Intravenous Immunoglobulins Modulate Neutrophil Activation and Vascular Injury Through FcγRIII and SHP-1

Jung-Eun Jang, Andrés Hidalgo, Paul S. Frenette

Rationale: Intravascular neutrophil recruitment and activation are key pathogenic factors that contribute to vascular injury. Intravenous immunoglobulin (IVIG) has been shown to have a beneficial effect in systemic inflammatory disorders; however, the mechanisms underlying IVIG’s inhibitory effect on neutrophil recruitment and activation are not understood.

Objective: We studied the mechanisms by which IVIG exerts protection from neutrophil-mediated acute vascular injury.

Methods and Results: We examined neutrophil behavior in response to IVIG in vivo, using real-time intravital microscopy. We found that an antibody that blocks both FcγRIII and its inhibitory receptor counterpart, FcγRIIB, abrogated the inhibitory effect of IVIG on leukocyte recruitment and heterotypic red blood cell (RBC) interactions with adherent leukocytes in wild-type mice. In the context of sickle cell disease, the blockade of both FcγRIIB and III abrogated the protective effect of IVIG on acute vaso-occlusive crisis caused by neutrophil recruitment and activation. Analysis of FcγRIIB- and FcγRIII-deficient mice revealed the predominant expression of FcγRIII on circulating neutrophils. FcγRIII mediated IVIG-triggered inhibition of leukocyte recruitment, circulating RBC capture, and enhanced Mac-1 activity, whereas FcγRIIB was dispensable. In addition, FcγRIII-induced IVIG anti-inflammatory activity in neutrophils was mediated by recruitment of Src homology 2 (SH2)-containing tyrosine phosphatase-1 (SHP-1). Indeed, the protective effect of IVIG on leukocyte recruitment and activation was abrogated in SHP-1-mutant mice.

Conclusions: FcγRIII, a classic activating receptor, has an unexpected inhibitory role on neutrophil adhesion and activation via recruitment of SHP-1 in response to IVIG. Our results identify SHP-1 as a therapeutic target in neutrophil-mediated vascular injury. (Circ Res. 2012;110:1057-1066.)

Key Words: neutrophils ■ vascular injury ■ FcγRIII ■ intravenous immunoglobulin ■ Src homology 2-containing tyrosine phosphatase-1 ■ endothelial cells ■ inflammation ■ adhesion molecules

Accumulation and recruitment of polymorphonuclear neutrophils are key pathogenic factors in the development of microvascular obstruction in cardiovascular disease, including sickle cell disease (SCD).1,2 Neutrophils are the major leukocyte subset recruited to inflamed venules, and the adherence of activated neutrophils to endothelial cells is a critical step that leads to reduction of the microcirculatory blood flow, ischemia, hypoxia, and tissue damage.3-6 Therefore, pharmacological approaches to inhibit neutrophil recruitment and activation represent important strategies to prevent vascular injury.

Intravenous immunoglobulin (IVIG) is a unique immune-modulating therapy that has a variety of effects on the immune system, depending on the underlying pathogenesis of given disease.7 The protective actions of IVIG in autoimmune diseases have been characterized, including modulation of immunoglobulin (Ig) Fc receptor expression, alteration of cytokine levels, complement inhibition, and modification of B-cell and T-cell functions.7,8 However, the molecular mechanisms by which IVIG exerts inhibition of neutrophil recruitment and activation in systemic acute inflammation remain to be understood. Direct observation of leukocyte recruitment by intravital microscopy has revealed that IVIG inhibits selectin-mediated leukocyte rolling and β2 integrin–dependent leukocyte adhesion to endothelium.9-12 In SCD mice, IVIG reverses acute vaso-occlusive crisis (VOC) by inhibit-
ing neutrophil adhesion to the endothelium and abrogating the direct interactions between adherent leukocytes and circulating red blood cells (RBCs).5,11

Fcy receptors (FcyRs) for IgG are expressed on a wide variety of hematopoietic cells, linking cellular and humoral immunity. The family of FcyRs has been categorized into 2 different classes: the activating (FcyRI, FcyRIII, and FcyRII) and inhibitory (FcyRIIB) receptors. Engagement of activating FcyRs associated with the common γ-chain triggers effector cell responses, such as antibody-dependent cell-mediated cytotoxicity, phagocytosis, reactive oxygen production, and release of inflammatory mediators, whereas the inhibitory FcyRIIB mediates the inhibition of activating FcyR-induced signal cascade.12 During inflammation, FcyRs play important roles in leukocyte recruitment and activation. FcyRIII mediates neutrophil tethering and adhesion in response to immune complexes in autoimmune disease.14,15 β2 integrins, particularly Mac-1, cooperate with FcyRs to sustain neutrophil adhesion.16 In addition, the common γ-chain containing immunoreceptor tyrosine-based activation motifs (ITAMs) is involved in the initial signaling events that are required to initiate E-selectin-mediated neutrophil slow rolling and outside-in signaling through β2-integrins in neutrophils.17,18 However, the mechanisms by which IVIG engagement to FcyRs modulates intravascular neutrophil recruitment and activation in the context of inflammation are incompletely defined.

We elucidate the mechanism by which IVIG exerts inhibition of intravascular accumulation and activation of neutrophils to localized inflamed area in vivo using real-time intravital microscopy. We show that engagement of IVIG to activating FcyRIII but not the inhibitory FcyRIIB inhibits leukocyte recruitment, abrogates heterotypic adherent leukocyte-RBC interactions, and reduces Mac-1 activity. In addition, we identify the protein tyrosine phosphatase SHP-1 as a critical downstream mediator involved in the FcyRIII-mediated inhibitory effects of IVIG on leukocyte recruitment and activation.

Methods
An expanded Methods section is available in the online-only Data Supplement.

Mice
Berkeley SCD mice [Tg(Hu-miniLcrat1α^y8β8) Hba^-/- Hbb^-/-] have been previously described.19 Fcgr2b^-/-20 and Fcgr3^-/-21 mice, generated by gene targeting, were purchased from The Jackson Laboratory (Bar Harbor, ME). Homozygous mice for the motheaten viable (me') mutation were established by mating C57BL/6-J-Ptn6me^-/- (+/me') heterozygous breeding pairs obtained from The Jackson Laboratory. Additional details of fully chimeric SCD and me'/me" mice refer to the online supplemental materials. All experimental procedures performed on mice were approved by the Animal Care and Use Committee of Mount Sinai School of Medicine and Albert Einstein College of Medicine.

