Supplemental Methods

Whole blood collection and plasma isolation

The University of Utah Institutional Review Board approved this study and all subjects provided informed consent. Human peripheral venous blood from healthy, medication-free adult subjects was drawn into acid-citrate-dextrose (pH 5.1, 1.4 ml ACD/8.6 ml blood) through standard venipuncture and used immediately upon collection. ACD had no effect on plasma pH level (**Supplemental Methods Figure I**). Plasma was harvested by sequential centrifugation of the whole blood at 150 x g for 20 minutes (min) followed by another spin at 1500 x g for 20 minutes and then once more at 13,000 x g for 2 min to remove remaining cell contaminants.

Whole Blood Clot Formation

For RNA-seq analysis, 9.5 mL of whole blood was recalcified (20 mM final) by adding whole blood to 500 uL of 400 mM $CaCl_2$ and gently inverting the tube. Recombinant tissue factor (1:20000, final Dade Innovin, Siemens, Tarrytown, NY) was then added and the whole blood was allowed to clot for 2 hours at 37^{o}C at 5%CO_2. The addition of $CaCl_2$ did not result in any visual hemolysis after plasma isolation. After two hours, the whole blood clot was dissociated with mechanical shear to remove the monocytes from the clot. Monocytes were isolated with ficoll-paque and positively selected for using CD14 magnetic beads (see below). Purified monocytes were counted and then lysed in Trizol for RNA isolation. Monocytes isolated from whole blood clots were compared to baseline monocytes (i.e. cells immediately isolated after whole blood was drawn) for RNA seq-analysis. For cytokine studies, whole blood was allowed to clot for 18 hours

before the blood was centrifuged at 13000 x g for 10 minutes. After the initial centrifugation, the supernatant was centrifuged again at 13000 x g for two minutes to remove residual cells. In indicated experiments, tissue-type plasminogen activator (tPA, American Diagnostica, Stamford, CT, USA) was added (1 μg/mL, final) at the initiation of clot formation.

Monocyte Isolation

Human monocytes were isolated by drawing human peripheral venous blood (500 ml) from healthy, medication-free donors. Blood was centrifuged at 150 x g for 20 min at 20^{o}C to separate platelet-rich plasma (PRP) from red and white blood cells (RBC/WBC). The RBC/ WBC mixture was resuspended with 0.9% sterile saline back to the original volume and layered over an equal volume of Ficoll-Paque Plus (GE Healthcare Biosciences, Piscataway, NJ, USA). The layered cells were then centrifuged for 30 min at 400 x g at 20^{o} C. After 30 min, the mononuclear leukocyte layer was removed and washed with Hank's Balance Salt Solution (Sigma-Aldrich, St. Louis, MO, USA) with 1% human serum albumin (HBSS/A) (University of Utah Hospital, Salt Lake City, UT, USA) and centrifuged for 10 min at 400 x g at 20^{o} C. The cell pellet was then resuspended and CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were added for 15 min at 4^{o}C. The cells were then washed with HBSS/A to remove any free CD14 microbeads and then resuspended in HBSS/A. The monocytes were then isolated by running the cell solution through an autoMACs cell separator (Miltenyi Biotec) using the PosselD2 program. Cells were then washed with HBSS/A and resuspended in M199

(BioWhitaker, Walkersville, MD, USA) and counted. The purity of monocytes was > 90%.

