Hemodynamic stress activates inflammatory responses and cell death through spectrin-dependent modulation of Store Operated Calcium Entry

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Abstract: Circulating blood cells, such as leukocytes, experience significant hemodynamic stresses. These stresses are significantly higher when a patient requires mechanical support of the circulation, such as cardiopulmonary bypass. To identify molecular mechanisms by which cells sense these stresses, we utilized genome wide CRISPRi and phosphoproteomics data. These screens identified non-erythroid spectrin 1 (SPTAN1) and RAF1 as effectors of hemodynamic stress in monocytes. We show that fluid stress induces SPTAN1/RAF1 signaling to promote STIM1 and ORAI1 interaction resulting in Store-Operated Calcium Entry (SOCE) thereby driving inflammation and cell death.

Blood cells, such as leukocytes, experience hemodynamic stresses as they circulate. These stresses are significantly increased when a patient requires mechanical support of the circulation or when the individual has narrowing of the cardiac valves/blood vessels. Exposure to higher hemodynamic stresses activates inflammatory pathways (*1*) that contribute to morbidity and mortality, such as is seen in patients recovering from cardiac surgery requiring cardiopulmonary bypass. Currently, the mechanisms by which hemodynamic stress stimulates non-adherent cells are elusive as compared to adherent cells. Previous work demonstrated that supraphysiologic stresses, such as present in cardiopulmonary bypass circuits, are sufficient to activate non-adherent monocytes into inflammatory phenotypes via a calcium dependent mechanism(*1*); however, we were not able to identify mechanisms by which hemodynamic stress stimulates calcium signaling in this previous work. To identify the pivotal molecular mechanisms by which non-adherent cells respond to shear stress, we have utilized a CRISPR-based genetic screen in conjunction with phosphoproteomics data to identify novel roles for SPTAN1, RAF1, and SOCE in the biomechanical activation of non-adherent monocytes.

CRISPR screen to identify genes involved in "sensing" hemodynamic stress. A genome wide CRISPR loss of function screen was used to gain insight into the molecular mechanisms that mediate the cellular response to flow stress in an unbiased manner (outlined in Figure 1A). We generated an *Interleukin-8-green fluorescent protein* (*IL8-GFP)* reporter in THP-1 cells, a human monocytic line. The *IL-8* promoter was used to drive *GFP* expression since exposure to hemodynamic stress of 2kPa, a supraphysiologic stress present during cardiopulmonary bypass and other clinical settings, is sufficient to activate *IL-8* expression by over 200-fold(*1*). *IL-8-GFP* cells were transduced with lentivirus CRISPR library(*2*) with a multiplicity of infection (MOI) of less than 1 with the goal of each infected cell expressing only one guide RNA (gRNA) thereby targeting one gene per a cell. Cells were flow sorted after exposure to fluid stress. The 10% cells with brightest GFP signal and the next 25% brightest cells were sorted into separate pools and sequenced along with unsheared infected cells. The MAgeckflute bioinformatic pipeline(*3*) was used analyze the sequencing data. Genes whose gRNAs were significantly underrepresented in the "bright" population as compared to the control populations, unsheared and the 25% less bright cells, were identified as candidate genes critical for shear activation of *IL-8* expression. 1628 candidate genes were identified with an adjusted $p<0.05$ (Figure 1C and Supplemental table 1). To our surprise genes identified as shear stress sensors in endothelial cells—such as *PECAM1*, *VEGFR2 (KDR)*, and *PIEZO1* (4-6)—were not identified as

candidates in the CRISPR screen. The absence of endothelial "flow sensor genes" suggests that circulating leukocytes utilize a different mechanism to sense shear stress as compared to adherent cells.

In an effort to gain deeper insight into candidate genes in the CRISPR screen, Metascape(*7*) analysis was used to identify gene ontology groups (GO-terms) that were enriched in the candidate gene list (key terms in Figure 1D and full list is in Supplemental table 2). Mitogen-activated protein (MAP) kinases (p-value=2.3×10^{-6}) were among the significantly enriched GO-terms. Several kinases in this subgroup—namely, MEKK2, MEK2, JNK3, and MEK—were previously identified as being critical to fluid stress activation of THP-1 cells(*1*), which helped validate the results of the screen.

