Targeting P-selectin and interleukin-1β in mice with sickle cell disease: effects on vaso-occlusion, liver injury and organ iron deposition

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Abstract

Continuous vaso-occlusive and inflammatory processes cause extensive end-organ damage in adults with sickle cell disease (SCD), and there is little evidence that long-term hydroxyurea therapy prevents this. In initial trials, P-selectin blockade with crizanlizumab reduced SCD vaso-occlusive crisis frequency, and interleukin (IL)-1β inhibition in SCD patients, using canakinumab, lowered inflammatory markers. We used murine SCD models to examine the effects of acute and chronic blockade of P-selectin and of IL-1β on vaso-occlusive events, their inflammatory profile and organ health. Both approaches improved impaired cutaneous microvascular perfusion in SCD mice by reducing TNF-α-induced vaso-occlusion. Acute P-selectin blockade markedly reduced TNF-α-induced neutrophil-platelet aggregate formation in SCD mice, and decreased leukocyte-rolling movements in the microvasculature, while acute IL-1β inhibition attenuated microvascular leukocyte adhesion. Six weeks of IL-1β-blocking immunotherapy improved the inflammatory profile of SCD mice, considerably reduced hepatic fibrosis and provided some relief from lung injury. In contrast, although P-selectin blockade reduced glomerular congestion, no significant benefit to overall organ pathology was observed. Unexpectedly, while combining the two immunotherapies reduced microvascular occlusion, their prolonged use caused acute liver injury. Notably, inhibition of IL-1β, but not of P-selectin, remarkably decreased hemosiderosis, in association with reduced tissue macrophage infiltration and the correction of biomarkers of dysregulated iron turnover. Our findings suggest that the attenuation of inflammation, as well as of vaso-occlusive processes, may be crucial for mitigating organ damage in SCD. Future trials should explore the ability of cytokine blockade to prevent multi-organ damage in patients with SCD, beyond evaluating vaso-occlusive crisis frequency.

Introduction

Sickle cell disease (SCD), an inherited disorder caused by the production of sickle hemoglobin (HbS),[1] is recognized as an inflammatory disease. SCD clinical complications are numerous, and can be classified as acute (e.g. painful vaso-occlusive crises [VOC]) or chronic (e.g. kidney disease, hepatopathy) in nature.[2] Characteristic vaso-occlusive processes in SCD arise from altered blood rheology, cellular activation, and adhesive interactions between red blood cells, leukocytes, platelets, and the endothelium. Inflammatory pathways drive the cellular activation that propagates this vaso-occlusion,[3] where amplification of the production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, promotes endothelial activation and facilitates the cell-cell adhesive interactions that occlude small vessels.[4-6] Hydroxyurea, presently the most commonly employed disease-modifying therapy in SCD, significantly reduces VOC frequency in SCD patients, with documented improvements in morbidity and mortality.[7] Advances in understanding SCD pathophysiology led to the development of newer therapeutics, such as crizanlizumab, voxelotor and L-glutamine. Crizanlizumab is a humanized monoclonal antibody that binds to P-selectin (CD62P) on the surface of activated endothelial cells and platelets and blocks its interaction with P-selectin glycoprotein ligand-1, predominantly on leukocytes,[8] thereby attenuating cellular recruitment and heterocellular aggregate formation in the vasculature.[9] A phase II trial (*clinicaltrials gov. Identifier: NCT01895361*) demonstrated that crizanlizumab significantly lowered the VOC rate in 198 SCD patients, aged 16 to 65 years, over 52 weeks of treatment,[8] leading to its Food and Drug Adminstration (FDA) approval for use in SCD patients aged over 16 years,[10] and later by other agencies. However, the recent STAND study (*clinicaltrials gov. Identifier: NCT03814746*) failed to confirm the efficiency of crizanlizumab for reducing SCD VOC frequency, compared to placebo, leading to revocation of its authorization in the European Union.[11] Inclacumab, another, potentially more potent, P-selectin-blocking molecule is currently under clinical development and in a phase III trial for preventing SCD VOC.[12]

Emerging therapies for SCD have primarily aimed to reduce VOC frequency and/or increase hemoglobin levels. However, advances in the clinical management of SCD have significantly improved the life expectancy of patients, and aging in SCD is worryingly associated with extensive end-organ damage,[13] which is challenging to analyze in clinical trials. Organs affected include the kidneys, brain, liver, heart and lung, with continuous vaso-occlusion (VO) and inflammatory processes in the vasculature being the most likely causes of such lesions.[3] At this time, there is a dearth of evidence that any of the available disease-modifying agents, including hydroxyurea, can sufficiently prevent end-organ damage in adults with SCD.[14,15] Exploring alternative anti-inflammatory therapeutic strategies to block other inflammatory molecules, or enhancing the benefits of P-selectin blocking therapy by using combination approaches, could further decrease vascular cellular interactions. This may improve VOC onset prevention and, potentially, prevent SCD organ damage.

Canakinumab is a humanized monoclonal antibody that blocks the activity of IL-1β, an inflammasome-processed cytokine that drives inflammatory responses and that is elevated in SCD.[5] A recent randomized, placebo-controlled, multicenter phase IIa study demonstrated that canakinumab was well tolerated in 25 sickle cell anemia (SCA) patients, aged 8 to 20 years, who were treated for 24 weeks.[16] While patients did not report significant reductions in average daily pain, progressive reductions in leukocyte counts and high-sensitivity C-reactive protein were observed, demonstrating the potential anti-inflammatory benefits of IL-1β neutralization. Accordingly, anti-IL1β antibody administration alleviated reperfusion injury, endothelial activation and flow stasis in NY1DD transgenic sickle mice exposed to hypoxia/reoxygenation.[17] Thus, hypothesizing that targeting both adhesive and inflammatory mechanisms could enhance VOC prevention in SCD, we first assessed both the individual and the combined effects of acute P-selectin blockage and IL-1β neutralization on TNF-α-induced VO-like processes in SCD mice. We then looked at the impacts of blocking these pathways, for 6 weeks, on the inflammatory profile and organ injury in SCD mice.

Methods

Antibodies

01BSUR was provided by Novartis BioMedical Research (Basel, Switzerland); RB40.34 and A110-1 IgG1 antibodies were from BD Biosciences (New Jersey, USA).

Animals and treatment protocols

Mice and treatment protocols are detailed in the *Online Supplementary Appendix*. All experimental procedures in animals were approved by the animal care and use committee of the University of Campinas (CEUA/ UNICAMP; Protocols 6079-1 and 6179-1), and by Novartis' animal care and use committee.

Intravital microscopy

Observations of the cremaster muscle microcirculation were made in anesthetized SCD mice, as previously described.[18] The microvascular rolling, adhesion and extravasation of leukocytes were quantified. See the *Online Supplementary Appendix*.

Laser Doppler flowmetry

Blood flow and perfusion in the cutaneous microcirculation of the pelvic region of mice was determined as described in the *Online Supplementary Appendix*.

Neutrophil-platelet aggregate quantification

Neutrophil-platelet ($CD45^+Ly6G^+CD41^+$) aggregates were determined in peripheral blood by flow cytometry, as described in the *Online Supplementary Appendix*, and calculated using FlowJo V10 software (see *Online Supplementary Figure S1*).

Histopathological evaluation

Organs were processed and histological staining/analyses were performed as described in the *Online Supplementary Appendix*.

Quantitative real time polymerase chain reaction

The extraction of mRNA from organs (stored at -80°C), cDNA synthesis, and the quantitative real time polymerase chain reaction protocol are described in the *Online Supplementary Appendix*, as is primer design (*Online Supplementary Table S1*).

Statistical analysis

See the *Online Supplementary Appendix*.