Purified Plasma Clot Formation

Whole blood was drawn into ACD (pH 5.1) in a ratio of 1:7 (ACD:Blood). Whole blood was then centrifuged for 20 minutes at 150 x g to isolate platelet-rich plasma (PRP). PRP was then centrifuged for 20 minutes at 1500 x g to generate platelet-poor plasma (PPP). Finally, PPP was centrifuged for 2 minutes at 12000 x g to generate cell-free plasma (CFP). CFP (450 μL) was added to a single well of a 24-well tissue culture treated plate and re-calcified with the addition of 25 μL of 400 mM $CaCl_2$. Recombinant tissue factor (Dade Innovin) was added at the same time (25 μL, 1:20000 dilution final) to trigger tissue factor dependent fibrin clot formation. Clot formation was allowed to occur for 30 minutes at 37°C in humidified incubator with 5% CO_2. Monocytes isolated using CD14 positive selection and resuspended with M199 without supplemental factors were added to the CFP clot at a final concentration of 2 x 10^6 total cells in a volume of 1000 μL. The final volume of clot and cells was 1500 μL. The cells and clot were allowed to incubate in a humidified incubator for 18 hours at 37°C at 5% CO_2. In experiments with heparin, plasma from the same individual was drawn into heparinized tubes and centrifuged in a similar manner. Heparinized plasma had only Innovin added back. Plasma was sometimes treated with cycloheximide (5 μg/mL,final)(Sigma-Aldrich), actinomycin D (5 μg/mL, final)(Sigma-Aldrich), lepirudin (University of Utah Pharmacy), or GPRP diluted in water (Pentapharm, 40 mg/mL, final). In some assays, tPA (America Diagnostica, 1 μg/ml, final) was added at the indicated time. Fibrinogen

deficient plasma was from Affinity Biologicals and was reconstituted with fibrinogen from Enzyme Research Laboratories at the indicated concentrations. FXIII deficient plasma was from Affinity Biologicals and was reconstituted with FXIIIa from Hematologic Technologies (10 μg/mL). The FXIII inhibitor, 1,3,4,5-Tetramethyl-2-[(2-oxopropyl)thio]imidazolium chloride (T101) was from Zedira (San Diego, CA, USA) and was added to plasma clots (5 μM, final) before the addition of tissue factor and calcium. Serum was made by adding thrombin (Sigma-Aldrich, 1.0 U/mL, final) to plasma and allowing the clot to form for an hour. After an hour, the clot was centrifuged for 10 minutes at 13000 x g to remove the clot. Thrombin-independent plasma clots were formed using Batroxobin (Pentapharm, 1 U/mL, final). In some experiments, monocytes were treated with the TLR4 inhibitor, CLI-095 (Invivogen, 1 μM, final), for 1 hour before addition to the plasma fibrin clot. In some experiments, a CD11b monoclonal antibody (M1/70 eBiosciences) or IgG (5 μg/mL, final) was pre-incubated with monocytes before addition to plasma clots. The inhibitory nature of the antibody was tested using Alexa-fluor 555 labeled fibrinogen (eBiosciences) and measuring fibrinogen binding by flow cytometry. In some experiments, monocytes (2 x 10^6, final concentration) resuspended in M199 without supplemental factors[1-5] and in a total volume of 1500 μL were stimulated with thrombin (0.1 U/mL, final) or lipopolysaccharide (100 ng/mL, final Sigma-Aldrich) and IL-8 and MCP-1 measured by ELISA. For purified fibrin clot formation, monocytes (2 x 10^6, final concentration) resuspended in M199 and in a total volume of 1000 μL were added to 500 μL of purified fibrin clots (2 mg/mL, final Enzyme Research Laboratories) generated by the addition of 1.0 U/mL thrombin (final).

FXIII crosslinking experiments

Plasma clots in the absence or presence of FXIII or in the absence or presence of T101 were formed as described above. After 60 minutes, crosslinking and clotting were stopped using a solution containing 50 mM DTT, 12.5 mM EDTA, 8 M urea. Samples were then incubated at 60°C for 1 hour. Samples were reduced, boiled, separated on 10% gels, and transferred to nitrocellulose membranes. Membranes were probed with rabbit anti-human fibrinogen polyclonal antibodies (Dako).

