase heme concentration in the blood [26]. A large fraction of extracellular heme is carried by the erythrocyte-derived microparticles and can be transferred to the endothelium, which further promote inflammation and vascular injury [27, 28]. Excessive reactive oxygen species, heme, and inflammatory cytokines produced by several leukocyte populations including monocytes and invariant natural killer (iNKT) cells, together produces a hypercoagulable, pro-inflammatory and pro-adhesive state in SCD that leads to progressive tissue damage [13, 23, 25, 29]. In addition, sRBCs are reported to exhibit an increased propensity for adhesion to vascular endothelium [5, 30, 31], which would remain unaffected by Mac-1 deletion. The cumulative effects of sRBC interactions with the endothelium, and the intermittent transient microvascular occlusions could also contribute to chronic organ damage in SCD.

Neutrophils also promote sickle cell pathophysiology by releasing neutrophil extracellular traps (NETs), which contribute significantly to SCD-related pulmonary dysfunction [30].

Cell-free heme has been identified as a potent inducer of NET formation upon inflammatory challenges in a SCD mouse model. DNase I and hemopexin treatment targeting NETs significantly alleviates acute pulmonary tissue damage in these mice [30]. These results therefore raise a possibility that NETs may contribute to progressive tissue damage associated with SCD. More recently, an aged subset of neutrophils has been identified to exhibit enhanced Mac-1 activation and NET formation in response to inflammatory challenges, and this subset expands in both SCD mouse model and patients [31]. Microbiota-derived signals drive the generation of aged neutrophils, and depletion of the microbiota with antibiotics administered through drinking water leads to a significant reduction of aged neutrophils and dramatic improvement of both acute vaso-occlusion and chronic organ damage [31]. Although these data suggest a role for neutrophils in chronic tissue damage, the present studies—inasmuch as the model reflects human disease—indicate that the inhibition of RBC-leukocyte interactions will not be sufficient to prevent these chronic complications. In addition, long-term interference of Mac-1-mediated leukocyte migration may lead to an increased number of aged and total neutrophils in the human circulation, which may enhance the risk of end-organ damage in SCD patients and should be carefully evaluated in future clinical studies.

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Figure 1. Mac-1 deficiency in SCD mice abrogates RBC-leukocyte interactions, improves blood flow and prolongs survival in TNF- α -induced acute VOC

(A) Breeding scheme for generating SCD.*Mac-1*^{-/-} mice. (B) Blood from SCD.*Mac-1*^{+/-} and SCD.*Mac-1*^{-/-} was collected and surface expression of Mac-1 on the polymorphonuclear neutrophil population gated on the basis of side- and forward-scatter properties and Gr-1-positive staining was examined by flow cytometry after anti-Mac-1 staining. (C) Leukocyte counts, determined by an ADVIA 120 Hematology System, in SCD.*Mac-1*^{+/-} (blue bar, n=8) and SCD.*Mac-1*^{-/-} (red bar, n=8) after the intravital microscopy experiment. Results are presented as mean $\pm$ SEM. (D) Numbers of adherent leukocytes of SCD.*Mac-1*^{+/-} (blue bar, n=6) and SCD.*Mac-1*^{-/-} (red bar, n=8) mice in cremasteric postcapillary venules between 90 to 180 minutes after TNF- α administration. Results are presented as mean $\pm$ SEM. (E) Number of interactions between RBCs and adherent leukocyte per minute in SCD.*Mac-1*^{+/-} (blue bar, n=6) and SCD.*Mac-1*^{-/-} (red bar, n=8) mice between 90 to 180 minutes after TNF- α administration. Results are presented as median with interquartile range. Mann-Whitney test. ** $p < 0.01$. (F) Blood flow rates (pL/sec) of SCD.*Mac-1*^{+/-} (blue bar, n=6) and SCD.*Mac-1*^{-/-} (red bar, n=8) mice between 90 to 180 minutes after TNF- α administration. Results are presented as mean $\pm$ SEM. ** $p < 0.01$. (G) Kaplan-Meier survival curves after TNF- α treatment and surgical trauma of SCD.*Mac-1*^{+/-} (blue line, n=8) and SCD.*Mac-1*^{-/-} (red line, n=8) mice. Survival was significantly improved in the absence of Mac-1; log-rank test, * $p < 0.05$.

Figure 2. Mac-1 deficiency in SCD mice does not prevent chronic organ damage

(A) Representative histology (Hematoxylin and eosin (H&E), top panels; Prussian blue, middle panels; trichrome, lower panels) of kidney of SCD.Mac-1^{+/-} (left panels) and SCD.Mac-1^{-/-} (middle panels) and C57BL/6 (right panels) mice. Black arrows indicate the areas with iron accumulation. (B) Quantification of urine osmolality of C57BL/6 mice (black bar, n=4), SCD mice (white bar, n=4), SCD.Mac-1^{+/-} (blue bar, n=3) and SCD.Mac-1^{-/-} (red bar, n=4). Results are presented as mean $\pm$ SEM. (C) Representative histology (H&E) of spleen of SCD.Mac-1^{+/-} (left panel) and SCD.Mac-1^{-/-} (middle panel) and C57BL/6 (right panel) mice. White pulp is indicated by white dashed line. (D) Representative histology of liver of SCD.Mac-1^{+/-} (left panels) and SCD.Mac-1^{-/-} (middle panels) and C57BL/6 (right panels) mice. In H&E staining (top panels), black arrows indicate the presence of intravascular sickle cell congestion and white arrowheads indicate areas with leukocyte infiltration. In Prussian blue staining (middle panels), black arrows indicate the areas with iron accumulation. Trichrome staining is shown in lower panels. (E) Representative histology of lungs of SCD.Mac-1^{+/-} (left panels) and SCD.Mac-1^{-/-} (middle panels) and C57BL/6 (right panels) mice. In H&E staining (top panels), black arrows indicate the presence of intravascular sickle cell congestion and yellow arrowheads indicate areas with leukocyte accumulation and aggregation. Trichrome staining is shown in lower panels.

Table 1Hematologic values for SCD.*Mac-I*^{+/-}, SCD.*Mac-I*^{-/-} and C57BL/6 mice

	RBC (×10 ⁶ cells/μl)	Hgb (g/dL)	Hct (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)
SCD. <i>Mac-I</i> ^{+/-} (n=4)	6.5±0.3	5.1±0.3	26.3±1.1	40.3±0.1	7.8±0.1	19.4±0.3
SCD. <i>Mac-I</i> ^{-/-} (n=4)	6.0±0.1	5.2±0.2	25.0±0.8	42.0±0.3	8.6±0.1	20.5±0.1
C57BL/6 (n=3)	9.8±0.1	13.2±0.1	50.3±1.2	51.7±0.6	13.6±0.1	26.4±0.4

RBC, red blood cell; Hgb, hemoglobin concentration; Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin content; MCHC, mean corpuscular hemoglobin concentration. Samples were obtained from animals at 6 months post bone marrow transplantation.

Values are shown as mean ± SEM. Mean for each parameter were significantly different between C57BL/6 and SCD (*Mac-I*^{+/-} or *Mac-I*^{-/-}) mice (*p*<0.0001)