Results

Acute inhibition of P-selectin and of IL-1β each improve cutaneous microvascular flow and perfusion in TNF-α-challenged SCD mice

Vaso-occlusive processes were induced in male Townes SCD mice by i.p. TNF-α administration (Figure 1A). The basal circulating concentrations of IL-1β are elevated in Townes SCD mice, therefore validating use of IL-1β neutralization therapy (Figure 1B). Significant P-selectin-dependent neutrophil-platelet aggregation formation was observed in the peripheral blood of TNF-α-treated Townes SCD mice, justifying P-selectin blockade use (Figure 1C). Laser Doppler flowmetry demonstrated that administration of TNF-α diminished both blood flow velocity (Figure 1D) and perfusion (Figure 1E) in the hindlimb superficial circulation of Townes SCD mice, compared to basal conditions. Alterations were indicative of the occurrence of VO and reduced tissue blood supply, respectively, in the cutaneous microcirculation. Anti-CD62P administration (anti-P-selectin; at 30 minutes [min] before TNF-α, and therefore at 3.5 hours [h] before analysis; Figure 1A) restored the TNF-α-induced decrease in

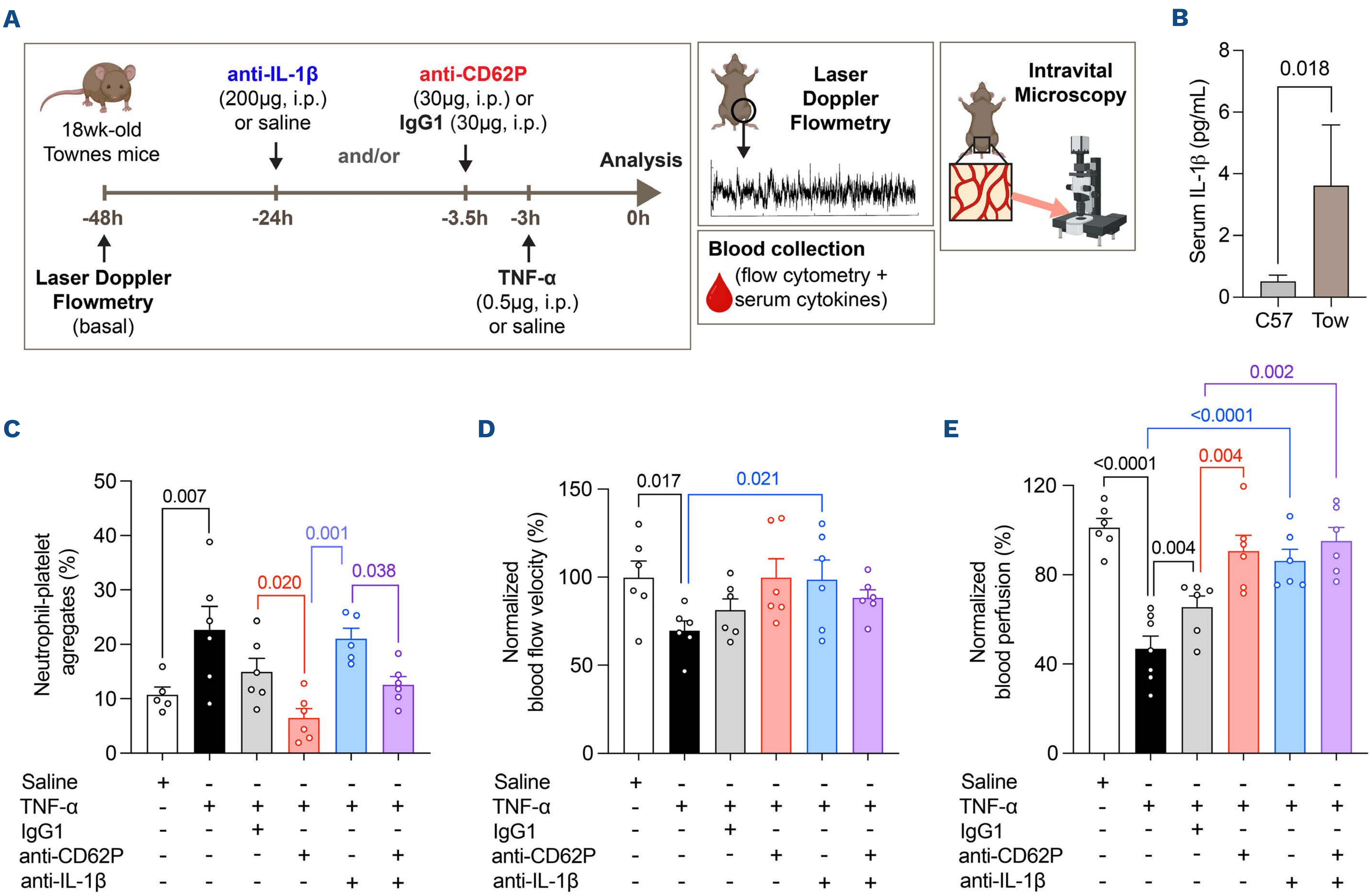

Figure 1. Acute P-selectin blockade and IL-1β neutralization in mice with sickle cell disease reverse TNF-α cytokine-induced cutaneous microvascular dysfunction, in association with differing effects on heterocellular aggregation formation. (A) Experimental design for acute protocols in sickle cell disease (SCD) mice. Townes SCD mice were treated intraperitoneally (i.p.) with antibodies targeting P-selectin (anti-CD62P) and/or IL-1β (anti-IL-1β), at 3.5 hours (h) or 24 h (respectively) prior to analysis, consisting of laser Doppler flowmetry of the skin, blood sampling or intravital microscopy. Mice were also subjected to laser Doppler flowmetry at 48 h before protocols to measure basal hemodynamics. Doses were administered as follows: saline (100 μL, 24 h before analysis, N=5-6); 30 μg/mouse IgG1 isotype control antibody (A110-1, 3.5 h before analysis, N=6); 30 μg/mouse anti-CD62P (RB40.34, 3.5 h before analysis, N=5-6); 200 μg/mouse anti-IL-1β (01BSUR, 24 h before analysis, N=5-6), and 30 μg/mouse anti-CD62P plus 200 μg/mouse anti-IL-1β (N=6). Inflammatory processes were induced in mice by the administration of TNF-α (TNF) at 3 h before flowmetry/blood sampling/intravital microscopy (0.5 μg, i.p.). (B) Basal levels of IL-1β were measured in the serum of C57BL/6J mice (C57, N=10) and Townes SCD mice (Tow, N=13) by enzyme-linked immunosorbant assay. (C) Flow cytometry was used to monitor neutrophil-platelet heterocellular aggregate formation in the blood of SCD mice. Platelet-neutrophil aggregates were determined as the percentage of $CD45^+Ly6G^+$ neutrophils in peripheral blood samples of mice that also labeled positive for the platelet marker, CD41. Laser Doppler flowmetry (PeriFlux 6000; Perimed) was used to measure blood flow (D) and perfusion (E) in the hindlimb superficial microcirculation of SCD mice. Data were normalized relative to basal measurements taken from the same mice, 48 h earlier. (B-E) Statistical comparisons are made between treatments and their mechanistic control, and between treatments.