Radiolabeled Protein Synthesis

Monocytes were resuspended in DMEM without methionine or cysteine and allowed to rest for 30 minutes. After 30 minutes, EasyTag™ EXPRESS^{35}S Protein Labeling Mix (0.06 mCI total) (Perkin Elmer) was added to each reaction and allowed to incubate overnight. The next day the monocytes were washed three times in complete media and then lysed in radioimmunoprecipitation assay buffer (RIPA) (1X PBS with 1% NP-40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate). Lysates were cleared and then Trichloroacetic acid (TCA) precipitated using 20% TCA on ice for 30 minutes. The precipitated proteins were loaded onto **a** Whatman grade GF/C glass microfiber filter (VWR) and washed five times with 10% TCA and five times with 95% ethanol. Filter papers were then read using a liquid scintillation counter.

RNA-Seq Analysis

Monocytes were purified using immune-magnetic positive selection as described previously[6, 7]. For global screening of RNAs (RNA-seq), monocytes were purified from individual donors. For the RNA-seq and real-time PCR analysis of mRNA expression, washed monocytes were lysed in Trizol and DNAse treated total RNA was isolated, as previously described (Rowley et al). An agilent bio-analyzer was used to Quality Control (QC) and quantitate RNA. RNA Integrity Number (RIN) scores were similar between all samples. RNA-seq libraries were prepared with TruSeq V2 with oligo-dT selection (Illumina, San Diego CA). For whole blood clots, 36 base pair (bp) paired end reads were sequenced on an Illumina GAIIx. For all other samples, 50 bp single end reads were sequenced on an Illumina Hiseq 2000. Reads were aligned (Novoalign) to the reference genome GRCh37/hg19 and a psuedotranscriptome containing splice junctions. The USeq analysis package was used to assign reads to composite transcripts (one per gene) and quantitate FPKMs as previously described[8-11]. Data are deposited in Sequence Read Archive (SRA) through NCBI (SRA number 2758937).

Patient Enrollment and Thrombus Retrieval

This was a prospective cohort study of patients referred to a university tertiary-care referral center with acute thrombosis. The local institutional review board approved this study and all patients provided informed consent (IRB# 42054). Inclusion criteria included patients with acute thrombosis undergoing surgical thrombectomy. Exclusion criteria included inability to provide informed consent or obtain a thrombus specimen. All thrombus specimens were removed by a vascular surgeon, placed immediately into

sterile specimen cups, and transported to the laboratory for processing. Demographic data, medical history, and clinical laboratory results were recorded for each patient.

Immunohistochemistry

Thrombi from patients were fixed with 4% paraformaldehyde for 30 minutes at room temperature before being placed in a 7.5% sucrose/4% paraformaldehyde solution for four hours. After four hours, the thrombi were placed in a 15% sucrose/4% paraformaldehyde solution overnight. The thrombi were then embedded into OCT mounting solution and froze at -80^{o}C. The thrombi were then sectioned in 5-20 μm thick sections and stained. Clots from the in vitro clot system were embedded with OCT mounting solution and then frozen at -80^{o}C. The clots were then sectioned in 5-20 μm thick sections and stained. Sections were permeablized using 0.1 X Triton followed by blocking with 10% goat serum. Staining was performed using a Rabbit anti-MCP-1 (Abcam 9669), Rabbit anti-IL8 (Abcam 7747), Mouse anti-CD14 (Abcam 63319), Mouse IgG (Santa Cruz 2025), and Rabbit IgG (Abcam 171870) in 10% goat serum overnight. The next day a goat anti-rabbit Alexa Fluor 546 and goat anti-mouse Alexa Fluor 488 were added and the slides were then imaged.

Imaging

High-resolution confocal reflection microscopy was performed with an Olympus IX81, FV300, and a FV1000 (Olympus) confocal-scanning microscope equipped with a 60 × /1.42 NA oil objective for viewing platelets. An Olympus FVS-PSU/IX2-UCB camera and scanning unit and Olympus Fluoview FV 300 and FV1000 Version 5.0 image acquisition software was used for recording. The images were further analyzed with the use of

Adobe Photoshop CS Version 8.0, and ImageJ Version 1.50b (National Institutes of Health).