The GO-term related to actin cytoskeleton was also one of the most significantly enriched (p-value=2.3×10^{-7}). *SPTAN1* (nonerythroid spectrin, p-value of 3×10^{-17}), *MYL2* (myosin II light regulatory chain, p=0.0003), *ACTA1* (α-actin, p = 0.001), *MYO1G* (myosin 1g, p = 0.01), *Septin8* (p=0.01), *ACTN1* (α-actinin, p = 0.02), *Septin6* (p=0.02), *Septin15* (p=0.03), and *Septin3* (p=0.04) are some of the candidate genes in this GO-term. We focused on SPTAN1, a component of the cell cortex(*8*), since it was member of the cytoskeleton GO-term that was among the top 50 candidates in the CRISPR screen based upon adjusted p-value.

SPTAN1 is important for the hemodynamic stress inflammatory responses of non-adherent cells.

The role of *SPTAN1* in mediating the response of non-adherent THP-1 cells to fluid stress was studied by using CRISPR to generate knockdown *SPTAN1* (*SPTAN1-KD*) cells. *IL8* expression, cellular necrosis, and ERK1/2 phosphorylation were used as readouts since these markers have been shown to be activated by hemodynamic stresses(*1*). We observed a significantly decrease in fluid stress induced *IL-8* expression *in SPTAN1-KD* as compared to control THP-1 cells (Figure 2A). We observed lower cellular necrosis in stressed *SPTAN1-KD* THP-1 cells as compared to control cells (Figure 2B-E). We also observed less flow mediated ERK1/2 phosphorylation in SPTAN1 deficient cells (Figure 2 F and G). Together, these data demonstrate SPTAN1 plays a key role in mediating fluid stress responses of non-adherent cells, which is a non-canonical function for SPTAN1.

RAF1 helps mediate SPTAN1's shear stress activation of non-adherent cells.

While previous studies have shown SPTAN1 can activate ERK signaling via a SRC dependent mechanism(*9*), we did not identify SRC as a candidate in our CRISPR screen (adj p-value=0.80) and inhibition of SRC signaling did not blunt the shear activation of *IL-8* expression (Supplemental figure 1). These data suggest an alternative pathway is involved in SPTAN1 modulation of ERK activity. We examined if KRAS/RAF1 signaling is a mechanism by which SPTAN1 stimulates ERK activity in the context of hemodynamic stress. *RAF1* is a candidate gene (adj p-value=7.88×10^{-15}) in the CRISPR screen that is a member of the MAPK GO-term(Figure 1D). In other contexts, RAF1 has been shown to activate ERK(*10*). In addition, *KRAS*, which is upstream of RAF1(*11*), had an adj-value of 0.06 in our CRISPR screen. We show KRAS

co-localized with SPTAN1 based upon immunocytofluorescence and proximity ligation assay (PLA) (Figure 3A and B). We also observe RAF1 colocalization with SPTAN1 (Figure 3C and D). *RAF1-knockdown* (*RAF1-KD*) cells have decreased flow mediated *IL-8* expression (Figure 3D) and necrosis (Figure 3F-I) as compared to wild-type cells. We also show flow mediated phosphorylation of ERK was decreased in RAF1 deficient cells by immunoblot (Figure 3J and K). Taken together, these data suggest a novel interaction between KRAS/RAF1 with SPTAN1 that plays an important role in fluid stress mediated cytokine expression and necrosis.

Hemodynamic stress activates store operated calcium entry via a SPTAN1/RAF1 mechanism.