Intravital Microscopy and Image Analyses
The cremasteric muscle was prepared as described in the Online Data Supplement Methods. Either IVIG (800 mg/kg) or an equivalent volume of control human albumin was intravenously infused by programmable syringe pump (PHD 4400, Harvard Apparatus, Holliston, MA) at the rate of 667 µL/kg per minute 3 hours after intrascrotal injection of 0.5 µg tumor necrosis factor (TNF)-α. Then, 20 minutes after IVIG or control albumin exposure, 8 to 12 venules were videotaped over a period of 60 minutes, with each venule recorded continuously for at least 2 minutes. To block endogenous FcγRIIB/II, we injected 1 mg/kg i.v. anti-FcγRIIB/II or control isotype rat IgG2b, before administration of either IVIG or control albumin. Bright-field intravital microscopy was performed, using video recordings, and all data were analyzed by playback assessment of video-captures as described in the Online Data Supplement Methods.

Hemodynamic Measurements
Arteriolar and venular diameter was measured with a video caliper before and after administration of either IVIG or control albumin. Centerline red cell velocities (V_RBC) were determined for each venule in real-time, using an optical Doppler velocimeter (Texas A&M, College Station, TX). Wall shear rate and blood flow rate were calculated as described in the Online Data Supplement Methods.

In Vivo Analysis of Mac-1 Activity
Albumin-coated fluospheres were intravenously injected into mice prepared for intravital microscopy as described in detail in the Online Data Supplement Methods. Images were captured for at least 30 seconds in the bright-field and FITC (for yellow-green fluospheres) channels and analyzed them with SlideBook software (Intelligent Imaging Innovations). Adherent leukocytes were visually identified in the bright-field channel and the number of fluospheres associated to each leukocyte was counted. The average number of albumin-coated fluospheres bound to adherent intravascular leukocytes in a given 100-µm-long venular segment was used as a measure of Mac-1 activity and was obtained from the formula: fluospheres/white blood cells (WBC)=total number of leukocyte-associated beads per venular segment/number of adherent leukocytes per venular segment, as previously described.22

Flow Cytometry Analyses
Blood samples were collected into sterile tubes containing 2 mmol/L ethylenediaminetetraacetic acid (EDTA) and lysed in 0.8% NH4Cl buffer and the remaining nucleated cells were washed twice in PBS containing 2 mmol/L EDTA and 0.5% BSA (PEB buffer). Primary blood leukocytes were stained by incubation with fluorescently labeled or biotinylated antibodies specific to mouse or corresponding with isotype controls. Biotinylated monoclonal antibody (mAb) was detected by incubation with Cy5-conjugated streptavidin (Jackson ImmunoResearch Laboratories). Stained samples were acquired with a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA) and an LSRII (BD) and then analyzed with FlowJo software (Tree Star, Inc). Leukocytes and neutrophils were gated on the basis of low forward-scatter and high side-scatter characteristics.
Immunoprecipitation and Western Blotting
Proteins were extracted from bone marrow neutrophils isolated from WT and FcγR2b−/− mice, and the association of SHP-1 with FcγRIII was analyzed by immunoprecipitation and Western blotting as described in the Online Data Supplement Methods.

Statistical Analyses
All data are presented as mean±SEM and analyzed using unpaired, 2-tailed Student t test or nonparametric Mann-Whitney U test, as appropriate. A probability value of P<0.05 was considered statistically significant.

Results
Low-Affinity Fcγ Receptors Alter Intravascular Cell Interactions in Wild-Type Animals
Low-affinity activating FcγRIII and its inhibitory counterpart, FcγRIIB, have been suggested to be involved in IVIG-mediated immunomodulation in diverse murine models of autoantibody-triggered immune diseases.23−25 We thus investigated whether FcγRIIB/III were required for IVIG’s suppressive effects on leukocyte recruitment in systemic acute inflammation in wild-type (WT) mice. To test this, either mAb against FcγRIIB/III or control isotype rat IgG2b (1 mg/kg) followed by IVIG or control albumin administration (800 mg/kg) 3 hours after administration of TNF-α (0.5 μg). Leukocyte behaviors were analyzed in cremasteric venules for 1 hour. Blood from WT mice was collected after an intravital microscopy experiment, and surface expression of FcγRII/III on the neutrophil population gated on the basis of side- and forward-scatter properties was examined by flow cytometry after PE-conjugated anti-FcγRIII staining. Numbers of circulating leukocytes. Percentages of monocytes, neutrophils and lymphocytes. Adherent leukocytes in venules. Number of circulating RBC-adherent leukocyte interactions per minute. Bars represent mean±SEM. **P<0.01, ***P<0.001 versus albumin.

The microvascular obstruction is a complex multicellular process involving endothelial activation, leukocyte adhesion to endothelium, and the direct interaction of circulating RBCs and adherent leukocytes (WBCs).26 In previous studies, we demonstrated that interactions between adherent leukocytes
and RBCs carrying normal hemoglobin (nRBC) occurred in inflamed venules under relatively low shear rates (<500 s⁻¹). We thus examined whether IVIG infusion could inhibit the interactions between adherent leukocytes and nRBCs. IVIG administration significantly reduced heterotypic interactions between adherent leukocytes and circulating nRBC interactions (67% reduction, 0.05 ± 0.02 RBC-WBC interactions/min; P < 0.001), whereas FcyRIIB/III blockade abrogated its inhibitory action on heterotypic interactions (0.16 ± 0.02 versus 0.15 ± 0.02 RBC-WBC interactions/min) under similar hemodynamic conditions (Figure 1D and Online Table II). These results demonstrate a clear requirement for FcyRIIB/III for the protective actions of IVIG on leukocyte recruitment and activation during acute inflammation, suggesting that IVIG might modulate their expression or function.

**FcyRIII But Not FcyRIIB Is a Major Fcy Receptor on Neutrophils**

In previous studies, we found that IVIG specifically targets the recruitment of neutrophils, which make up the majority of adherent leukocytes in inflamed venules. We therefore examined the cell surface expression of both FcyRIIB and FcyRIII on circulating neutrophils using myeloid lineage-specific markers (ie, Gr-1, CD115, and F4/80). Gr-1⁺ and CD115⁺ neutrophils were labeled by a dual-specific antibody to FcyRIIB/III, but barely by one directed at FcyRIIB, compared with expression levels of positive CD115⁺ monocyte and negative CD3⁺ T-cell controls (Figure 2A). Because the high degree of homology between FcyRIIB and FcyRIII has prevented the generation of specific antibodies against individual low-affinity Fcy receptors, we used FcyRIII-deficient (Fcgri⁻/⁻) mice to examine further the surface expression of FcyRIIB on neutrophils. In Gr-1⁺/CD115⁺ neutrophils, the binding of the antibody recognizing FcyRIIB/III in Fcgri⁻/⁻ mice was virtually undetectable, compared with WT mice (Figure 2B and Online Figure II), suggesting that mouse neutrophils constitutively express FcyRII but little or no FcyRIIB in the steady state.

