Supplemental Materials

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

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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] me^v/me^v and control WT chimeras were generated by transplanting 5 X 10^6 donor bone marrow cells from me^v/me^v homozygous or C57BL/6 (CD45.2) mice into lethally irradiated 8-week-old C57BL/6 Ly5.2 (CD45.1) male mice, respectively. Blood of me^v/me^v 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 CI: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% N_2 and 5% CO_2. 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, $\gamma = 2.12\ (8V_{mean}) / D_v$, where D_v is the venular diameter, V_{mean} is estimated as $V_{RBC}/\ 1.6$, 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 $Q = V_{mean}\pi d^2/4$, 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 LumPlanFI 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.[5] 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

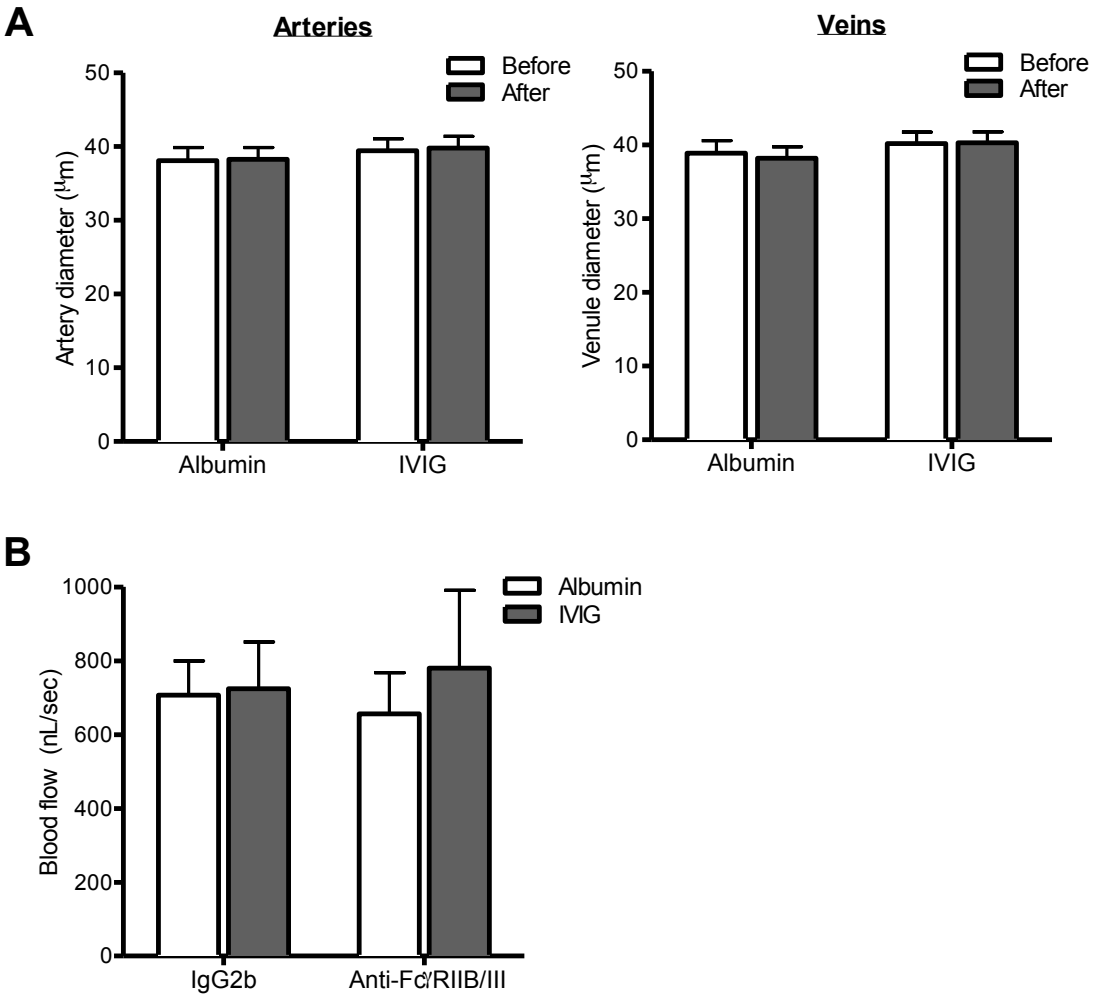
Bone marrow neutrophils (BMNs) isolated from WT and *Fcgr2b*$^{-/-}$ mice were incubated in either IVIG (6.7 mg/ml) or control albumin for 15 min at 37°C. Resting, albumin-, or IVIG-treated neutrophils were lysed in TN1 buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 10 mM NaF, 1% Triton X-100, 125 mM NaCl, 10 mM Na_3VO_4) with proteases inhibitor (Sigma) and phosphatase inhibitor cocktail (Thermo Scientific). Lysates were then incubated overnight with 5 µg anti-FcγRIIB/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).