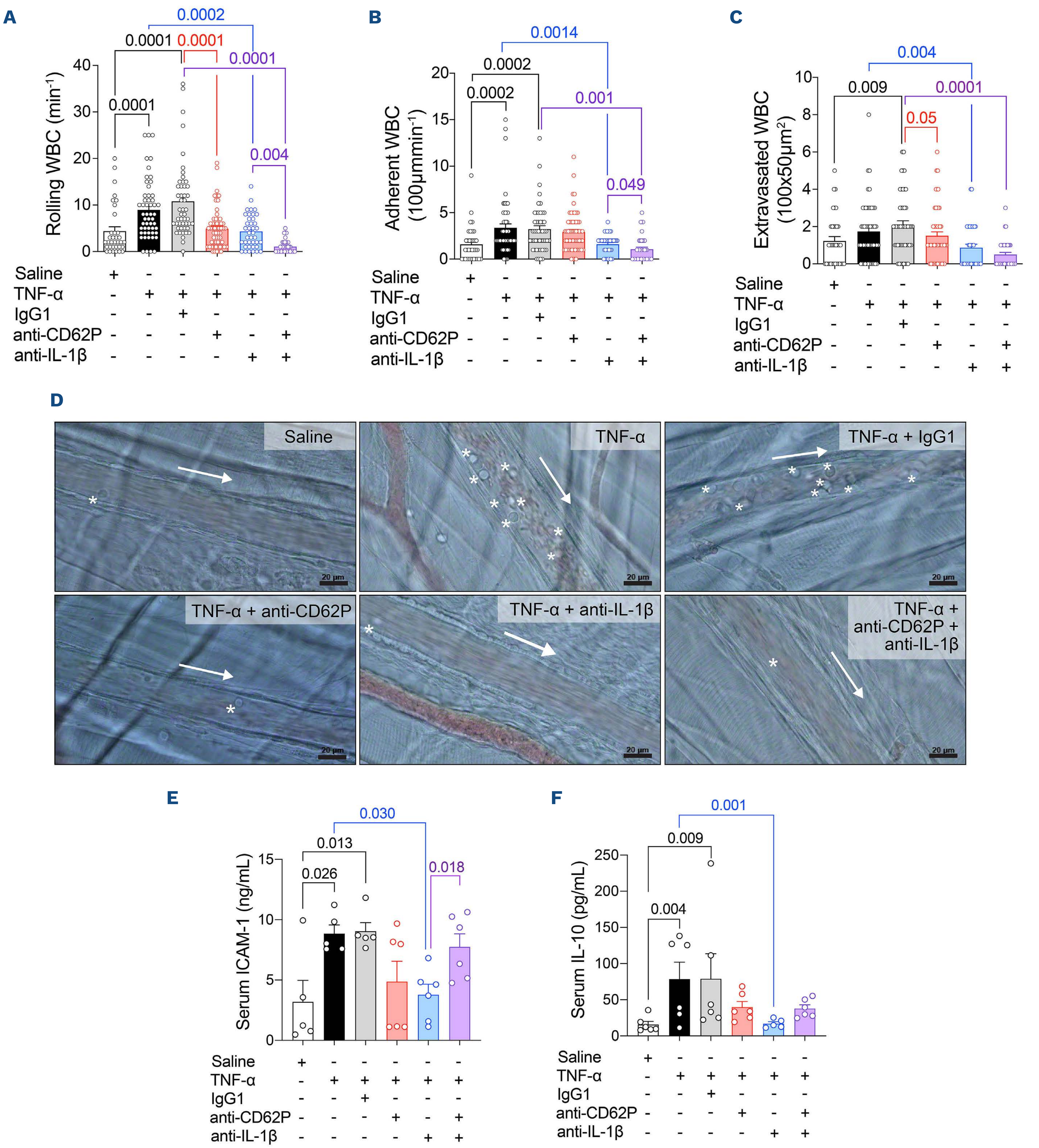

Figure 2. Acute P-selectin blockade and IL-1β neutralization modulate TNFα-induced leukocyte recruitment and vaso-occlusive-like processes in mice with sickle cell disease. Townes sickle cell disease (SCD) mice were treated intraperitoneally (i.p.) with antibodies targeting P-selectin (anti-CD62P) and/or the inflammasome-processed cytokine, IL-1β (anti-IL-1β), at 3.5 or 24 hours (h), respectively, prior to intravital microscopy (N=5-6 per group) (see Figure 1A). Vaso-occlusive processes were induced in mice by the administration of TNF-α (TNF, 0.5 μg, i.p.): leukocyte (white blood cell [WBC]) recruitment was quantified in 5-9 venules of the cremaster muscle of each mouse at 180 minutes (min) after TNF. Graphs depict WBC rolling along the venule walls (100 μm) (A), WBC adhesion (B), and WBC extravasation (C). Circles represent values for each venule. (D) Representative photo-

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micrographs from a cremaster venule in each treatment group; bars represent 20 μm. Note the visible improvement in venular blood flow in animals treated with anti-CD62P, anti-IL-1β, or their combination, compared to the venules of TNF-challenged mice (and IgG1/TNF-treated mice), where individual cells (indicated by *) can be distinctly observed, indicating vaso-occlusion. White arrows indicate the direction of blood flow. Serum concentrations of (E) soluble ICAM-1 (sICAM-1), and (F) IL-10 were determined by enzyme-linked immunosorbant assay. (A-C) and (E-F), statistical comparisons are made between treatments and their mechanistic control, and between treatments.

cutaneous microvascular blood flow to baseline levels and significantly improved perfusion in mice (Figure 1D, E), as did anti-IL-1β administration (21 h before TNF-α, and therefore at 24 h before analysis; Figure 1A). The administration of a combination of both anti-CD62P and anti-IL-1β immunotherapies (30 min and 21 h before TNF-α, respectively) also improved cutaneous blood perfusion in TNF-α-stimulated mice. Of note, the administration of an IgG1 isotype control (mechanistic control for anti-CD62P) slightly, but significantly, increased blood perfusion in TNF-α-induced SCD mice (Figure 1E), but less effectively than P-selectin blockade ($P<0.05$). Previous prolonged-administration studies have shown that the intravenous administration of high dose immunoglobulin (800 mg/kg) can protect mice from neutrophil-mediated vascular injury by blocking FcγRIII,[19] possibly explaining why the low dose of control IgG1 used (approximately 1.2 mg/kg) may slightly improve cutaneous perfusion.

Acute inhibition of P-selectin, but not of IL-1β, abolishes TNF-α-induced heterocellular aggregate formation in SCD mice

Anti-CD62P administration to SCD mice completely abolished the formation of neutrophil-platelet aggregates in TNF-α-stimulated SCD mice (compared to control IgG1 administration), consistent with the known role for P-selectin in the formation of these aggregates (Figure 1C).[20] In contrast, administration of anti-IL-1β antibody did not modulate heterocellular aggregate formation, and combined anti-CD62P and anti-IL-1β administration did not decrease aggregate formation further, compared to anti-CD62P alone.

Acute inhibition of IL-1β potentiates the effects of P-selectin blockade on microvascular TNF-α-induced leukocyte recruitment in SCD mice

Increased leukocyte recruitment in the microvasculature is believed to initiate TNF-α-induced vaso-occlusive processes in SCD mice,[21] thereby reducing blood flow velocity (Figure 1D). TNF-α induced significant leukocyte recruitment in the cremaster microcirculation, increasing leukocyte rolling and adhesion to venule walls (Figure 2A, B). Administration of anti-CD62P abrogated TNF-α-induced leukocyte rolling in venues (compared to IgG1; Figure 2A), but did not alter venular leukocyte adhesion (Figure 2B). P-selectin blockade also reduced leukocyte extravasation in the microcirculation (Figure 2C). In contrast, anti-IL-1β administration significantly reduced TNF-α-induced leukocyte adhesion, as well as rolling and extravasation in the SCD mice (compared to saline administration; Figure 2A-D). Importantly, the combination of both anti-CD62P and anti-IL-1β immunotherapies potentiated the inhibition of TNF-α-induced leukocyte recruitment, further decreasing leukocyte rolling and adhesion (compared to anti-IL-1β alone).

TNF-α administration significantly augmented the serum concentrations of the soluble ICAM-1 (sICAM-1) (Figure 2E) and IL-10 (Figure 2F) inflammatory markers. While the abrogating effects of acute P-selectin blockade on circulating sICAM-1 and IL-10 were not significant, acute IL-1β neutralization significantly decreased the TNF-α-induced elevations in serum sICAM-1 and IL-10 in the SCD mice, however combination therapy with the two agents failed to significantly reduce sICAM-1.

Effects of prolonged P-selectin and IL-1β blocking immunotherapies on platelet-leukocyte aggregate formation and the inflammatory profile in Berkeley SCD mice

For prolonged-administration studies, Berkeley mice (4-months old) were treated for 6 weeks with anti-CD62P or anti-IL-1β monotherapies, or their combination. Control animal groups were treated with saline, or an IgG1 control antibody (Figure 3A). Mice were not subjected to TNF-α inflammatory stimulus in these experiments.

Similarly to findings in Townes SCD mice, basal circulating concentrations of IL-1β were significantly elevated in Berkeley mice, compared to C57BL/6 mice (Figure 3B). Likewise, as observed in acute conditions, anti-CD62P immunotherapy significantly reduced neutrophil-platelet aggregate formation in SCD mice, compared to the IgG1 group (Figure 3C), while IL-1β neutralization had no effect. Anti-IL-1β immunotherapy significantly altered the serum inflammatory profile in SCD mice, decreasing TNF-α (Figure 3D) and IL-6 (Figure 3E) concentrations, but not interferon-γ (Figure 3F). In contrast, anti-CD62P immunotherapy had no significant effect on the levels of inflammatory molecules measured; however, its combination with anti-IL-1β immunotherapy reversed the effect of anti-IL-1β monotherapy on serum IL-6 (Figure 3E).

Effects of prolonged P-selectin and IL-1β blockade on markers of liver and kidney damage in Berkeley SCD mice

Following treatment of Berkeley SCD mice for 6 weeks with saline, IgG1 or immunotherapies, serum biochemical markers of liver damage and of hemolysis were quantified (*Online Supplementary Table S2*). Serum alanine aminotransferase (ALT) levels were significantly higher in control saline/IgG1-treated Berkeley mice, compared to untreated age-matched hemizygous (SA) mice, while aspartate aminotransferase (AST) was not significantly altered. Neither monotherapy significantly altered AST or ALT in SCD mice. However, combined anti-CD62P/anti-IL-1β therapy elevated ALT to levels that were significantly higher than those observed for mice that received anti-IL-1β monotherapy.

Total, direct bilirubin, and indirect bilirubin were significantly higher in control saline/IgG1-treated Berkeley SCD mice,