It has been proposed that the benefits of IVIG may result from the induction of expression of inhibitory FcyRIIB on tissue-infiltrating macrophages, thereby raising the threshold required for triggering activating FcyRs in autoimmune disease. Thus, we sought to determine whether low affinity FcyRIIB/III expression on neutrophils is changed after IVIG administration. We found that the expression levels of FcyRIIB/III on neutrophils did not change 1 hour after administration of IVIG or control albumin into control WT, FcyRIIB-deficient (Fcgri⁻/⁻), and Fcgri⁻/⁻ mice (Figure 2C). These results indicate that IVIG administration does not induce inhibitory FcyRIIB expression on neutrophils and that the activating FcyRIII is a major receptor in murine neutrophils.

**IVIG Inhibition of Leukocyte Recruitment and Activation Is Mediated by FcyRIII**

In mice, both inhibitory FcyRIIB and activating FcyRIII are important for IVIG-mediated amelioration of inflammation. To determine the differential contribution of FcyRIIB and FcyRIII to IVIG-mediated protection, we assessed leukocyte recruitment and heterotypic interactions between adherent leukocytes and circulating RBCs after...
IVIG or control albumin administration in the context of systemic acute inflammation in control WT, Fcgr2b−/−, or Fcgr3−/− mice. As compared with control albumin, IVIG administration significantly reduced leukocyte adhesion to endothelium by approximately 30% to 40% in both control WT (1108 ± 88 versus 1439 ± 117 adherent WBCs/mm², *P < 0.05) and Fcgr2b−/− mice (890 ± 84 versus 1523 ± 112 adherent WBCs/mm², **P < 0.01). Strikingly, its inhibitory effect on leukocyte recruitment was absent in Fcgr3−/− mice (1201 ± 87 versus 1145 ± 51 adherent WBCs/mm²; Figure 3A and Online Table III). Furthermore, IVIG administration significantly reduced RBC interactions with adherent leukocytes by 50% under relatively low shear rates (<500 s⁻¹) in both control WT (0.12 ± 0.02 versus 0.23 ± 0.03, RBC-WBC interactions/min; *P < 0.01) and Fcgr2b−/− (0.10 ± 0.02 versus 0.19 ± 0.03 RBC-WBC interactions/min; **P < 0.05) mice but not Fcgr3−/− (0.30 ± 0.04 versus 0.26 ± 0.05, RBC-WBC interactions/min) mice compared with control albumin administration (Figure 3B and Online Table IV).

Activated Mac-1 integrin microdomains at the leading edge of neutrophils, a phenomenon that is triggered by E-selectin expressed on the inflamed endothelium, drive vascular damage in SCD through heterotypic interactions. Twenty-two We thus assessed the effect of IVIG on Mac-1 activity in adherent leukocytes using a previously validated in vivo fluosphere bead-binding assay. Twenty-two This specifically assay measure Mac-1 activity on adherent neutrophils, since albumin-coated fluospheres captured by adherent Gr-1pos F4/80neg neutrophils have been shown to be absent in Mac-1-deficient (Igcam−/−) mice. Twenty-two Overlay of images acquired in bright-field and fluorescence channels revealed that the capture of albumin-coated fluorescent beads by adherent leukocytes was strongly inhibited in IVIG-infused mice (Figure 3C). In parallel with the reduction in heterotypic interactions with RBCs seen after IVIG treatment, IVIG administration significantly reduced Mac-1 activity in leukocytes compared with control albumin administration in both control WT and Fcgr2b−/− mice. However, IVIG-induced reduction of Mac-1 activity was abrogated in Fcgr3−/− mice (Figure 3D). Taken together, these results indicate that FcγRIII mediates IVIG-induced inhibition of leukocyte adhesion to endothelium, heterotypic RBC interactions with adherent leukocytes, and Mac-1 integrin activation.

**Involvement of SHP-1 in the Inhibitory Effect of IVIG on Leukocyte Recruitment**

Previous studies have provided evidence that activating FcγRs associated with ITAMs can induce inhibitory signaling mediated by recruitment of Src homology 2 (SH2)-containing tyrosine phosphatase-1 (SHP-1). Twenty-eight Twenty-nine The tyrosine phosphatase SHP-1 can act as a negative regulator that dephosphorylates multiple immunoreceptor-regulated substrates, leading to cell inactivation. Thirty We hypothesized that IVIG induces the recruitment of SHP-1 to FcγRIII in neutrophils, thereby inactivating ITAM-mediated activating signal cascade through dephosphorylation. To assess whether SHP-1 is recruited to FcγRIII in response to IVIG, bone marrow neutrophils isolated from control WT mice were incubated with either IVIG or control albumin and then analyzed the association of SHP-1 with FcγRIII using bone marrow neutrophils. IVIG treatment induced the recruitment of SHP-1 to FcγRIII in neutrophils from WT mice (Figure 4A). In addition, IVIG-induced SHP-1 recruitment to
FcyRIIB/III was also observed in neutrophils from Fcgr2−/− mice (Online Figure III). This indicates SHP-1 associates with FcγRIII in response to IVIG, suggesting that SHP-1 may be involved in FcγRIII-mediated inhibitory pathways induced by IVIG.

We next gauged the role of SHP-1 in IVIG’s anti-inflammatory effect on leukocyte recruitment and activation by assessing motheaten viable (me/me) mice that have reduced phosphatase activity due to a mutation in SHPTP1.31 Neutrophils from me/me mice show increased oxidative production, surface expression of β2 integrins, and leukocyte adhesion in vitro,31 suggesting a significant role for SHP-1 in modulating the tyrosine phosphorylation signaling pathways that regulate neutrophil activation. To analyze the contribution of SHP-1 in the hematopoietic lineage, we generated chimeras by transplantation of WT and me/me bone marrow donors into WT recipients (Online Figure IV, A and B). In contrast to WT chimeras, administration of IVIG in me/me bone marrow chimera did not alter leukocyte recruitment, RBC interactions with adherent leukocytes, and Mac-1 activity (Figure 4B through 4D and Online Table V and VI). Taken together, these results suggest that IVIG mediates its inhibitory effect on leukocyte recruitment and activation via recruitment of SHP-1 to FcγRIII.