We sought to identify the mechanisms by which hemodynamic stress modulates calcium signaling since extracellular calcium is necessary for fluid stress activation of non-adherent cells(*1*). Using phosphoproteomics, we identified proteins that were phosphorylated in response to flow stress (Supplemental table 3). In store-operated calcium entry (SOCE), Stromal Interacting Molecule (STIM) family members (STIM 1 and 2), migrate to the inner surface of the cell membrane to form a complex with ORAI Calcium Release-Activated Calcium Modulator (ORAI) family member channels and facilitate calcium entry. STIM1 phosphorylation between amino acids 616-634 increased by over 80% after 30 minutes of shear stress exposure ($p<7x10^{-60}$). ERK1/2 phosphorylation of this STIM1 region STIM1 has been shown to be crucial for SOCE(*12*). STIM2 phosphorylation between amino acids 717-730 increased by over 23% after 30 minutes of fluid stress exposure ($p<5.24x10^{-58}$) and *STIM2* is a candidate in CRISPR screen (p-value=$5.98x10^{-6}$).

In order to demonstrate that shear activates SOCE, we utilized a bimolecular fluorescence complementation STIM1/ORAI1 Venus reporter(*13*) in which Venus signal develops when tagged STIM1 and ORAI1 are in close proximity. Sheared STIM1/ORAI1 VENUS THP-1 cells have increased VENUS signal (10.6% +/- 0.7 Venus positive) as compared to static control (1.8% +/- 0.2)(Figure 4A-B). STIM1 and ORAI1 are important for the hemodynamic activation of *IL8* expression and necrosis since knocking down *STIM1* or *ORAI1* significantly reduced hemodynamic stress responsiveness as compared control cells (Figure 4C-G).

Given that ERK1/2 mediated phosphorylation of STIM1 is important for SOCE and our data that SPTAN1 and RAF1 modulate ERK1/2 signaling, we examined if SPTAN1 and RAF1 are involved in the shear activation SOCE by using the STIM1/ORAI1 VENUS cells. *SPTAN1* and *RAF1-KD* cells have decreased shear mediated VENUS signal demonstrating that SPTAN1 and RAF1 are upstream of SOCE in the context of fluid stress (24% reduction and 40.3% reduction, respectively)(Figure 4H). These data demonstrate that hemodynamic stress activation of STIM1/ORAI1 involve SPTAN1 and RAF1 signaling. In addition, we observed that SOCE is important for the flow mediated gene expression and cell death.

Based upon these data we propose the model (Figure 4I) wherein hemodynamic stress imposes a load on the cell cortex resulting in Spectrin activating RAF1. RAF1 in turn

facilitates SOCE. SOCE is important for the flow activation of *IL8* expression and cell death. To our knowledge, our data demonstrate novel roles for SPTAN1 and SOCE in the transduction of hemodynamic stress stimuli and novel interactions between STPAN1 and KRAS/RAF1. More broadly, these data, especially the CRISPR screen, provide an intellectual toehold into elucidating the mechanisms by which non-adherent cells “sense” and respond to hemodynamic stress. In addition to shedding light on a fundamental question in cell biology, our findings may have translational impact by opening the door to novel treatments to ameliorating the inflammation seen in situations where circulating leukocytes are exposed to supraphysiologic hemodynamic stresses.