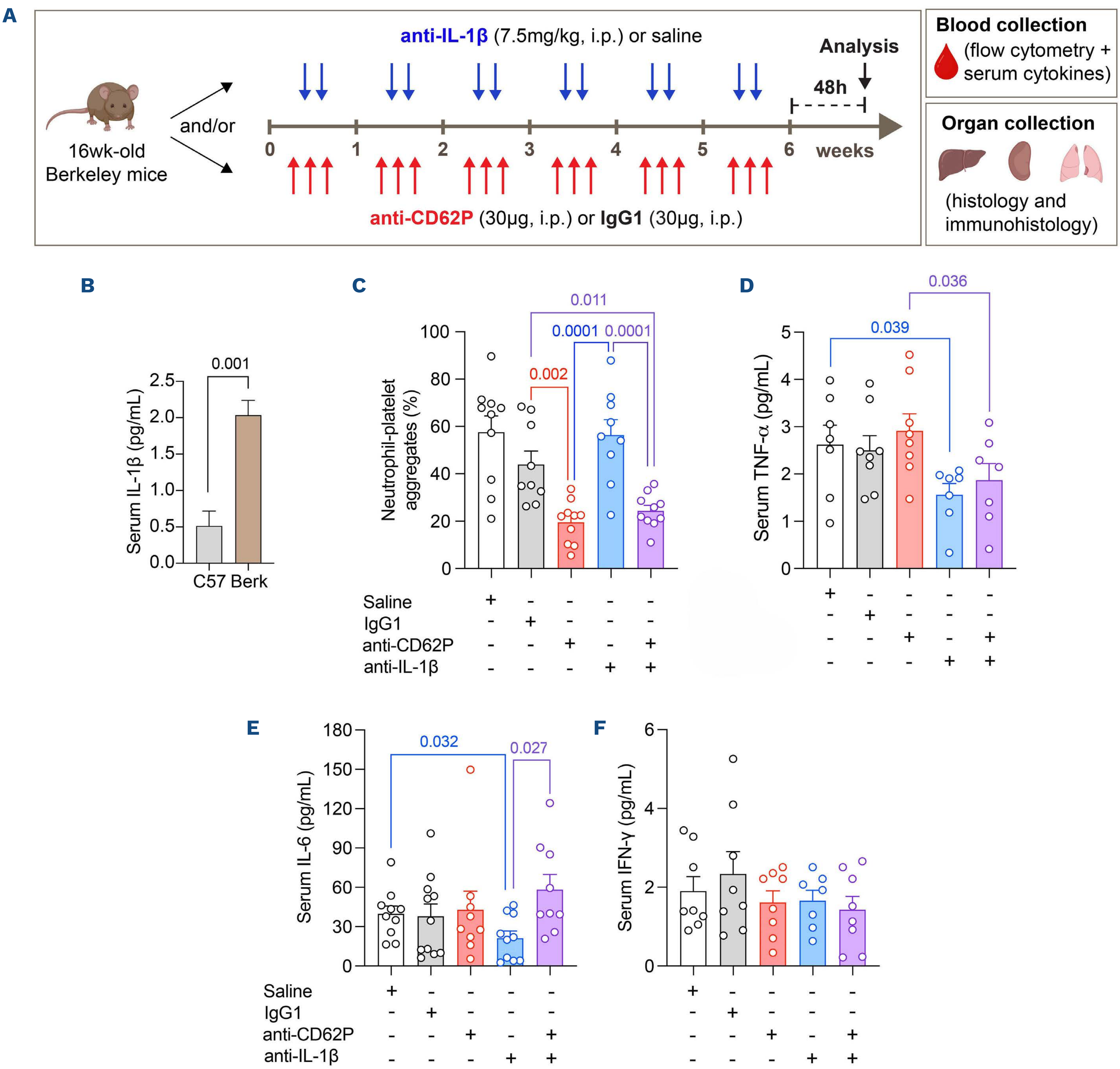

Figure 3. Anti-inflammatory effects of chronic administration (6 weeks) of immunotherapies that block P-selectin and/or IL-1β in sickle cell disease mice. (A) Experimental design: Berkeley sickle cell disease (SCD) mice (4-months old) were treated with intraperitoneal (i.p.) injections of 30 µg/mouse anti-CD62P (N=9-10) or equivalent IgG1 (N=9), three times a week, and/or 7.5 mg/kg of anti-IL1β antibody (N=7-9) or saline (N=10), twice a week, for 6 weeks. At 48 hours (h) after administration of the final intervention dose, peripheral blood samples and organs of mice were collected and appropriately stored for processing. (B) Basal levels of IL-1β were measured in the serum of C57BL/6J mice (C57, N=10) and Berkeley SCD mice (Berk, N=7) by enzyme-linked immunosorbant assay (ELISA). (C) Platelet-neutrophil aggregates were monitored in peripheral blood samples by flow cytometry, and serum concentrations of (D) TNF-α, (E) IL-6 and (F) IFN-γ were determined by ELISA. Platelet-neutrophil aggregates are expressed as the percentage of neutrophils ($CD45^+/Ly6G^+$) that also displayed labeling for the platelet marker, CD41. (C-F) Statistical comparisons are made between treatments and their mechanistic control, and between treatments.

compared to hemizygous mice, indicative of accentuated hemolysis and liver dysfunction. A further increase in indirect (unconjugated) bilirubin, in the absence of any alteration in direct bilirubin levels, occurred only in the mice that received the anti-CD62P/anti-IL-1β combination therapy, suggestive of a further increase in hemolysis in these animals.[22] Urine creatinine was significantly lower in IgG1-treated Berkeley SCD mice, compared to aged-matched hemizygous mice, suggestive of renal damage; however, the elevations in urine creatinine observed in the groups treated with anti-CD62P were not statistically significant.

Effects of prolonged P-selectin and IL-1β blockade on hepatic tissue alterations in Berkeley SCD mice

Following the 6-week administration protocols, organs were collected from Berkeley SCD mice euthanatized at 48 hours after the final administration. Histopathological analysis showed that, compared to aged-matched hemizygotes, the livers of homozygous SCD Berkeley mice treated with saline (or control IgG1) presented vessel and sinusoidal congestion, intralobular necrosis, and pericentral fibrotic bands (Figure 4, hematoxylin and eosin [H&E]; *Online Supplementary Figure S3* highlights histopathological features). Collagen deposition, indicative of fibrosis, was also observed in the liver of saline-treated SCD mice (Figure 4, Masson). Accentuated iron accumulation was also observed, particularly associated with tissue macrophages (Figure 4, Perls; *Online Supplementary Figure S4*), together with increased $CD68^+$ cell immunolabeling (Figure 4).

Prolonged anti-CD62P therapy did not significantly alter the congested vessel number or the observations of vessel and sinusoidal congestion in the livers of SCD mice (Figure 4; Figure 6A, B). Furthermore, hepatocyte degeneration and fibrosis were not significantly decreased by anti-CD62P therapy (Figure 6C, E), when compared to IgG1 treatment. Some improvement in sinusoidal/vessel congestion was observed in response to IL-1β neutralization (Figure 4; Figure 6A, B) in SCD mice, when compared to anti-CD62P or saline treatment. This decreased congestion was associated with ameliorations in fibrosis and parameters of acute liver injury (hepatocyte degeneration and lobular necrosis) in the anti-IL1β-treated SCD mice (Figure 4; Figure 6C-E). Combining the anti-CD62P and anti-IL-1β therapies did not potentiate the effects of anti-IL-1β upon vessel congestion and, indeed, appeared to reverse some of this monotherapy's benefits, with increased hepatocyte injury observed (ballooning and steatosis) (Figure 6A-E; *Online Supplementary Figure S3A*).

Anti-CD62P therapy had no significant effect on the extensive hepatic iron deposition observed in SCD mice (Figures 4, 6F and 8A). In contrast, and importantly, iron accumulation was almost completely abolished in the livers of mice treated with anti-IL-1β monotherapy, or with the combination of anti-CD62P and anti-IL-1β (Figures 4, 6F and 8A). The presence of $CD68^+$ cells was significantly decreased in mice that received anti-CD62P and/or anti-IL-1β immunotherapies (Figures 4 and 6G), although anti-IL-1β monotherapy decreased the macrophage infiltration most effectively.

Effects of prolonged P-selectin and IL-1β blockade on renal and pulmonary tissue alterations in Berkeley SCD mice

Comparing the kidney histopathology of homozygous SCD Berkeley mice treated for 6 weeks with saline, or non-specific IgG1, to that of aged-matched hemizygotes, glomerular and capillary congestion, and interstitial inflammation with apparent sickled red cells were observed (Figure 5, H&E, and highlighted in *Online Supplementary Figure S3B*). Renal collagen deposition close to vessels and the medulla was considered normal in saline/IgG1-treated SCD mice (Figure 5, Masson), however, iron accumulation was extensive, especially in the proximal tubules, and $CD68^+$ macrophage immunostaining was increased (Figure 5).

Anti-CD62P therapy reduced renal glomerular and capillary congestion, as well as the number of apparent sickled red cells, compared to IgG1-treated SCD mice (Figures 5 and 7A, B). In contrast, anti-IL-1β therapy had no significant effect on vessel congestion (Figure 5; Figure 7A, B). Kidney sections from animals receiving combination therapy (anti-CD62P/anti-IL-1β) displayed nephrosclerosis, and glomerular congestion, as well as occasional protein droplet reabsorption, indicating further renal deterioration (*Online Supplementary Figure S3B*). Again, anti-IL-1β monotherapy, and its combination with anti-CD62P, completely abolished renal iron deposition in the Berkeley SCD mice (Figure 7D). Notably, $CD68^+$ macrophage infiltration was significantly decreased only in the group treated with anti-IL-1β monotherapy (Figures 5 and 7E).

The histopathological observations of the lungs of saline-treated SCD mice indicated alveolar congestion, atelectasis (septal thickening) and some peribronchial inflammation (*Online Supplementary Figures S2* and *S3*, H&E, Masson), when compared to hemizygote mice. No iron deposition was observed in the lungs of 22-week old SCD mice. While some improvements in the histopathological features of the lungs of SCD mice occurred following the administration of control IgG1 (*Online Supplementary Figure S2*; Figure 7G), P-selectin-blocking therapy did not significantly alter SCD lung histopathology. In contrast, Masson staining indicated discrete correction of pulmonary septal thickening in SCD mice that received the anti-IL-1β monotherapy and the combined therapies (*Online Supplementary Figures S2* and *S3*; Figure 7H).