**FcγRIII Mediates the Protective Effect of IVIG Against Acute VOC**

Acute VOC is the most common complication of SCD and is a major cause of morbidity and mortality for SCD patients.33 Previous studies have established that IVIG can reverse acute VOC in a humanized murine model of SCD via specific inhibition of neutrophil recruitment and their interactions with circulating RBCs.9,11 We thus tested whether engagement of the low-affinity receptors FcγRIIB/III mediates the protective effect of IVIG on acute VOC in SCD similar to systemic acute inflammation in WT mice. We injected mice with a mAb against FcγRIIB/III or control isotype rat IgG2b, before IVIG or control human albumin administration in TNF-α–treated SCD mice (Figure 5A). Injection of 1 mg/kg FcγRIIB/III mAb was sufficient to saturate endogenous FcγRIIB/III antigens on neutrophils by >96%, compared with control IgG2b injection (Figure 5B). Consistent with previous studies,9,11 IVIG administration significantly reduced leukocyte adhesion to endothelium (~30% reduction, 1303±96 versus 1961±123 adherent WBCs/mm²; P<0.01) and heterotypic adherent leukocyte-RBC interactions (>98% reduction, 0.01±0.001 versus 0.80±0.3 RBC-WBC interactions/min; P<0.001) in control IgG2b–treated SCD mice (Figure 5C and D). Although IVIG administration did not cause vasodilatory response in both arteries and veins, its infusion significantly improved blood flow rates, a surrogate measure for vaso-occlusion, compared with the albumin-infused control group (P<0.01, Figure 5E and Online Figure V, A and B), as well as the mean centerline RBC velocity (V_{RBC}) and wall shear rate in venules (P<0.01, respectively; Table). Importantly, blockade of endogenous FcγRIIB/III abrogated IVIG’s alteration of leukocyte adhesion to endothelium (2100±134 versus 2026±181 adherent WBCs/mm²), heterotypic adherent leukocyte-RBC interactions (0.26±0.06 versus 0.28±0.10 RBC-WBC interactions/min), and blood flow rates (271±16 versus 265±15 mL/s) (Figure 5C through 5E). Furthermore, IVIG-induced inhibitory effect on leuko-
cyte recruitment led to reduced sickling, resulting in protection from intermittent vaso-occlusion in postcapillary venules, whereas FcγRIIB/III blockade abrogated IVIG’s suppressive effect on acute vaso-occlusion (Figure 5F and Online Movies I to IV). In addition, IVIG significantly prolonged the survival time of SCD mice compared with the albumin-infused control group (P < 0.05, log-rank test). Taken together, endogenous FcγRIIB/III blockade negated the protective effect of IVIG on acute VOC in SCD mice, thus demonstrating that the beneficial effects of IVIG during neutrophil-mediated acute injury result from the interaction with, and signaling through, FcγRIII.

Discussion

IVIG is an effective therapeutic agent in a variety of autoimmune diseases or chronic inflammatory disorders. The mechanisms of IVIG action have been most thoroughly investigated in autoantibody-mediated diseases, but the exact mechanism by which IVIG prevents leukocyte recruitment and activation during acute vascular injury remains poorly understood. Using intravital microscopy, we have directly studied the roles of IVIG in intravascular neutrophil recruitment and activation in TNF-α–induced acute inflammation. Our data demonstrate that IVIG inhibits leukocyte adhesion to endothelium and heterotypic RBC interactions with adherent leukocytes by reducing Mac-1 activity on neutrophils. In addition, our studies identify FcγRIII as the FcγR required for mediating IVIG’s anti-inflammatory activity. Engagement of IVIG to FcγRIII induces recruitment of the protein tyrosine phosphatase, SHP-1, which subsequently inhibits leukocyte recruitment and activation (Online Figure VI).

The anti-inflammatory activity of high-dose IVIG can be attributed to the minor fraction of dimeric or sialylated IgG in

Figure 5. Blockade of FcγRIIB and III abrogates the protective actions of IVIG against acute vaso-occlusion in SCD mice. A, Experimental scheme. Three hours after administration of TNF-α (0.5 μg), SCD mice (n=8 per group) were injected with either anti-FcγRIIB/III monoclonal antibody (mAb) or isotype rat IgG2b, (1 mg/kg) before administration of IVIG or an equivalent volume of human albumin (800 mg/kg). B, Blood from SCD mice was collected after an intravital experiment, and surface expression of FcγRIIB/III on the neutrophils was examined with PE-conjugated anti-FcγRIIB/III mAb by flow cytometry. C, Adherent leukocytes in venules. D, Number of circulating sickle RBC-adherent leukocyte interactions per minute. E, Blood flow rates. Bars represent mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 versus albumin. F, Representative images of each group after IVIG or control albumin administration showing leukocyte recruitment and heterotypic interactions. Scale bars, 20 μm. G, Kaplan-Meier survival curves for individual SCD mice. P = 0.03, log-rank test, IVIG versus albumin in IgG2b-treated group; P = 0.93, log-rank test, IVIG versus albumin in anti-FcγRIIB/III mAb-treated group.
a murine model of immune thrombocytopenic purpura that macrophages are responsible for the clearance of autoantibody-coated platelets.23,34,35 These studies have suggested that IVIG increases expression of the inhibitory receptor FcγRIIB on the surface of inflammatory macrophages, thereby resulting in suppression of autoantibody-triggered inflammation.23,34 Thus, macrophages containing FcγRIIB in autologous-meditated disorders are the effector cells in the anti-inflammatory response mediated by IVIG. Because neutrophils represent the vast majority of adherent leukocytes in inflamed venules and are specifically targeted by IVIG in SCD,9 we have examined the surface expression of FcγRIIB/III on IVIG-treated neutrophils. Our results show that neutrophils predominantly express FcγRIII but not FcγRIIB in the steady state. Interestingly, IVIG administration did not alter the surface expression of either inhibitory FcγRIIB or activating FcγRIII on neutrophils. Based on our data and previous studies, IVIG may therefore differentially act on distinct effector cells or potentially affect various leukocyte subsets through different mechanisms of action.

Neutrophil recruitment during inflammation is classically attributed to a multistep cascade involving selectin-mediated initial tethering and rolling along the vessel wall, followed by β2 integrin–mediated firm adhesion to the vascular endothelium.37 During leukocyte recruitment, IVIG modulates these adhesion molecules, including P-selectin (PSGL-1), Mac-1 (αMβ2), and LFA-1 (αβ2) on leukocytes.10,38 Moreover, analyses of leukocyte recruitment by intravital microscopy have also revealed that IVIG can inhibit P-selectin–dependent leukocyte rolling, E-selectin–mediated slow rolling, and β2 integrin–dependent leukocyte adhesion to the endothelium in vivo suggesting that IVIG directly targets leukocytes.9–11 The receptors and signaling pathways potentially involved in this direct inhibitory effect remain poorly understood. We found that blockade of endogenous FcγRIIB/III abrogated IVIG’s anti-inflammatory activity during leukocyte recruitment. In addition, the inhibitory effects of IVIG on leukocyte recruitment and activation were abolished in Fcgr3−/− mice, indicating the requirement of FcγRII to mediate IVIG’s anti-inflammatory activity during leukocyte recruitment.