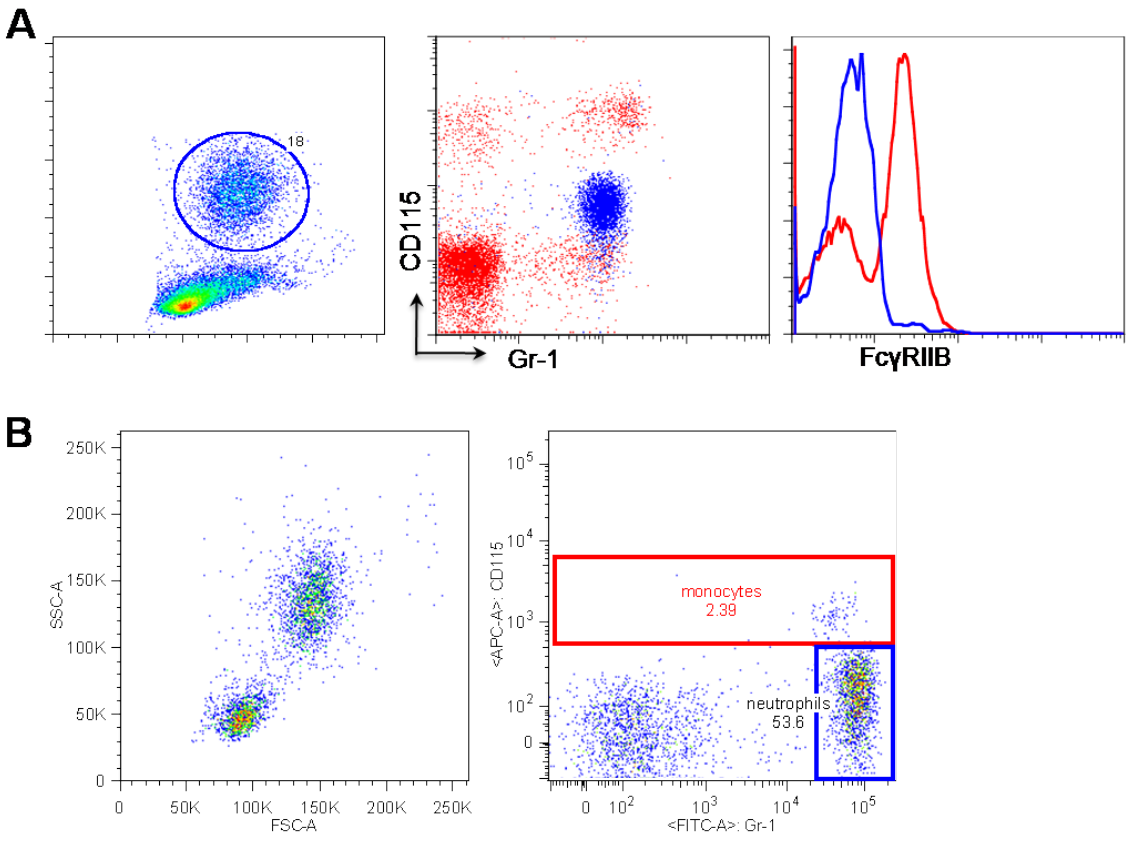
Reference

1. Turhan A, Weiss LA, Mohandas N, Coller BS, Frenette PS. Primary role for adherent leukocytes in sickle cell vascular occlusion: A new paradigm. *Proc Natl Acad Sci U S A*. 2002;99:3047-3051
2. Jang JE, Hod EA, Spitalnik SL, Frenette PS. Cxcl1 and its receptor, cxcr2, mediate murine sickle cell vaso-occlusion during hemolytic transfusion reactions. *J Clin Invest*. 2011;121:1397-1401
3. Chiang EY, Hidalgo A, Chang J, Frenette PS. Imaging receptor microdomains on leukocyte subsets in live mice. *Nature methods*. 2007;4:219-222
4. Hidalgo A, Chang J, Jang JE, Peired AJ, Chiang EY, Frenette PS. Heterotypic interactions enabled by polarized neutrophil microdomains mediate thromboinflammatory injury. *Nat Med*. 2009;15:384-391