Effects of prolonged P-selectin and IL-1β blockade on the gene expressions of organ damage markers and iron regulation proteins in Berkeley SCD mice

The gene expressions of tissue damage markers and iron regulation proteins were quantified by quantitative polymerase chain reaction in the organs of SCD mice following treatments (Figure 3A). In SCD mice that received

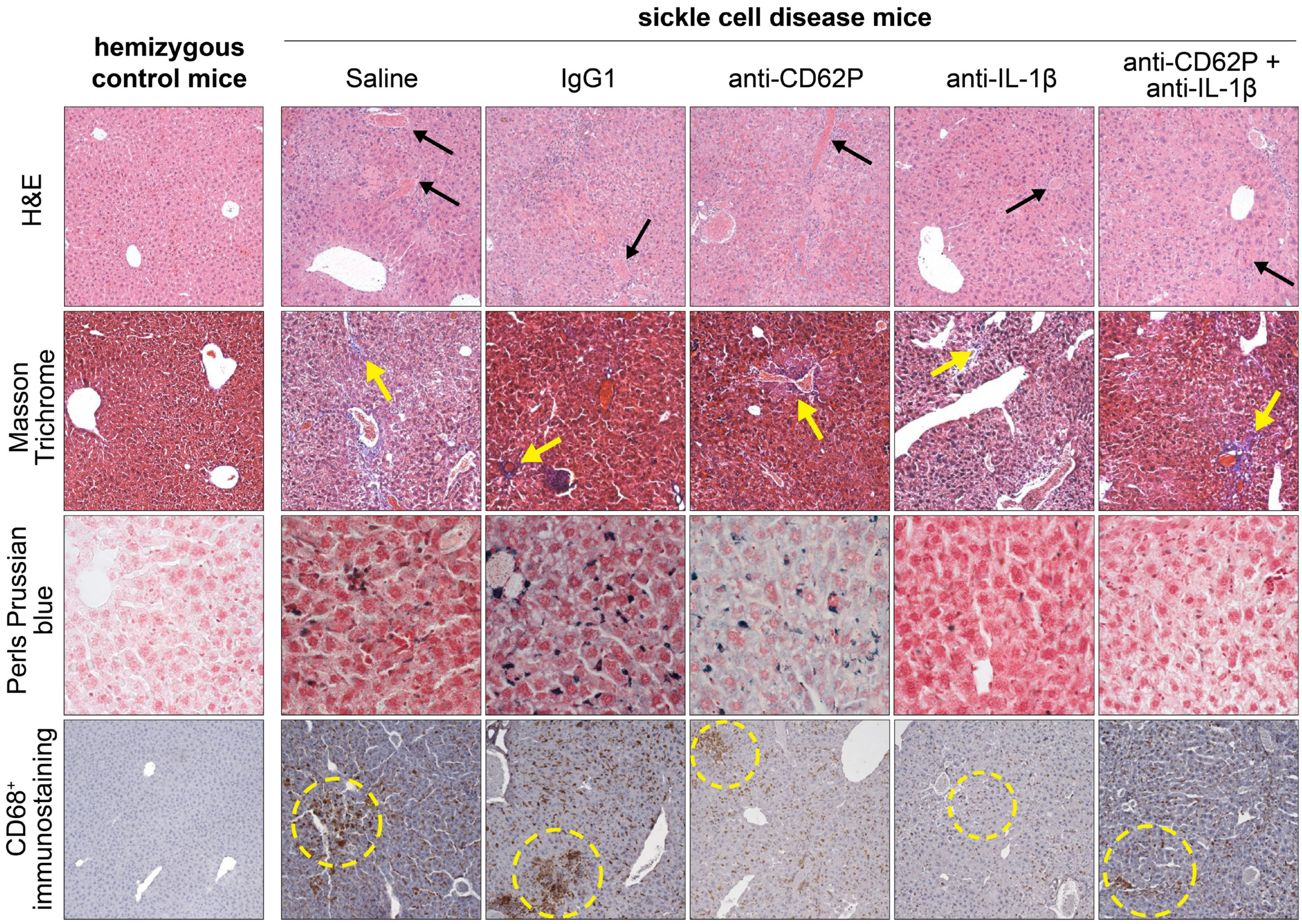

Figure 4. Effects of 6-weeks administration of anti-CD62P and anti-IL-1β immunotherapies on liver histology, injury, and iron accumulation in sickle cell disease mice. Berkeley sickle cell disease (SCD) mice (4-months old) were treated with immunotherapies, or not, as described in Figure 3A, for 6 weeks. At 48 hours (h) after administration of the final intervention dose, livers were dissected and processed for histological analysis (hematoxylin and eosin [H&E] staining, 20x magnification [mag.]), analysis of fibrosis (Masson Trichrome, 20x mag.), iron accumulation (Perls Prussian blue, 63x mag.), and $CD68^+$ cells (anti-CD68 immunohistochemical staining, 20x mag.). Image depicts representative photographs of stained sections from each treatment group and comparison with liver sections from hemizygous Berkeley control mice of the same age. Photographs are representative of 4 images per section (2-4 sections per mouse). Black arrows: congested vessels; yellow arrows: collagen deposits; yellow circles: areas of macrophage infiltration. Photomicrographs taken with a Zeiss Axio Imager D2.

saline, liver expressions of genes encoding collagen type 3, ICAM-1, TIMP-1, TIMP-2, TGF-β, and HIF-1α were significantly increased compared to hemizygote mice (*Online Supplementary Figure S5A*). Immunotherapies targeting P-selectin and IL-1β did not affect the expressions of these genes. In the kidneys, the expression of the gene encoding NGAL, a kidney damage marker, was significantly elevated in saline-treated SCD mice, compared to hemizygotes, while the expression of the KIM-1 gene (*Havcr1*), which has a renal anti-inflammatory role, was unchanged. Immunotherapies did not alter these gene expressions in SCD mouse kidneys (*Online Supplementary Figure S5B*).

As significant amelioration of hepatic and renal siderosis was observed in Berkeley SCD mice following anti-IL-1β therapy, we examined the hepatic gene expressions of proteins involved in iron regulation in these mice. In saline-treated SCD mice, the hepatic expressions of genes encoding bone morphogenetic gene-6 (BMP-6) and the transferrin receptor-1 (TrF-1), which mediates iron uptake by cells, were significantly increased compared to age-matched hemizygote controls (Figure 8C, D). After 6 weeks of IL-1β blockade, the expressions of the genes encoding BMP-6 and hepcidin were suppressed in the SCD mouse liver, compared to saline-treated SCD mice (Figure 8B, C).

Discussion

Clinical trials of several disease-modifying therapies in development for SCD have concentrated on assessing VOC frequency and patient-reported pain as primary outcomes.[8,16,23] While reducing VOC frequency in SCD is paramount, there is an urgent need to simultaneously halt irreversible organ damage progression, which significantly impacts morbidity and