Activating FcγRIII is associated with the common γ-chain, which contains an ITAM motif. Although ITAMs are used by multiple receptors to activate immune cells, recent studies have suggested that ITAMs can paradoxically function to propagate inhibitory signals under specific conditions.39 ITAM-mediated inhibition downstream of activating FcγRs can occur when a low-affinity ligand favors the recruitment of a signaling effector such as protein tyrosine phosphatase SHP-1 with inhibitory potential instead of activation of signal-promoting kinases.28,29 In agreement with this contention, our data show that the protein tyrosine phosphatase SHP-1 is recruited to FcγRIII in IVIG-treated neutrophils compared with either resting or albumin-treated neutrophils, suggesting that IVIG induces recruitment of SHP-1 to FcγRIII in neutrophils.

IVIG significantly increases leukocyte rolling velocities, suggesting that it alters adhesion pathways involving E-selectin.9 Interestingly, E-selectin engagement cannot induce LFA-1–dependent slow rolling in the absence of ITAM-associated with immunoreceptor in leukocytes.40 E-selectin–mediated intracellular signaling pathways in neutrophils indeed exhibit strong similarities to that of FcγR or β2 integrin–mediated outside-in signaling. Binding of neutrophils to E-selectin on inflamed endothelium activates Src family kinases, which in turn activates ITAM-dependent pathways, such as spleen tyrosine kinase, phosphoinositide-3-kinase, and p38 mitogen-activated protein kinase, resulting in LFA-1–dependent slow rolling and Mac-1 activation at the leading edge of adherent neutrophils.18,22,40–42 Since protein tyrosine phosphatases such as SHP-1 can switch off ITAM-induced activating signaling cascades,40 our results suggest the possibility that the recruitment of SHP-1 in response to IVIG inactivates ITAM-mediated signals, including E-selectin– and FcγRIII-induced pathways. This results in alteration in neutrophil responses triggered by these receptors: Mac-1 activation, reduced β2 integrin–dependent neutrophil arrest, and reduced Mac-1–dependent RBC interactions. Therefore, signaling pathways controlled by SHP-1 phosphatase may become therapeutic targets to control neutrophil functions in inflammatory disease.

Of particular relevance, we assessed the contribution of FcγRs in IVIG-induced protection from VOC in the context of SCD. Consistent with the central role of integrin activation and RBC capture by neutrophils during vascular occlusion, blockade of FcγRIIB/III before IVIG delivery prevented all the beneficial effects associated with this treatment. In addition, IVIG inhibits ROS-producing neutrophils in a experimental model of transfusion-related acute lung injury, in which mechanisms similar to those leading to vaso-occlusion promote vascular and organ injury (data not shown).32 These results suggest that other types of vascular disease could also be benefit from the insights on IVIG’s mode of action reported herein. For example, neutrophils have been recently shown to participate in chronic arterial disease, including atherosclerosis43 or restenosis.44 One could envision that prolonged neutrophil activation, through mechanisms partly shared with the acute processes described here, contributes to vascular injury in large vessels.

Table. Hemodynamic Parameters After IVIG or Control Albumin Administration Following Injection of Anti-FcγRIIB/III mAb or IgG2b Control in TNF-α–Treated SCD Mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mice n</th>
<th>Venule, μm</th>
<th>Venular Diameter, μm</th>
<th>Venular Velocity, mm/s</th>
<th>Centerline Velocity, mm/s</th>
<th>Shear Rate, s⁻¹</th>
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<td>IgG2b</td>
<td>6</td>
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<td>22±0.6</td>
<td>1.3±0.1</td>
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<tr>
<td>IVIG</td>
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<td>76</td>
<td>20±0.2</td>
<td>1.9±0.1*</td>
<td>990±60*</td>
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<td>Anti-FcγRIIB/III</td>
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<tr>
<td>Albumin</td>
<td>7</td>
<td>61</td>
<td>21±0.2</td>
<td>1.2±0.1</td>
<td>622±27</td>
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<tr>
<td>IVIG</td>
<td>8</td>
<td>56</td>
<td>21±0.3</td>
<td>1.2±0.1</td>
<td>601±44</td>
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Hemodynamic parameters were analyzed from intravital microscopy recording of venules used for the results shown in Figure 5C and 5D. Data are presented as mean±SEM.

*P<0.01 versus albumin.
Neutrophil FcyRIII Mediates IVIG Activity

In summary, this study reveals an inhibitory role for FcyRIII in response to IVIG, resulting in recruitment of SHP-1 and preventing neutrophil adhesion and activation in areas of vascular injury. Therefore, elucidating the target signals affected by IVIG-induced SHP-1 recruitment will provide novel insights for designing therapeutic strategies that prevent vascular disease.

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Disclosures
None.

References

**Novelty and Significance**

**What Is Known?**
- Immunoglobulin G (IgG) are potent immune modulators acting through Fc receptors (Fc-γR).
- Intravenous immunoglobulins (IVIG) are widely used to treat a variety of autoimmune disease and inflammatory disorders.
- IVIG inhibits neutrophil recruitment that mediates vascular damage and occlusion during sickle cell disease crises.

**What New Information Does This Article Contribute?**
- Activating Fc-γRIII, rather than the classical inhibitory Fc-γRIIB, on neutrophils mediates IVIG-induced inhibition of neutrophil recruitment and activation.
- Src homology 2 (SH2)-containing tyrosine phosphatase-1 (SHP-1) is a critical downstream mediator involved in Fc-γRIII-mediated IVIG’s anti-inflammatory activity.
- SHP-1 is a potential therapeutic target in neutrophil-mediated vascular injury.

The recruitment and activation of neutrophils in the microvasculature are key pathogenic factors that contribute to vascular injury, including vaso-occlusive episodes in sickle cell disease. IVIG treatment exerts anti-inflammatory activity by reducing neutrophil recruitment, but the mechanisms underlying its modulation on neutrophil function remain unclear. Understanding the mechanisms by which IVIG dampens neutrophil recruitment and activation will provide valuable insights for designing therapeutic strategies aimed at preventing neutrophil-mediated vasculopathies. We show that engagement of IVIG to activating Fc-γRIII but not the inhibitory Fc-γRIIB inhibited neutrophil recruitment and activation. Furthermore, the Fc-γRIII-induced IVIG anti-inflammatory activity in neutrophils was mediated by recruitment of protein tyrosine phosphatase SHP-1. This study demonstrates an unexpected inhibitory role of Fc-γRIII on neutrophil adhesion and activation in response to IVIG and implicates SHP-1 in the therapeutic efficacy of IVIG. Thus, elucidating the target signals affected by IVIG-induced SHP-1 recruitment will provide novel insight for designing therapeutic strategies to prevent vascular disease.
Intravenous Immunoglobulins Modulate Neutrophil Activation and Vascular Injury Through Fc γRIII and SHP-1

Jung-Eun Jang, Andrés Hidalgo and Paul S. Frenette

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Supplemental Materials

Intravenous immunoglobulins modulate neutrophil activation and vascular injury through FcγRIII and SHP-1

Jung-Eun Jang, MS; Andrés Hidalgo, PhD; Paul S. Frenette, MD

From Department of Medicine, Mount Sinai School of Medicine, New York, NY, USA
Ruth L. and David S. Gottesman Institute for Stem Cell and Regenerative Medicine Research, Albert Einstein College of Medicine, Bronx, NY, USA (J-E.J., P.S.F.), and Department of Epidemiology, Atherothrombosis and Imaging, Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain (A.H.).