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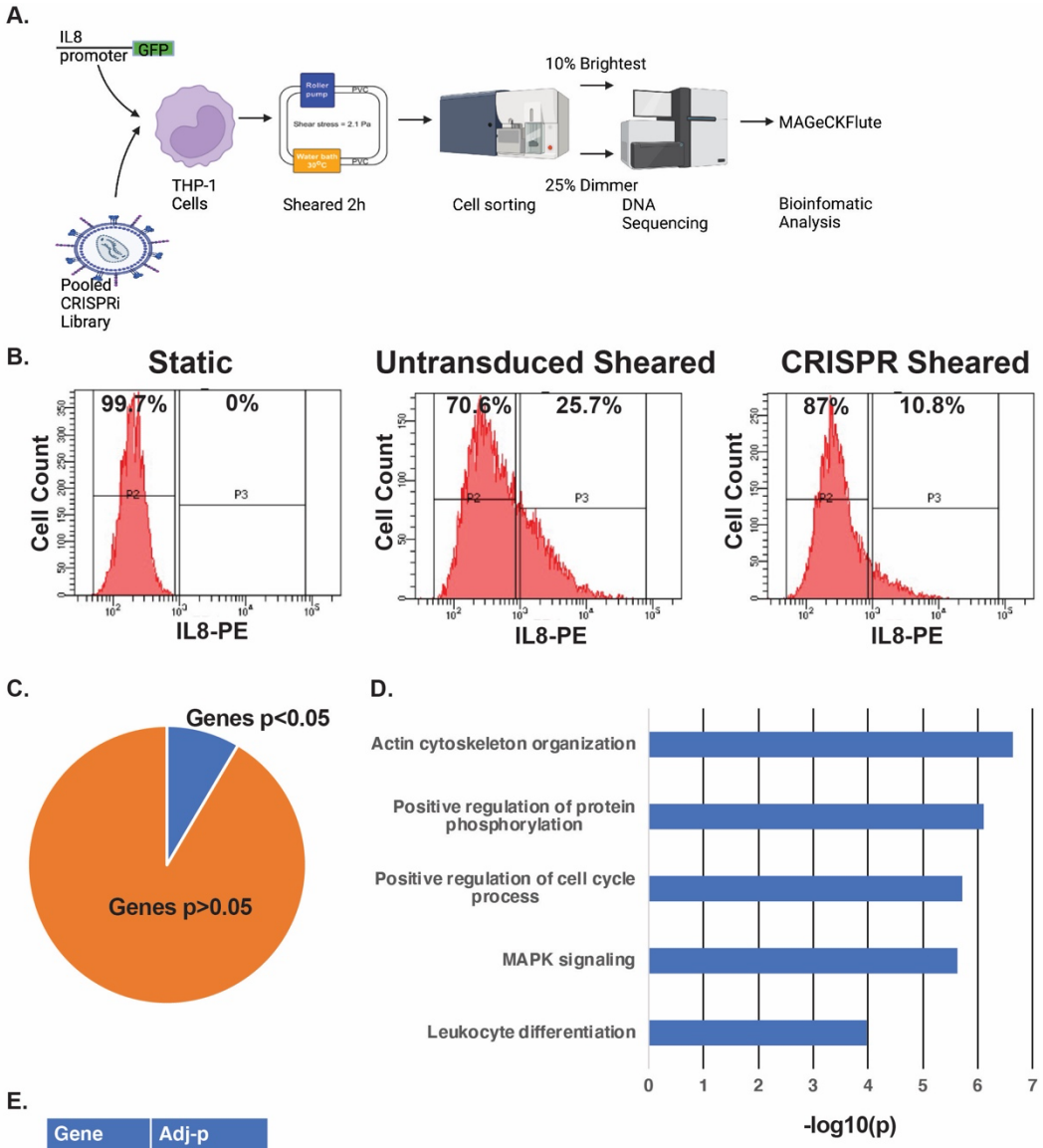
Author contributions. L.S., L.N.T, and J.S. performed the experiments. M.G. performed the mass spec experiments and related bioinformatics. A.T. performed the

bioinformatic for the CRISPR screen. R.S. supervised J.S. and helped edit the manuscript. V.N. conceptualized, supervised, and wrote the manuscript.

Data and material availability. The CRISPR screen data is being submitted to GEO.



E.

Gene	Adj-p value
SPTAN1	3.26x10-17
MYL2	0.0003
ACTA1	0.001
MYO1G	0.01
SEPTIN8	0.01
ACTN1	0.02
SEPTIN6	0.02
SEPTIN15	0.03
SEPTIN3	0.04

Figure 1. Pooled CRISPRi screen identifies candidate genes important for the hemodynamic stress activation of non-adherent cells. A. Experimental design of the CRISPRi screen. An *IL-8* promoter was used to drive GFP expression in THP-1 cells. Then, the cells were transduced with the Brunello CRISPRi lentivirus library. The transduced *IL8-GFP* THP-1 cells were exposed to supraphysiologic shear stress then

rested for 24 h. FACS was used to separate the 10% of the cells with the brightest GFP signal and next 25% brightest cells. DNA was isolated from these pools of cells and sequenced. The sequencing data was processed using the MAGeCKFlute bioinfomatic pipeline. **B.** IL-8 staining of control and sheared THP-1 demonstrates that transduction with the CRISPR library reduces the shear-mediated IL-8 staining. **C.** 8.5% (1628) genes' gRNAs were underrepresented in the 10% GFP bright cells with an adjusted-p value <0.05. **D.** Selected key GO-terms from the Metascape analysis of the candidate gene list. **E.** Candidate genes associated with cell cortex GO-term.