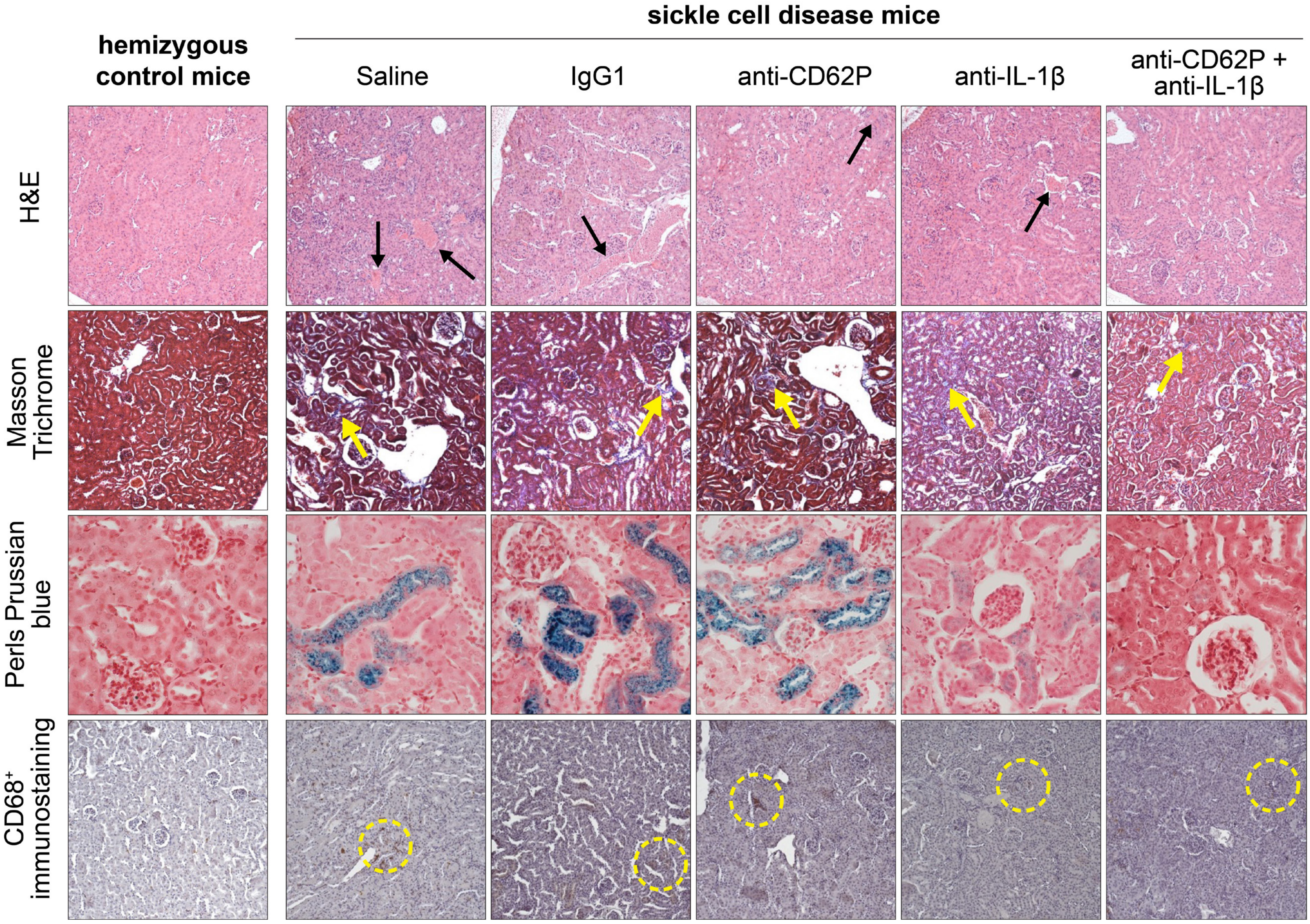

Figure 5. Effects of 6-weeks administration of anti-CD62P and anti-IL-1β immunotherapies on kidney histology, injury, and iron accumulation in sickle cell disease mice. Berkeley sickle cell disease (SCD) mice (4-months old) were treated, or not, with immunotherapies, as described in Figure 3A, for 6 weeks. At 48 hours (h) after administration of the final intervention dose, kidneys were dissected and processed for histological analysis (hematoxylin and eosin [H&E] staining, 20x magnification [mag.]), analysis of fibrosis (Masson Trichrome, 20x mag.), iron accumulation (Perls Prussian blue, 63x mag.), and $CD68^+$ cell infiltration (anti-CD68 immunohistochemical staining, 20x mag.). Image depicts representative photographs of stained sections from each treatment group and comparison with kidney sections from hemizygous Berkeley control mice of the same age. Photographs are representative of 4 images per section (2-4 sections per mouse). Black arrows: congested vessels; yellow arrows: collagen deposits; yellow circles: areas of macrophage infiltration. Photomicrographs taken with a Zeiss Axio Imager D2.

mortality in patients during adulthood.[24] While early clinical trials in SCD patients showed modest success of P-selectin blocking immunotherapy for decreasing painful VOC,[8] and some reported success for treating SCD priapism,[25,26] its effectiveness for reducing organ damage is less clear. Studies in murine SCD models have shown that blocking P-selectin activity and neutrophil-platelet aggregate formation can resolve pulmonary arteriole microembolisms[9] and inhibit heme-induced acute chest syndrome development.[27] In the acute setting, we found that inhibition of P-selectin inhibited leukocyte rolling, but not adhesion, in the cremaster microcirculation of TNF-challenged Townes SCD mice, in association with inhibition of heterocellular aggregate formation in peripheral blood. Leukocyte rolling is a crucial initial step in the adhesion and recruitment of leukocytes to the endothelium, and inhibiting these mechanisms restored blood perfusion and flow velocity in the cutaneous microcirculation of these inflamed mice. Conversely, acute P-selectin blockade did not significantly improve the inflammatory profile of these mice. Furthermore, although anti-P-selectin therapy reduced VO processes in SCD mice, 6 weeks of anti-CD62P administration did not modify the inflammatory cytokine profile of Berkeley SCD mice. Notably, while some improvement in renal vessel congestion occurred, hepatocyte degeneration and hepatic injury in these mice were not mitigated by anti-P-selectin therapy. A previous study also reported that P-selectin deficiency in Townes SCD mice did not prevent progressive liver injury.[28]

Acute IL-1β neutralization also successfully restored cutaneous microvascular perfusion and blood flow in TNF-challenged Townes SCD mice. However, unlike the effects of P-selectin inhibition, this improved microvascular blood flow was associated with an inhibition of leukocyte rolling and adhesive interactions, without modulation of heterocellular

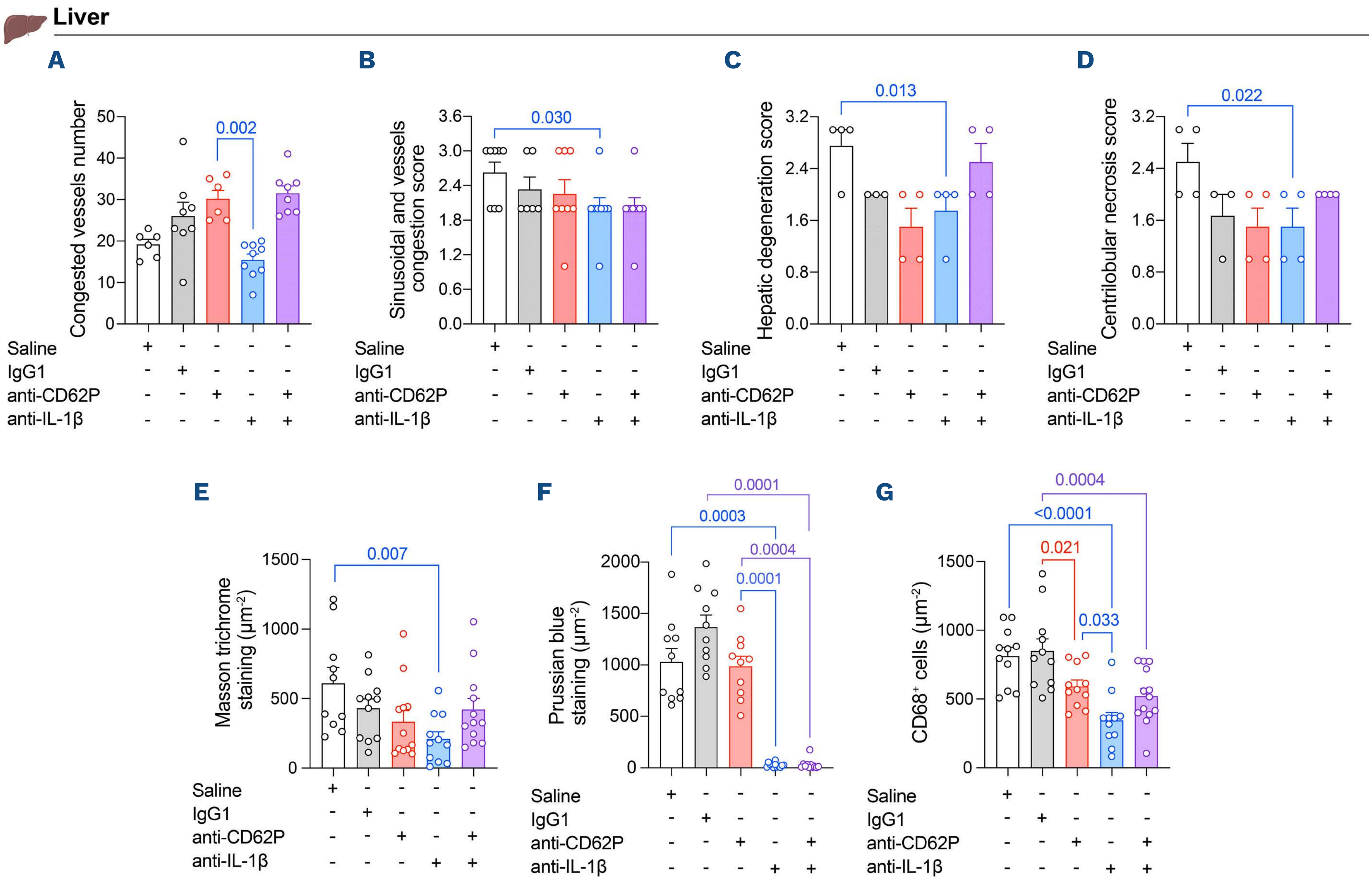

Figure 6. Morphometric analysis, histopathological evaluation and quantification of collagen, iron and macrophage infiltration in the livers of Berkeley sickle cell disease mice after 6 weeks of immunotherapies. Berkeley sickle cell disease (SCD) mice (4-months old) were treated, or not, with immunotherapies, as described in Figure 3A, for 6 weeks. At 48 hours (h) after administration of the final intervention dose, livers were dissected and processed for analysis in 2-4 histological slices per animal (N=4/group). The following analyses were performed in hematoxylin and eosin-stained sections: (A) morphometric analysis of the number of congested vessels (count μm^{-2}); (B) congestion in sinusoids and vessels; (C) hepatic degradation score; (D) centrilobular necrosis score. (E) Analysis of collagen fiber deposition (Masson Trichrome; AU staining intensity μm^{-2}). (F) Hemosiderin quantification (Perls Prussian blue staining; staining intensity μm^{-2}), and quantification of (G) monocyte/macrophage infiltration (anti-CD68 immunohistochemical staining; AU of staining intensity μm^{-2}).