Supplementary Methods

Mice
Chimeric SCD mice were generated by transplanting ~3 X 10^6 bone marrow nucleated cells from Berkeley SCD mice into lethally irradiated C57BL/6 animals to generate genetically identical age- and gender-matched cohorts of SCD mice. Fully chimeric male SCD mice (expressing >97% human globin, including βS) were subjected to intravital microscopy 3-5 months after bone marrow transplantation, as previously described.1 meν/meν and control WT chimeras were generated by transplanting 5 X 10^6 donor bone marrow cells from meν/meν homozygous or C57BL/6 (CD45.2) mice into lethally irradiated 8-week-old C57BL/6 Ly5.2 (CD45.1) male mice, respectively. Blood of meν/meν and WT chimera showed >95% donor chimerism as assessed by flow cytometry 3 weeks after transplantation. C57BL/6 and C57BL/6 Ly5.2 were obtained from the National Cancer Institute. All mice were genotyped using PCR amplification of tail DNA.
Reagents and antibodies

Human γ-globulin (IVIG; 10% caprylate/chromatography purified Gammunex) was purchased from Talecris Biotherapeutics (Clayton, NC) and human albumin (5% and 25% Buminate) from Baxter health care corporation. Gr-1 (Ly6G/C; clone RB6-8C5), CD115 (clone AFS98) and control isotype (IgG2b,κ) were obtained from eBioscience and F4/80 (clone Cl:A3.1) from AbD Serotec, FcγRII/III (clone 2.4G2) from BD Pharmingen. FcγRIIB (anti-Ly17.2) was a gift from Dr. Jeffrey Ravetch. The anti-MHC-I antibodies directed at the H2d (clone 34-1-2s; mouse IgG2a, κ) haplotypes were purified from hybridoma supernatants (ATCC).

Intravital microscopy (IVM)

Wild-type and SCD mice were anesthetized by intraperitoneal (i.p.) injection of a mixture of 2% chloralose (Sigma-Aldrich, St Louis, MO) and 10% urethane (Sigma-Aldrich) in PBS. A polyethylene tube was inserted into the trachea 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. We performed brightfield intravital microscopy using video recordings as previously reported.² Venules were visualized with a custom-designed intravital microscope (MM-40, Nikon), using a 60x water immersion objective (Nikon). Images were recorded using a charge-coupled device video camera (Hamamatsu, Bridgewater, NJ) and video recorder (Sony SVHS, SVO-9500). Survival time, defined as the time interval from TNF-α injection to the death of mouse, was recorded.

Image analyses for brightfield intravital microscopy

Adherent WBCs were defined as those remaining stationary for at least 30 seconds over
a 100-µm venular segment. RBCs were identified by their size and shape (discoid and sickle-shaped cells). An interaction between RBCs and adherent WBCs was defined as the arrest of an RBC on an adherent WBC for more than 2 video frames (> 0.07 second), a time interval that allows to discern adhesion events when the video segments are played in real time.

**Hemodynamic measurements**

Wall shear rate (γ) was calculated based on Poiseuille’s law for a Newtonian fluid, $\gamma = 2.12 \left(8V_{\text{mean}}\right) / D_v$, where $D_v$ is the venular diameter, $V_{\text{mean}}$ is estimated as $V_{\text{RBC}}/1.6$, and 2.12 is a median empirical correction factor obtained from actual velocity profiles measured in microvessels *in vivo*. Blood flow rate (Q) was calculated as $Q = V_{\text{mean}}\pi d^2/4$, where d is venule diameter, and is expressed as nL/s.

**In vivo analysis of Mac-1 activity**

Yellow-green fluosphere® beads (1 µm in diameter, with excitation/emission of 505/515 nm, respectively; Molecular Probes) were incubated with 1 mg/ml bovine serum albumin (BSA) (Fisher Bioreagents) for at least 2 h in phosphate-buffered saline (PBS) and sonicated for 15 min in a water-bath sonicator (Laboratory Supplies Co.) immediately before use. Albumin-coated fluospheres ($10^9$) were intravenously injected into mice prepared for intravital microscopy as indicated above, 20 min after administration of IVIG or control albumin. Images were captured 10 min after injection to allow clearance of fluosphere aggregates, which rapidly occurs in the first minutes. Images were then acquired with an Olympus BX61WI workstation using a LumPlanFl 60x water objective with numerical aperture 0.90, as previously described. ³,⁴

**Blood and white blood cell (WBC) differential counts**
Blood samples were collected from the venous plexus after IVM examination and were used to determine automated peripheral blood counts using a Coulter (Beckman, Fullerton, CA) or ADVIA 120 Hematology Analyzer System (Bayer, Holliston, MA). Peripheral blood smears were prepared for Wright-Giemsa staining. The differential counts were determined morphologically under light microscopy or by ADVIA 120 Hematology Analyzer System.

Isolation of bone marrow neutrophils (BMNs)
Neutrophils were isolated from bone marrow cells by Percoll (GE Healthcare) gradient centrifugation as described. Briefly, bone marrow cells harvested by flushing femur and tibia in RPMI using 21-gauge needle were dissociated into a single-cell suspension by gently passing the flushed marrow through the needle several times. Neutrophils were then separated from the remaining cells by centrifugation over discontinuous (52%, 65%, and 75%) Percoll gradient at 1000 x g for 20 min at 4°C. Mature neutrophils (band and segmented) were recovered at the interface of the 65-75% fractions and were >90% pure and >95% viable in the neutrophil-rich fraction as determined by trypan blue and Wright-Giemsa staining and flow cytometry.