5. Jaillon S, Peri G, Delneste Y, Fremaux I, Doni A, Moalli F, Garlanda C, Romani L, Gascan H, Bellocchio S, Bozza S, Cassatella MA, Jeannin P, Mantovani A. The humoral pattern recognition receptor ptx3 is stored in neutrophil granules and localizes in extracellular traps. *J Exp Med*. 2007;204:793-804

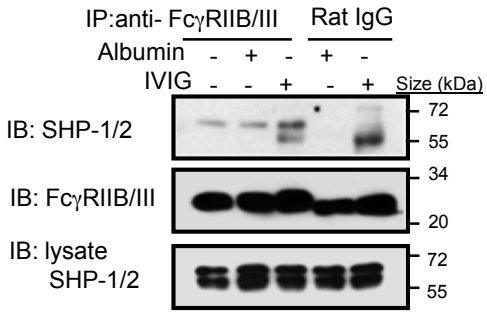
Online Figures



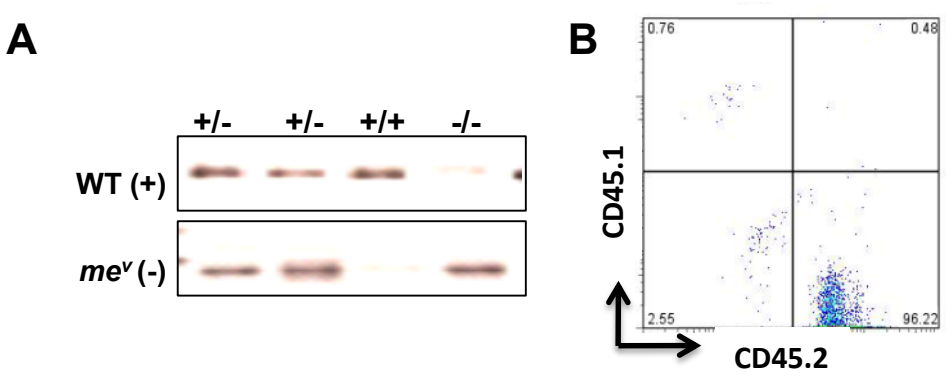
Online Figure I. **Effect of IVIG on vasodilation and blood flow in WT mice.** (A) Arteriolar and venular diameters were monitored before and after either IVIG or control albumin administration in WT mice treated with TNF-α (n= 20 vessels from two mice per group). (B) Blood flow rates (n= 8 mice per group). Bars represent mean ± SEM.