aggregate formation. Importantly, acute IL-1β neutralization reduced circulating sICAM-1; endothelial ICAM-1 is critical for leukocyte anchoring and typically clusters on TNF-activated endothelium to enhance leukocyte recruitment efficiency.[29] Antibody blockade of IL-1β reduces the endothelial activation induced by chimeric antigen receptor (CAR) T, tumor cells, and myeloid cells,[30] and it may be postulated that acute IL-1β neutralization restores microvascular blood flow in TNF-challenged SCD mice by blocking endothelial activation. These data suggest that differing mechanistic approaches of inhibition may have similar resulting beneficial effects on occlusion in the microvasculature.

In contrast to the effects of P-selectin blockade, both acute and longer-term IL-1β neutralization ameliorated the inflammatory profile of SCD mice. Prolonged anti-IL-1β administration reduced serum concentrations of TNF-α and IL-6, both major players in molecular inflammatory responses that, along with IL-1β, contribute to liver and renal necrosis and subsequent organ fibrosis.[31-33] Advanced liver injury is implicated in up to 11% of deaths in individuals with SCD, and severe hepatopathy significantly affects SCD outcome.[34] While depletion of the leukocyte MAC-1 integrin was not previously associated with reduced organ damage in SCD mice,[35] neutralization of IL-1β abrogated hepatic injury in these mice. Anti-IL-1β therapy was also associated with a decrease in septal thickening in the lungs of SCD mice. In contrast, significant renal fibrosis was not apparent in any of the SCD mice at the age studied.

Regarding non-histological biomarkers of organ damage, ALT levels were elevated in SCD Berkeley mice, compared to hemizygote mice, accompanied by an increase in direct bilirubin levels, indicating liver dysfunction and hepatobiliary injury. Although blockade of neither P-selectin nor IL-1β improved these parameters, these data combined with histological

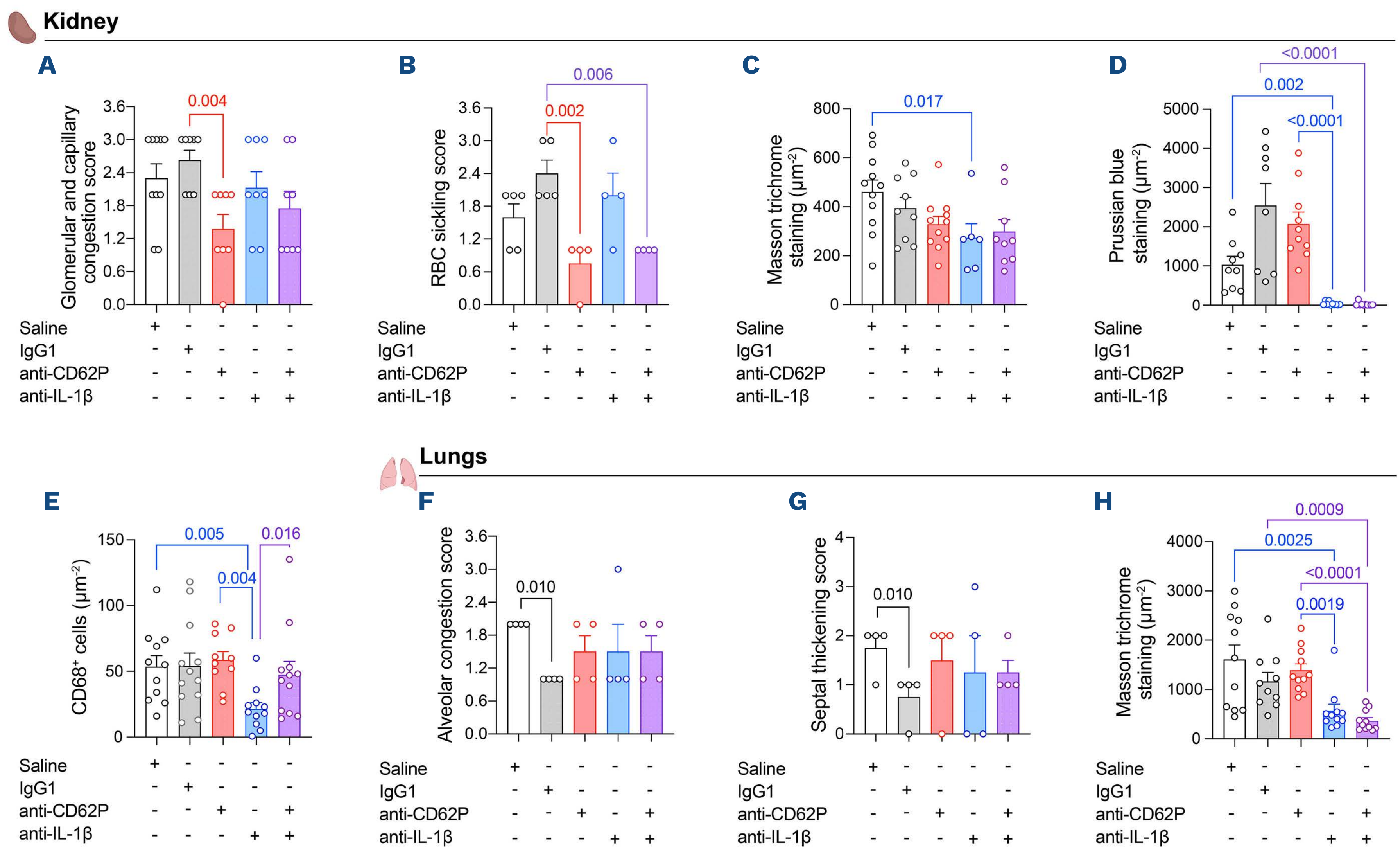

Figure 7. Histopathological evaluation and quantification of collagen, iron and macrophage infiltration in the kidneys and lungs of Berkeley sickle cell disease mice after 6 weeks of immunotherapies. Berkeley sickle cell disease (SCD) mice (4-months old) were treated, or not, with immunotherapies, as described in Figure 3A, for 6 weeks. At 48 hours (h) after administration of the final intervention dose, kidneys, and lungs were dissected and processed for analysis in 2-4 histological slices per animal (N=4/group). Kidneys: (A) congestion in glomerular vessels and capillaries; (B) red blood cell (RBC) sickling score. (C) Analysis of collagen fiber deposition (Masson Trichrome; AU staining intensity μm^{-2}). (D) Hemosiderin quantification (Perls Prussian blue; staining intensity μm^{-2}). (E) Analysis of monocyte/ macrophage infiltration (anti-CD68 immunohistochemical staining; AU of staining intensity μm^{-2}). Lungs, hematoxylin and eosin staining: (F) congestion in alveolar vessels; (G) septal thickening score. (H) Analysis of collagen fiber deposition, mainly in the interalveolar septa (Masson Trichrome; AU staining intensity μm^{-2}). Statistical comparisons are made between treatments and their mechanistic control, and between treatments.

observations suggest that, while not reversed, a slowing of organ damage progression occurred. Additionally, although anti-IL-1β therapy tended to improve hepatic ICAM-1 gene expression in SCD mice, no changes occurred in the expressions of genes associated with liver fibrosis or pathology.

Combining the two blocking immunotherapies in the acute protocol reduced microvascular leukocyte recruitment more effectively than either approach alone. This was accompanied by improved cutaneous blood perfusion following TNF challenge and reduced heterocellular aggregate formation. Curiously, the effect of IL-1β neutralization on reducing circulating sICAM-1, suggestive of a beneficial effect on endothelial activation, was abolished when combined with P-selectin inhibition. Furthermore, hepatopathy was exacerbated following prolonged combined immunotherapy, as demonstrated by histological and liver biomarker analysis, and the combination of therapies failed to diminish circulating IL-6. This surprising finding suggests a potential risk for combining these approaches for prolonged use. It is unclear why this hepatopathy occurs during combined therapy; however, the sustained circulation of damaging aged neutrophils,[36] due to their inability to extravasate to sites of inflammation, coupled with continued endothelial activation, may exacerbate hepatic injury.[37] Indeed, processes of sterile inflammation contribute significantly to tissue and organ damage in SCD.[38] A possible role for tissue macrophage accumulation in this hepatotoxicity is further discussed below.