Immunoprecipitation and western blotting
Bone marrow neutrophils (BMNs) isolated from WT and Fcgr2b−/− mice were incubated in either IVIG (6.7 mg/ml) or control albumin for 15 min at 37°C. Resting, albumin-, or IVIG-treated neutrophils were lysed in TN1 buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 10 mM Na₄P₂O₇, 10 mM NaF, 1% Triton X-100, 125 mM NaCl, 10 mM Na₃VO₄) with proteases inhibitor (Sigma) and phosphatase inhibitor cocktail (Thermo Scientific). Lysates were then incubated overnight with 5 µg anti-FcyRIIB/III ab or control isotype rat IgG with protein A agarose beads (Millipore). Immune complexes bound to beads were washed in
TN1 buffer and bound proteins eluted in SDS sample buffer. Proteins were separated by SDS-PAGE (10% gel), transferred onto PVDF membranes (Millipore), probed with rabbit anti-SHP-1/2 ab (clone NL213) (Upstate), followed by peroxidase-conjugated anti-rabbit IgG, light chain specific (Jackson ImmunoResearch). Blots were developed with the Super-Signal substrate (Pierce Chemical Co).

Reference

Online Figure I. Effect of IVIG on vasodilation and blood flow in WT mice. (A) Arteriolar and venular diameters were monitored before and after either IVIG or control albumin administration in WT mice treated with TNF-α (n= 20 vessels from two mice per group). (B) Blood flow rates (n= 8 mice per group). Bars represent mean ± SEM.
Online Figure II. Circulating neutrophils do not express FcγRIIB. Circulating leukocytes were stained for FcγRIIB, Gr-1, CD115 expression after RBC lysis. (A) Neutrophil population gated on the basis of side and forward scatter properties by flow cytometry (left panel) expressed Gr-1$^{hi}$ and CD115$^{low}$ (middle panel, blue), and had low binding to anti-FcγRIIB (clone Ly17.2) (right panel, blue) compared to other circulating leukocytes (right panel, red) in WT mice. (B) Gating strategy for Figure 2B and 2c showing neutrophils (Gr-1$^{hi}$/CD115$^{low-neg}$) and monocytes (Gr-1$^{low-hi}$/CD115$^{hi}$) in TNF-α-treated mice.
Online Figure III. Recruitment of SHP-1/2 to FcγRIII in response to IVIG in FcγRIIB-deficient mice. SHP-1 association with FcγRIII in response to IVIG in neutrophils isolated from Fcgr2b−/− mice. Bone marrow neutrophils (BMNs) isolated from control Fcgr2b−/− mice (n= 2-3 per group) were incubated with IVIG or albumin (6.7 mg/ml) at 37 °C for 15 min, and then lysates were prepared. Lysates were immunoprecipitated with anti-FcγRIIB/III or control isotype rat IgG2b followed by immunoblotting (IB) with anti-SHP-1/2 ab.

Online Figure IV. Genotype of mev/mev mice and chimerism in transplanted mice. (A) mev/mev mice were produced from matings of +/-mev heterozygous mating, respectively. Homozygous mev/mev mice were identified by genotype and used as donor for generating chimeric mev/mev mice. (B) Representative flow cytometry profile of circulating leukocytes from lethally irradiated Ly5.2 (CD45.1) host reconstituted with Ly5.1 (CD45.2) mev/mev donor bone marrow cells.
Online Figure V. Effect of IVIG on vasodilation in SCD mice. Arteriolar and venular diameters were monitored before and after either IVIG or control albumin administration (n= 20 vessels from two mice per group) in SCD mice treated with TNF-α. Bars represent mean ± SEM. Representative images of each group show size of (A) artery and (B) venule before and after either IVI or control albumin administration. Scar bars, 10 µm.
Online Figure VI. Proposed mechanistic pathway of IVIG’s inhibition of neutrophil activation. E-selectin and FcγRIII can trigger intracellular signaling events through activation of Src family kinases, ITAM adapters and Syk upon ligand engagement, subsequently leading to increase β2 integrin affinity for circulating RBC capture and leukocyte adhesion. The recruitment of SHP-1 induced by the engagement of IVIG on FcγRIII may modulate ITAM-mediated pathways in both FcγRIII and E-selectin signaling cascades resulting in increased leukocyte rolling velocity, reduced leukocyte arrest and Mac-1-dependent heterotypic interactions with circulating RBCs.
Online Tables

Online Table I. Hemodynamic parameters analyzed for leukocyte adhesion to endothelium after control albumin or IVIG administration in anti-FcγRIIB/III or IgG2b-treated WT mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mice (n)</th>
<th>Venule (n)</th>
<th>Venular diameter (µm)</th>
<th>Centerline velocity (mm/s)</th>
<th>Shear rate (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG2b Albumin</td>
<td>14</td>
<td>136</td>
<td>31 ± 2</td>
<td>1.4 ± 0.04</td>
<td>527 ± 42</td>
</tr>
<tr>
<td>IVIG</td>
<td>12</td>
<td>124</td>
<td>30 ± 2</td>
<td>1.5 ± 0.1</td>
<td>560 ± 35</td>
</tr>
<tr>
<td>Anti-FcγRIIB/III Albumin</td>
<td>11</td>
<td>110</td>
<td>30 ± 2</td>
<td>1.4 ± 0.1</td>
<td>533 ± 30</td>
</tr>
<tr>
<td>IVIG</td>
<td>11</td>
<td>105</td>
<td>31 ± 3</td>
<td>1.4 ± 0.1</td>
<td>525 ± 55</td>
</tr>
</tbody>
</table>

Online Table II. Hemodynamic parameters analyzed for RBC-leukocyte interactions after control albumin or IVIG administration in anti-FcγRIIB/III or IgG2b-treated WT mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mice (n)</th>
<th>Venule (n)</th>
<th>Venular diameter (µm)</th>
<th>Centerline velocity (mm/s)</th>
<th>Shear rate (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG2b Albumin</td>
<td>14</td>
<td>70</td>
<td>36 ± 1</td>
<td>1.3 ± 0.03</td>
<td>382 ± 10</td>
</tr>
<tr>
<td>IVIG</td>
<td>12</td>
<td>50</td>
<td>37 ± 1</td>
<td>1.4 ± 0.05</td>
<td>407 ± 9</td>
</tr>
<tr>
<td>Anti-FcγRIIB &amp;III Albumin</td>
<td>11</td>
<td>52</td>
<td>34 ± 1</td>
<td>1.2 ± 0.04</td>
<td>407 ± 11</td>
</tr>
<tr>
<td>IVIG</td>
<td>11</td>
<td>46</td>
<td>38 ± 2</td>
<td>1.4 ± 0.06</td>
<td>393 ± 9</td>
</tr>
</tbody>
</table>