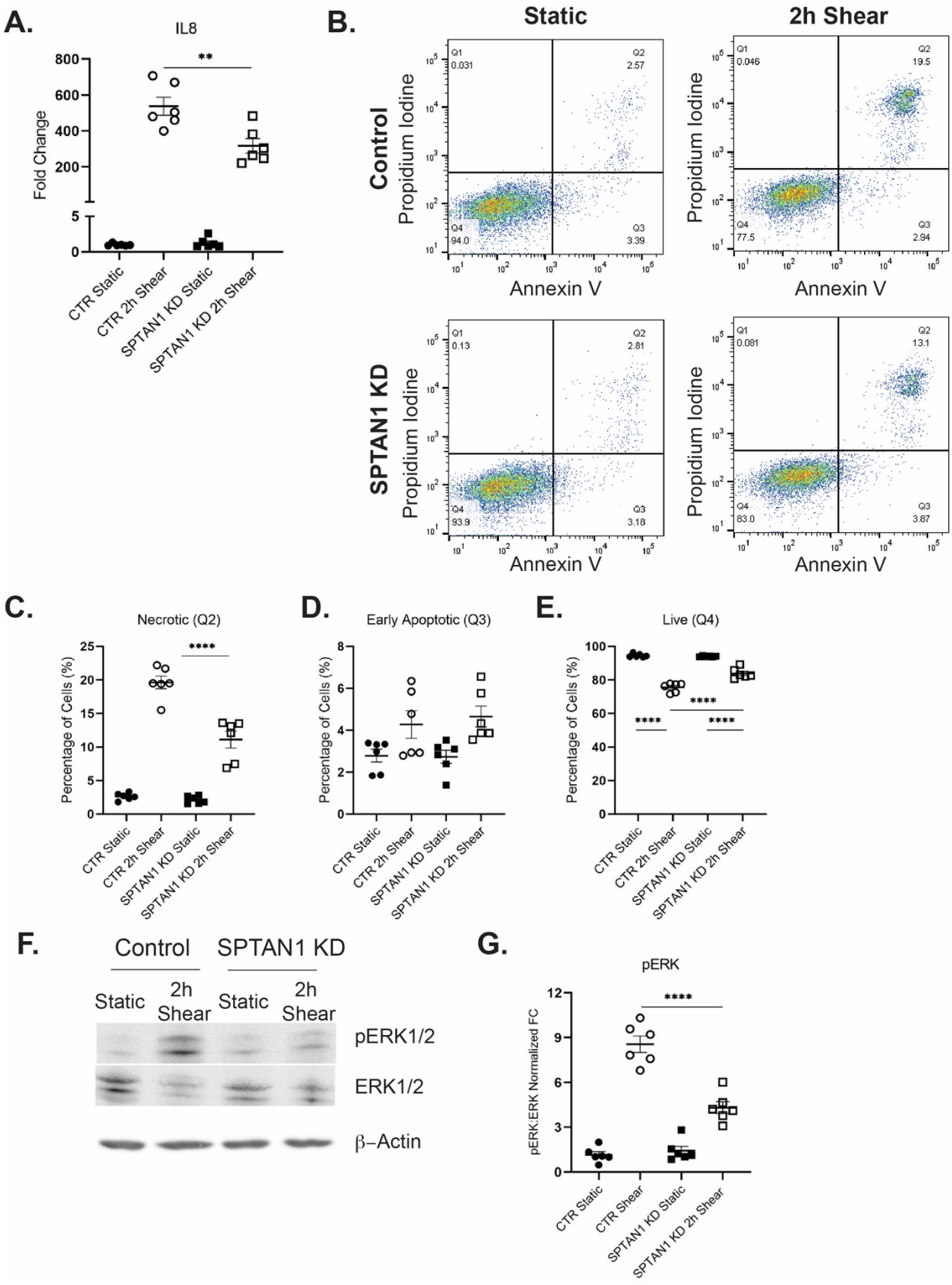

A.
IL8
Fold Change
800
600
400
200
5
0
**
CTR Static
CTR 2h Shear
SPTAN1 KD Static
SPTAN1 KD 2h Shear
B.
Static
2h Shear
Control
SPTAN1 KD
Propidium Iodine
Annexin V
Q1 0.031
Q2 2.57
Q4 94.0
Q3 3.39
Q1 0.046
Q2 19.5
Q4 77.5
Q3 2.94
Q1 0.13
Q2 2.81
Q4 93.9
Q3 3.18
Q1 0.081
Q2 13.1
Q4 83.0
Q3 3.87
C.
Necrotic (Q2)
Percentage of Cells (%)
****
D.
Early Apoptotic (Q3)
E.
Live (Q4)
F.
Control
SPTAN1 KD
Static
2h Shear
pERK1/2
ERK1/2
β–Actin
G.
pERK
pERK:ERK Normalized FC
CTR Static
CTR Shear
SPTAN1 KD Static
SPTAN1 KD 2h Shear

Figure 2. SPTAN1 is necessary for shear stress activation of THP-1 cells. A. *SPTAN1-KD* THP-1 cells have 41.2% reduction in the shear-mediated activation of *IL-8* expression as compared to sheared WT THP-1 cells based upon qPCR. N=6. **B.** Representative flow cytometry data using PI and Annexin V staining demonstrate decreased shear mediated cellular necrosis (Q3) in *SPTAN1-KD* THP-1 cells as compared to WT control cells. **C-E.** Quantification of cellular necrosis, early apoptosis, and live cells demonstrates a 43.4% reduction in cellular necrosis and 10.3% increase in live cells after shear in the *SPTAN1-KD* THP-1 cells as compared to control sheared cells. N=6. **F and G.** *SPTAN1-KD* cells have decreased shear-mediated ERK1/2 phosphorylation as compared to control THP-1 cells. N=3. **<0.01, ****<0.001.

Figure 3.