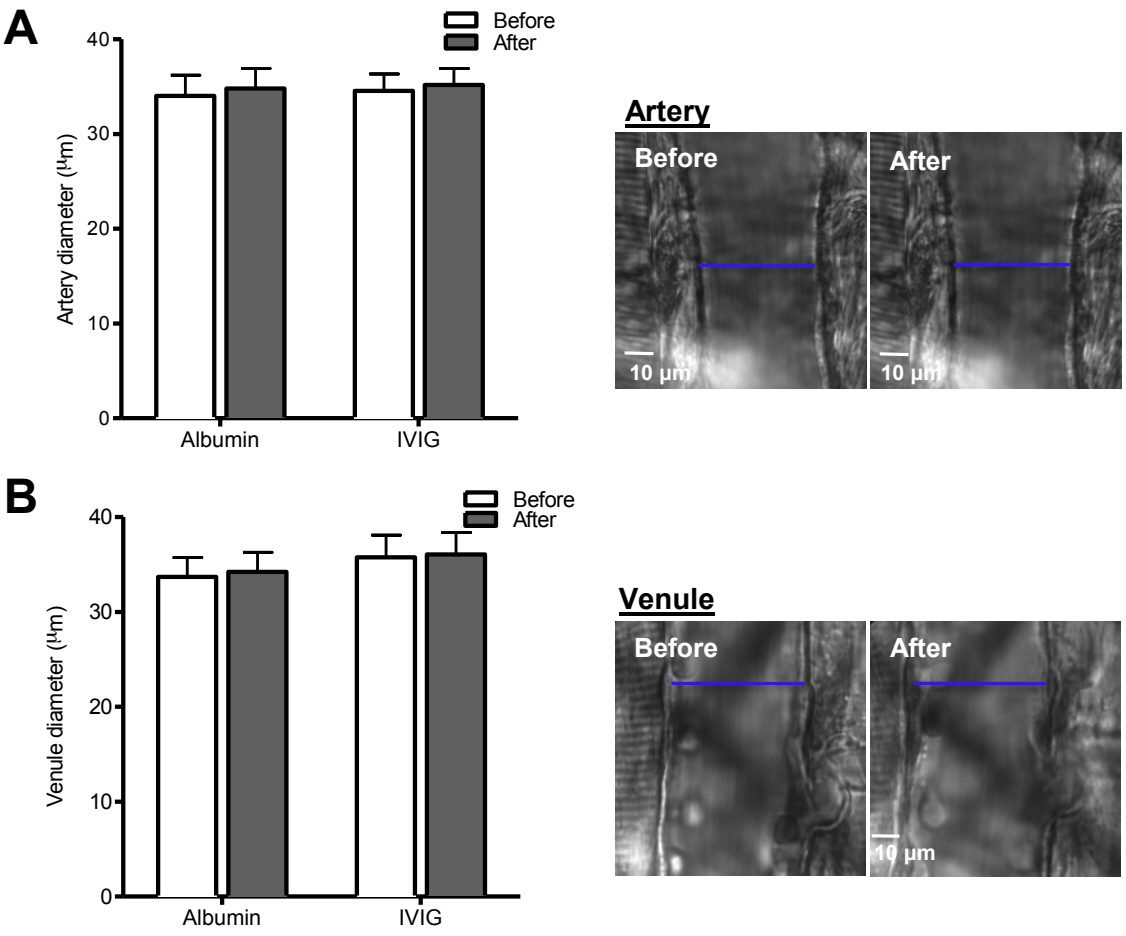
Online Figure II. Circulating neutrophils do not express FcγRIIB. Circulating leukocytes were stained for FcγRIIB, Gr-1, CD115 expression after RBC lysis. (A) Neutrophil population gated on the basis of side and forward scatter properties by flow cytometry (left panel) expressed Gr-1^{hi} and $CD115^{low}$ (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}/$CD115^{low\text{-}neg}$) and monocytes (Gr-$1^{low\text{-}hi}$/$CD115^{hi}$) in TNF-α-treated mice.