Hemodynamic parameters were analyzed from intravital microscopy recording of venules used for the result shown in Figure 1C and 1D. Data are presented as mean ± SEM.
Online Table III. Hemodynamic parameters analyzed for leukocyte adhesion on the endothelium in control WT, Fcgr2b\textsuperscript{\textminus/\textminus}, and Fcgr3\textsuperscript{\textminus/\textminus} mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Mice (n)</th>
<th>Venule (n)</th>
<th>Venular diameter (µm)</th>
<th>Centerline velocity (mm/s)</th>
<th>Shear rate (s\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>Albumin</td>
<td>6</td>
<td>34</td>
<td>29 ± 1</td>
<td>1.0 ± 0.04</td>
<td>378 ± 20</td>
</tr>
<tr>
<td></td>
<td>IVIG</td>
<td>7</td>
<td>53</td>
<td>28 ± 1</td>
<td>1.4 ± 0.11 *</td>
<td>542 ± 41*</td>
</tr>
<tr>
<td>Fcgr2b\textsuperscript{\textminus/\textminus}</td>
<td>Albumin</td>
<td>5</td>
<td>33</td>
<td>28 ± 1</td>
<td>1.0 ± 0.05</td>
<td>369 ± 22</td>
</tr>
<tr>
<td></td>
<td>IVIG</td>
<td>6</td>
<td>36</td>
<td>28 ± 1</td>
<td>1.3 ± 0.10*</td>
<td>492 ± 55</td>
</tr>
<tr>
<td>Fcgr3\textsuperscript{\textminus/\textminus}</td>
<td>Albumin</td>
<td>5</td>
<td>36</td>
<td>28 ± 1</td>
<td>1.4 ± 0.10</td>
<td>561 ± 61</td>
</tr>
<tr>
<td></td>
<td>IVIG</td>
<td>6</td>
<td>29</td>
<td>28 ± 2</td>
<td>1.5 ± 0.13</td>
<td>586 ± 68</td>
</tr>
</tbody>
</table>

*P<0.05 vs albumin

Online Table IV. Hemodynamic parameters analyzed for RBC-leukocyte interactions in control WT, Fcgr2b\textsuperscript{\textminus/\textminus}, and Fcgr3\textsuperscript{\textminus/\textminus} mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Mice (n)</th>
<th>Venule (n)</th>
<th>Venular diameter (µm)</th>
<th>Centerline velocity (mm/s)</th>
<th>Shear rate (s\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>Albumin</td>
<td>6</td>
<td>36</td>
<td>39 ± 2</td>
<td>1.0 ± 0.03</td>
<td>302 ± 10</td>
</tr>
<tr>
<td></td>
<td>IVIG</td>
<td>6</td>
<td>31</td>
<td>41 ± 1</td>
<td>1.0 ± 0.03</td>
<td>268 ± 10</td>
</tr>
<tr>
<td>Fcgr2b\textsuperscript{\textminus/\textminus}</td>
<td>Albumin</td>
<td>5</td>
<td>37</td>
<td>37 ± 1</td>
<td>1.0 ± 0.03</td>
<td>295 ± 9</td>
</tr>
<tr>
<td></td>
<td>IVIG</td>
<td>6</td>
<td>34</td>
<td>41 ± 1</td>
<td>1.1 ± 0.03</td>
<td>300 ± 13</td>
</tr>
<tr>
<td>Fcgr3\textsuperscript{\textminus/\textminus}</td>
<td>Albumin</td>
<td>6</td>
<td>31</td>
<td>42 ± 2</td>
<td>1.2 ± 0.05</td>
<td>310 ± 14</td>
</tr>
<tr>
<td></td>
<td>IVIG</td>
<td>6</td>
<td>30</td>
<td>46 ± 2</td>
<td>1.2 ± 0.05</td>
<td>279 ± 11</td>
</tr>
</tbody>
</table>

Hemodynamic parameters were analyzed from intravital microscopy recording of venules used for the result shown in Figure 3A and 3B. Data are presented as mean ± SEM.
Online Table V. Hemodynamic parameters analyzed for leukocyte adhesion on the endothelium in control WT or me\(^v/me\(^v\) mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Mice (n)</th>
<th>Venule (n)</th>
<th>Venular Diameter (µm)</th>
<th>Blood flow (nL/sec)</th>
<th>Centerline Velocity (mm/s)</th>
<th>Shear rate (s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>Albumin</td>
<td>5</td>
<td>36</td>
<td>26 ± 1</td>
<td>456 ± 37</td>
<td>1.4 ± 0.1</td>
<td>570 ± 78</td>
</tr>
<tr>
<td></td>
<td>IVIG</td>
<td>5</td>
<td>35</td>
<td>26 ± 1</td>
<td>532 ± 55</td>
<td>1.6 ± 0.2</td>
<td>661 ± 66</td>
</tr>
<tr>
<td>me(^v/me(^v)</td>
<td>Albumin</td>
<td>4</td>
<td>30</td>
<td>27 ± 2</td>
<td>725 ± 149</td>
<td>2.0 ± 0.3</td>
<td>793 ± 125</td>
</tr>
<tr>
<td></td>
<td>IVIG</td>
<td>4</td>
<td>27</td>
<td>28 ± 2</td>
<td>642 ± 68</td>
<td>1.7 ± 0.2</td>
<td>638 ± 109</td>
</tr>
</tbody>
</table>

Online Table VI. Hemodynamic parameters analyzed for RBC-leukocyte interactions in control WT or me\(^v/me\(^v\) mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Mice (n)</th>
<th>Venule (n)</th>
<th>Venular diameter (µm)</th>
<th>Centerline velocity (mm/s)</th>
<th>Shear rate (s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>Albumin</td>
<td>5</td>
<td>24</td>
<td>43 ± 2</td>
<td>1.1 ± 0.1</td>
<td>288 ± 17</td>
</tr>
<tr>
<td></td>
<td>IVIG</td>
<td>5</td>
<td>27</td>
<td>42 ± 1</td>
<td>1.1 ± 0.1</td>
<td>288 ± 14</td>
</tr>
<tr>
<td>me(^v/me(^v)</td>
<td>Albumin</td>
<td>5</td>
<td>17</td>
<td>39 ± 2</td>
<td>1.2 ± 0.1</td>
<td>332 ± 19</td>
</tr>
<tr>
<td></td>
<td>IVIG</td>
<td>4</td>
<td>16</td>
<td>35 ± 2</td>
<td>1.2 ± 0.1</td>
<td>395 ± 33</td>
</tr>
</tbody>
</table>

Hemodynamic parameters were analyzed from intravital microscopy recording of venules used for the result shown in Figure 4B and 4C. Data are presented as mean ± SEM.
Online movie legends

Movie I. Representative video segment showing acute vaso-occlusion in post-capillary venule of SCD mice after control albumin administration.

Movie II. Representative video segment showing leukocyte recruitment in post-capillary venule of SCD mice after IVIG administration.

Movie III. Representative video segment showing leukocyte recruitment and RBC-leukocyte interactions after control albumin administration in SCD mice with blockade of FcγRIIB/III.

Movie IV. Representative video segment showing leukocyte recruitment and RBC-leukocyte interactions after IVIG administration in SCD mice with blockade of FcγRIIB/III.