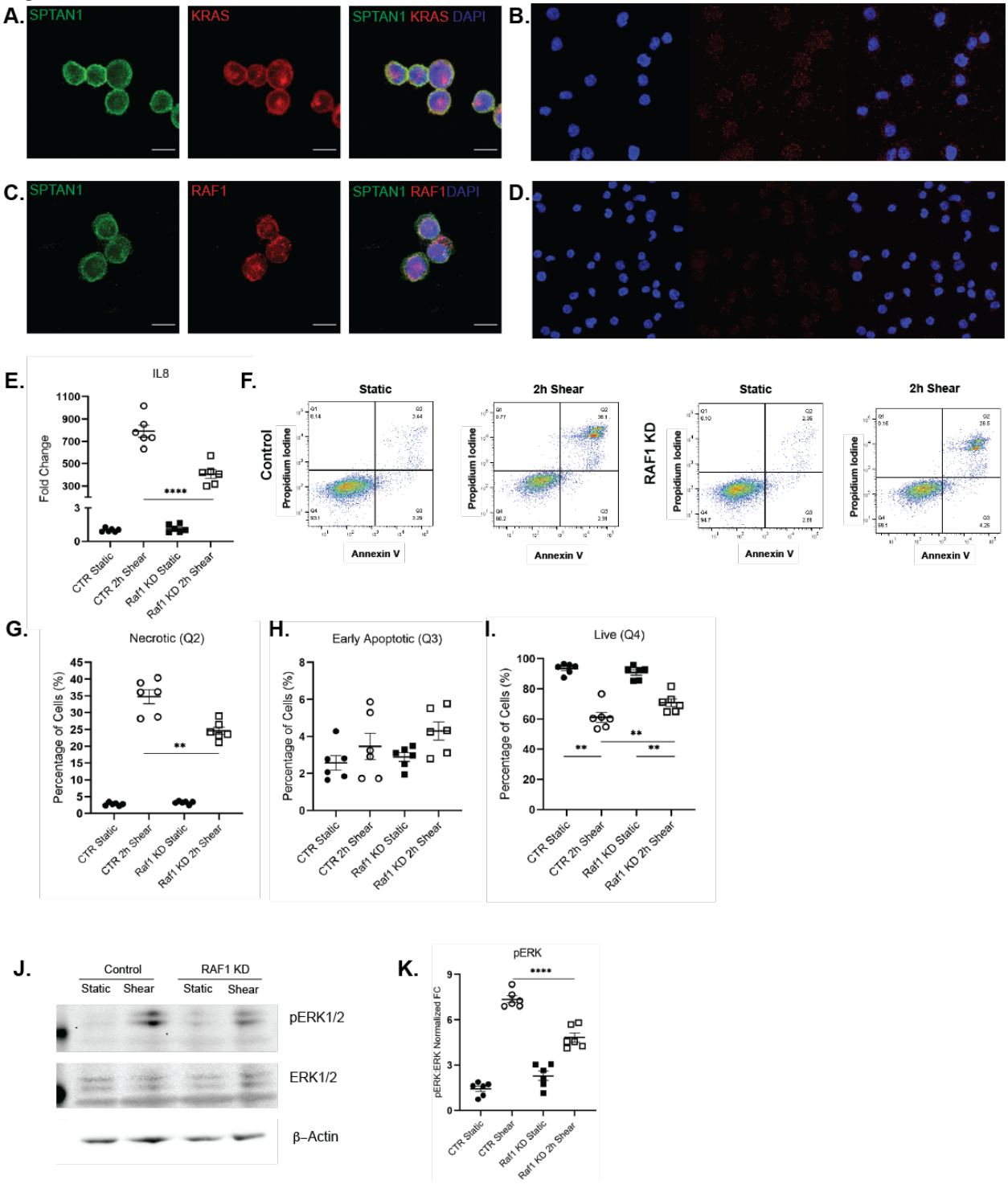


Figure 3. RAF1 colocalizes with SPTAN1 and contributes to the shear activation of non-adherent THP-1 cells. A and B. KRAS colocalizes with SPTAN1 in THP-1 cells based upon immunocytofluorescence (A) and PLA assay (B). **C and D.** RAF1 colocalizes with SPTAN1 based upon PLA (C) and ICC. **E.** *RAF1-KD* cells have 48.5% reduction in shear-mediated *IL8* expression as compared to sheared control cells. (n=6).

F-I. Flow cytometry data demonstrate that *RAF1-KD* cells undergo 29.3% less shear mediated necrosis and have 15.8% increased cell survival as compared to control THP-1 cells (N=6). **J and K.** Knockdown of *RAF1* decreases the shear-mediated ERK1/2 phosphorylation as compared to control THP-1 cells. N=3. *$p<0.05$, **$p<0.01$, ****$p<0.001$.