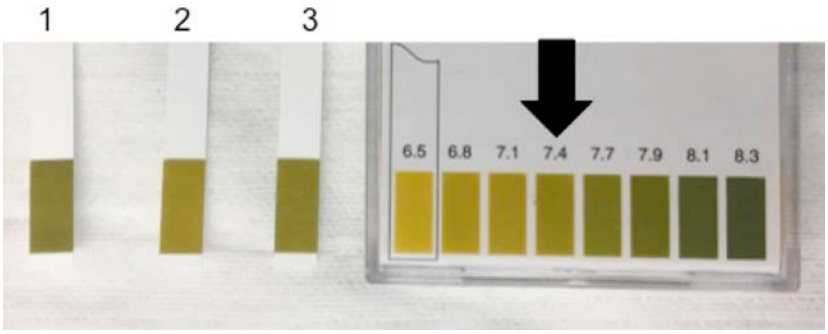
For the ultrastructural analyses, monocytes embedded in clots were fixed in 2.5% glutaraldehyde in PBS buffer. The samples were subsequently washed and postfixed with 2% osmium tetroxide, rewashed, dehydrated by a graded series of acetone concentrations (50%, 70%, 90%, 100%; 2 × 10 minutes each), and embedded in Epon. Thin sections were counterstained (ie, uranyl acetate and lead citrate), viewed with a JEOL JEM-1011 electron microscope (JEOL), and digital images were captured with a side-mounted Advantage HR CCD camera (Advanced Microscopy Techniques).

Quantification of Chemokine and Cytokine Protein Expression

To assess the response of monocytes embedded in plasma clots, we used a multiplexed sandwich capture assay for the quantification of 13 chemokines and cytokines developed at the ARUP Institute for Experimental and Clinical Pathology at the University of Utah as previously described[12]. The chemokines or cytokines assayed included: CD40 ligand, interferon-γ, IL-10, IL-12, IL-13, IL-1β, IL-2, IL-2 receptor, IL-4, IL-5, IL-6, IL-8, and tumor necrosis factor-α (TNF-α). To confirm and extend the chemokine results obtained using the multiplex sandwich capture assay, quantification of IL-8 and MCP-1 in supernatants and cell lysates was performed by ELISA (R&D Systems, Inc.) as per the manufacturer's instructions. Whole blood cytokine analysis was also examined in cell-free supernatants using a Human Cytokine Array Kit (R&D Systems, Inc.)

Statistical methods

For RNA-seq analysis, Deseq2 was used to identify differentially expressed transcripts. To focus on robustly expressed transcripts, only transcripts with per group average > 3 FPKM were included in the analysis. For the plasma clot samples, a q value < .05 and fold change of 4-fold were used as thresholds for differential expression. For the whole blood clot model, assignment of differentially expressed transcripts was based on fold change of 4-fold. For relevant studies, we calculated the mean ± SEM and performed ANOVAs to identify differences among multiple experimental groups. If significant differences existed, a Student Newman-Keuls posthoc procedure was used to determine the location of the difference between groups. When single comparisons were performed, a Student's t-test was employed. Statistical significance was set at $P < .05$.



Supplemental Methods Figure I. Plasma isolated from whole blood drawn into ACD has a physiologic pH. Whole blood was drawn into ACD (pH 5.1; ratio 1:7 ACD to blood) and centrifuged for 20 minutes at 150 x g to isolate platelet rich plasma. Cell free plasma was then generated by sequential centrifugation for 20 minutes at 1500 x g followed by an additional 2 minutes at 12,000 x g. The pH of isolated cell free plasma was determined using pH strips. Shown are pH strips (1, 2, 3) from three independent experiments with the reference guide on the right. The color of these three experiment test strips approximated physiologic pH (i.e. pH 7.4, black arrow).

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