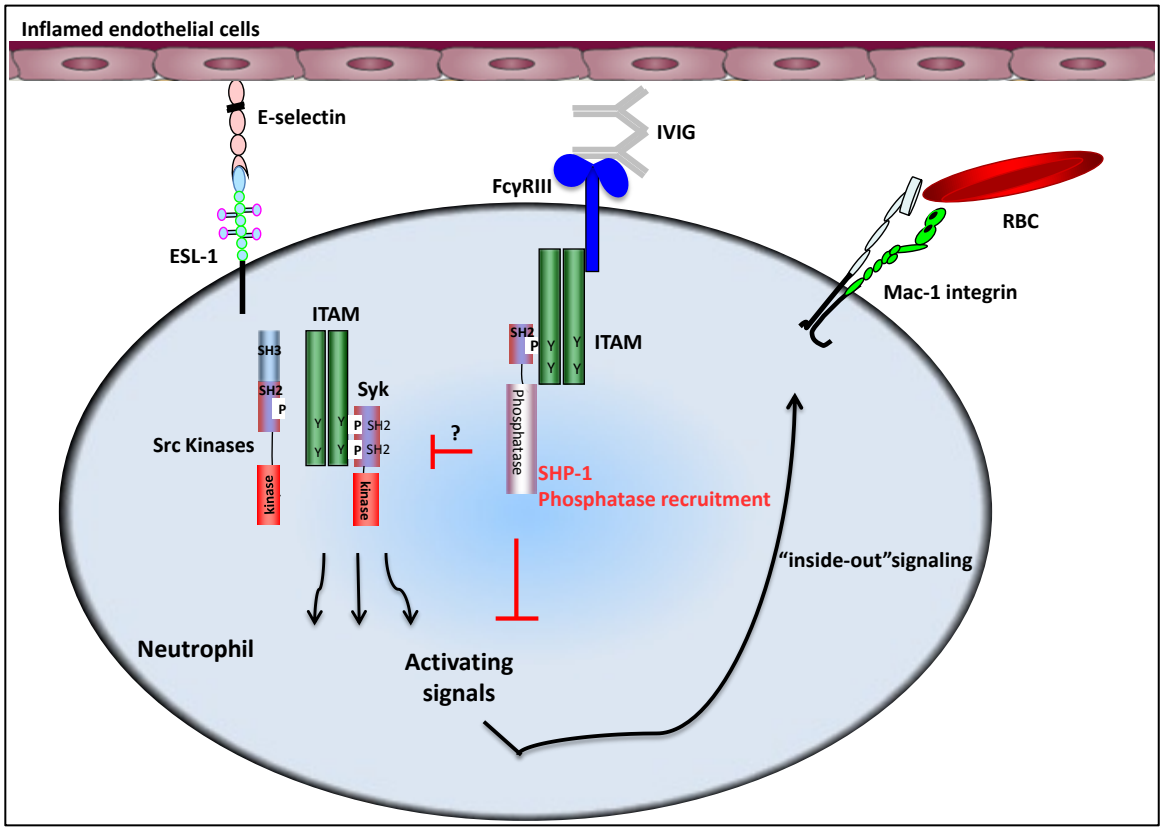
Online Figure III. Recruitment of SHP-1/2 to FcγRIII in response to IVIG in FcγRIIB-deficient mice. SHP-1 association with FcγRIII in response to IVIG in neutrophils isolated from *Fcgr2b*$^{-/-}$ mice. Bone marrow neutrophils (BMNs) isolated from control *Fcgr2b*$^{-/-}$ mice (n= 2-3 per group) were 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 *me*^{v}*/me*^{v} mice and chimerism in transplanted mice. (A) *me*^{v}*/me*^{v} mice were produced from matings of +/*me*^{v} heterozygous mating, respectively. Homozygous *me*^{v}*/me*^{v} mice were identified by genotype and used as donor for generating chimeric *me*^{v}*/me*^{v} mice. (B) Representative flow cytometry profile of circulating leukocytes from lethally irradiated Ly5.2 (CD45.1) host reconstituted with Ly5.1 (CD45.2) *me*^{v}*/me*^{v} 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.