The analyses of organ iron deposition, or hemosiderosis, in SCD mice treated with immunotherapies yielded remarkable findings. Mice treated with saline or the control IgG1 displayed significant and extensive hemosiderosis in the liver sinusoids and renal proximal tubules, with this iron accumulation primarily associated with macrophages. CD68⁺ immunostaining demonstrated augmented immune cell presence in tissues,

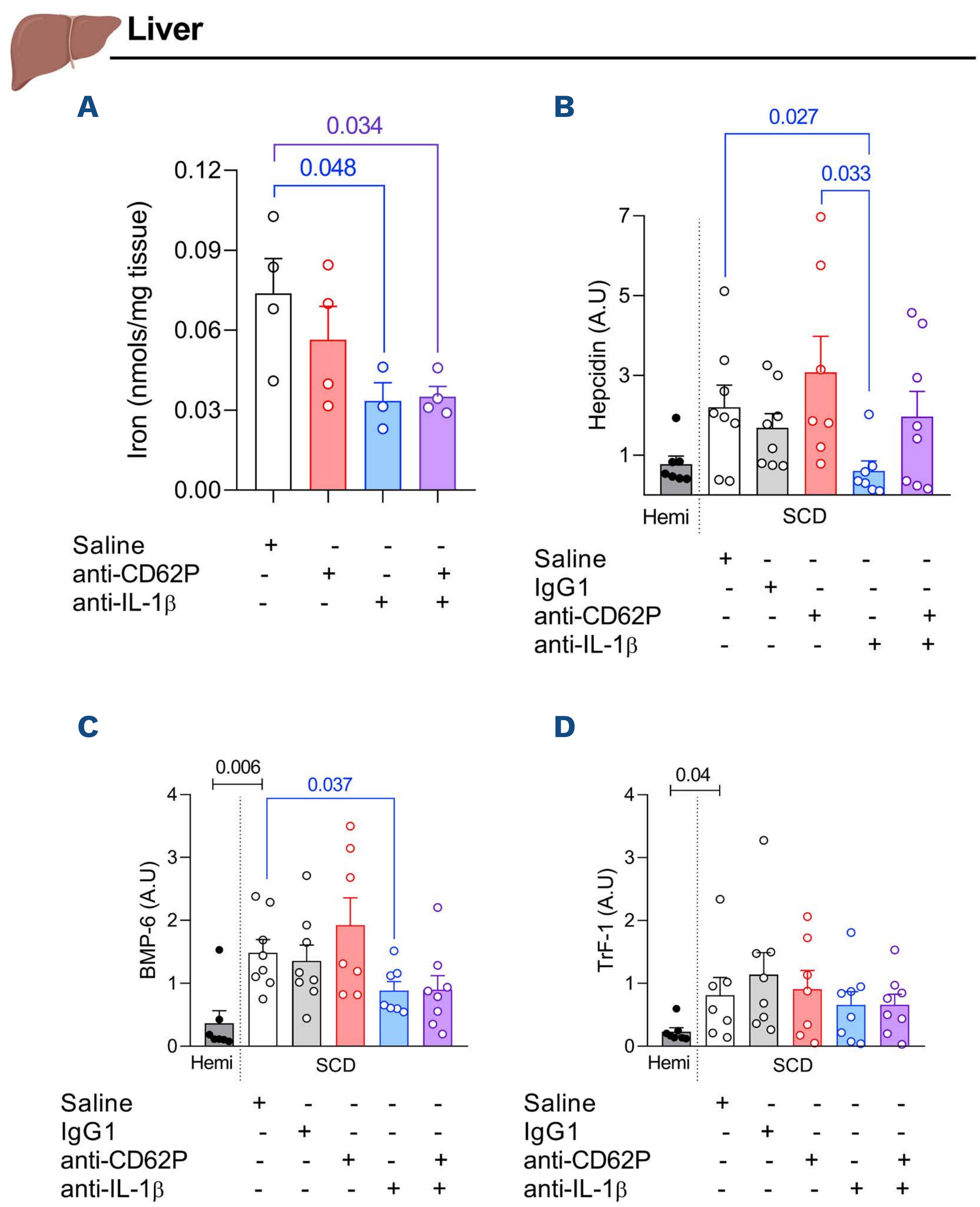

Figure 8. Effects of 6-weeks administration of anti-CD62P and anti-IL-1β immunotherapies on liver iron content and the gene expression of markers of iron regulation in livers from sickle cell disease mice. Berkeley sickle cell disease (SCD) mice (4-months old) were treated, or not, with immunotherapies, as described in Figure 3A, for 6 weeks. At 48 hours (h) after administration of the final intervention dose, livers were dissected and processed for iron measurement and analysis of gene expression by quantitative polymerase chain reaction (PCR). (A) Total and free iron in liver homogenates (nMols/mg tissue). (B-D) Organs from aged-matched hemizygous littermate mice (Hemi) were used for histopathological comparisons. Expressions of the genes encoding (B) hepcidin (*Hamp*), (C) bone morphogenetic protein-6 (BMP-6, *Bmp6*) and (D) transferrin receptor-1 (TrF-1, *Tfrc*) were normalized to the expression of *Actb* and *Gapdh*. Statistical comparisons are made between treatments and their mechanistic control, and between treatments.

where such leukocytes observed in the SCD liver have been previously described as infiltrated monocytes, or macrophages, or resident Küpffer cells.[28] Iron deposition (but not necessarily iron overload) in organs is a characteristic of SCD, even in the absence of regular transfusions,[39,40] and mice models of SCD replicate this condition.[41,42] Such iron accumulation contributes to oxidative stress and inflammation, particularly in the liver.[43,44] Besides reducing liver fibrosis, 6 weeks of IL-1β neutralization practically eliminated hepatic and renal iron accumulation. Iron quantification confirmed a significant decrease in liver iron content in these mice. Aside iron chelation and iron restriction therapies,[44,45] other therapeutic approaches reported to reduce liver iron deposition in SCD mice have demonstrated relatively discrete improvements.[46,47] The observed effect of IL-1β neutralization on hemosiderosis in SCD mice was associated with the suppression of hepatic gene expressions of hepcidin and BMP-6 (which regulates hepcidin expression). Hepcidin plays a pivotal role in regulating iron metabolism, and our observations are consistent with the correction of iron homeostasis and increased efflux (or downregulated iron uptake) by macrophages and hepatocytes.[48,49]

Although prolonged P-selectin blockade modestly reduced $CD68^+$ cells in the SCD liver, as previously shown after P-selectin deletion in SCD mice,[28] this was not associated with any decrease in organ iron deposition. Decreased macrophage infiltration in the organs of SCD mice treated with anti-IL-1β therapy may be reflective of a reduced necessity to recycle iron. However, while combined anti-CD62P/anti-IL-1β therapy also abolished iron deposition in SCD mice, the presence of renal and hepatic macrophages was greater ($P<0.05$) than in mice subjected to anti-IL-1β monotherapy. It may be that the persistent presence of such macrophages, which are presumably inflammatory in phenotype, in addition to continued endothelial activation and reduced P-selectin-mediated neutrophil recruitment, in the organs of combined therapy-treated SCD mice, contributes to the exacerbated tissue damage in the liver and the sustained inflammatory profile in these mice. Indeed production of IL-6, produced extensively by inflammatory macrophages, was sustained in these mice, as was hepatic hepcidin gene expression, possibly reflective of elevated IL-6.[50]

In conclusion, we investigated the impact of P-selectin and/or IL-1β blockade on vaso-occlusive processes and organ damage in SCD mice, with a summary of findings presented in *Online Supplementary Table S3*. While P-selectin blockade successfully reduced microvascular occlusion, restoring blood flow, its effect on organ injury was essentially limited to mitigating vessel congestion. It should be highlighted that no further progression of organ damage occurred during chronic anti-P-selectin monotherapy. IL-1β neutralization reduced vaso-occlusion and decreased hepatic injury by controlling inflammation and reducing organ iron accumulation. Unexpectedly, prolonged combined therapy exacerbated hepatic injury, possibly due to sustained inflammatory processes and altered macrophage activity, serving as a reminder that combination therapies should be carefully evaluated in preclinical models, and in clinical trials. Our findings emphasize the importance of targeted therapies that simultaneously address both vaso-occlusion and chronic inflammation to improve SCD outcomes. Anti-IL-1β immunotherapy warrants further clinical investigation of its potential to decrease inflammatory and organ damage biomarkers in SCD patients.

Disclosures

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Contributions

SA and NC conceived the study. EMFG, SA, LST, JK and NC designed the study. EMFG, LST, LFSG, PLM, and FCL performed experimental work. EMFG, LST, BCZ, CHP and FFC analyzed the data. All authors interpreted the data. EMFG, LST, BCZ, FFC and NC wrote the paper. All authors reviewed and contributed to the final manuscript.

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Data-sharing statement

Research datasets for the study are available upon reasonable request from NC.

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