Treatment		Mice (n)	Venule (n)	Venular diameter (μm)	Centerline velocity (mm/s)	Shear rate (s^{-1})
IgG2b	Albumin	14	136	31 ± 2	1.4 ± 0.04	527 ± 42
	IVIG	12	124	30 ± 2	1.5 ± 0.1	560 ± 35
Anti-FcγRIIB/III	Albumin	11	110	30 ± 2	1.4 ± 0.1	533 ± 30
	IVIG	11	105	31 ± 3	1.4 ± 0.1	525 ± 55

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.

Treatment		Mice (n)	Venule (n)	Venular diameter (μm)	Centerline velocity (mm/s)	Shear rate (s^{-1})
IgG2b	Albumin	14	70	36 ± 1	1.3 ± 0.03	382 ± 10
	IVIG	12	50	37 ± 1	1.4 ± 0.05	407 ± 9
Anti-FcγRIIB &III	Albumin	11	52	34 ± 1	1.2 ± 0.04	407 ± 11
	IVIG	11	46	38 ± 2	1.4 ± 0.06	393 ± 9

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*$^{-/-}$, and *Fcgr3*$^{-/-}$ mice.

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

*P<0.05 vs albumin

Online Table IV. Hemodynamic parameters analyzed for RBC-leukocyte interactions in control WT, *Fcgr2b*$^{-/-}$, and *Fcgr3*$^{-/-}$ mice.

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

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.

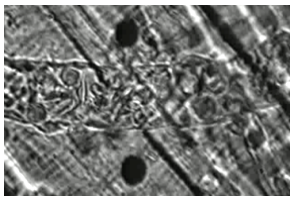
Group	Treatment	Mice (n)	Venule (n)	Venular Diameter (μm)	Blood flow (nL/sec)	Centerline Velocity (mm/s)	Shear rate (s^{-1})
Wild type	Albumin	5	36	26 ± 1	456 ± 37	1.4 ± 0.1	570 ± 78
	IVIG	5	35	26 ± 1	532 ± 55	1.6 ± 0.2	661 ± 66
me^{v}/me^{v}	Albumin	4	30	27 ± 2	725 ± 149	2.0 ± 0.3	793 ± 125
	IVIG	4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

Online Table VI. Hemodynamic parameters analyzed for RBC-leukocyte interactions in control WT or *me^{V}/me^{V}* mice.

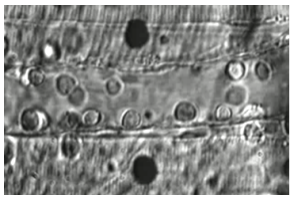
Group	Treatment	Mice (n)	Venule (n)	Venular diameter (μm)	Centerline velocity (mm/s)	Shear rate (s^{-1})
Wild type	Albumin	5	24	43 ± 2	1.1 ± 0.1	288 ± 17
	IVIG	5	27	42 ± 1	1.1 ± 0.1	288 ± 14
me^{v}/me^{v}	Albumin	5	17	39 ± 2	1.2 ± 0.1	332 ± 19
	IVIG	4	16	35 ± 2	1.2 ± 0.1	395 ± 33

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.

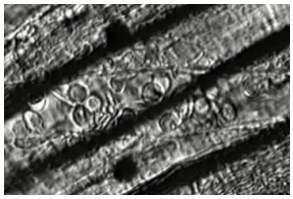
Online movie legends



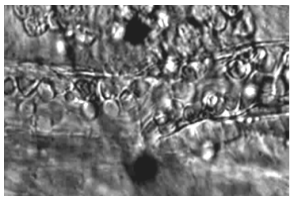
Movie I. Representative video segment showing acute vaso-occlusion in post-capillary venule of SCD mice after control albumin administration.



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.