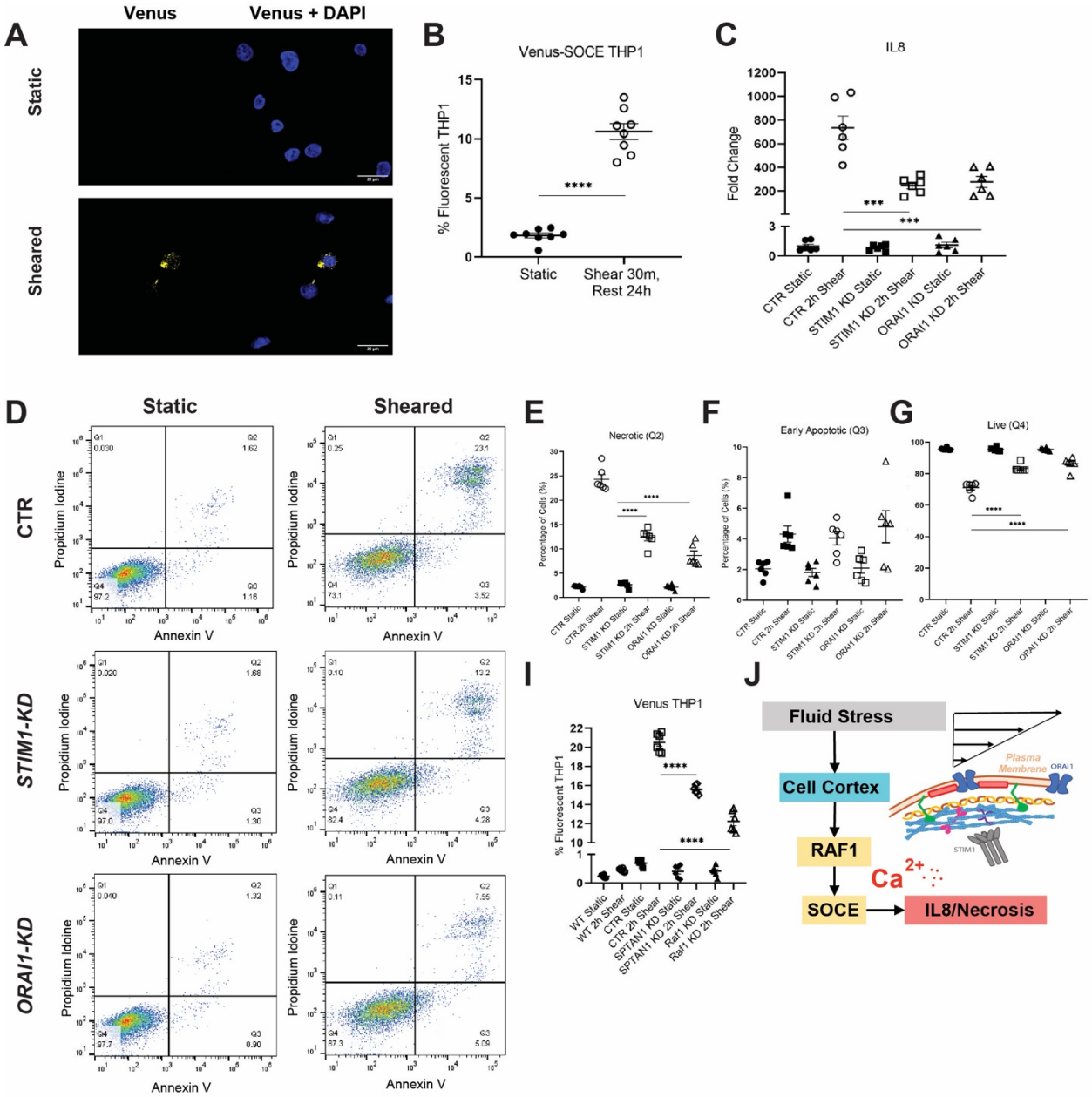


Figure 4. Hemodynamic stress activates STIM1/ORAI1 signaling via SPTAN1/RAF1 signaling. A and B. Shear stress increases STIM1/ORAI1 interaction based upon the increased Venus signal in split Venus STIM1/ORAI1 THP-1 cells(10.6%

+/-0.7% Venus positive in the sheared cells as compared to 1.8% +/-0.2% in static controls). **C.** *STIM1-KD* and *ORAI1-KD* cells have decreased shear mediated *IL8* expression as compared to control THP-1 cells. N=6. **D-G.** Flow cytometry data demonstrate that *STIM1-KD* and *ORAI1-KD* cells have decreased shear mediated cellular necrosis and have increased cell survival. N=6. **H.** *SPTAN1-KD* and *RAF1-KD* cell have decreased STIM1/ORAI1 interaction based upon decreased split Venus STIM1/ORAI1 signal. N=6. **I.** Based upon our data, we propose the model that shear stress imposes a load on the cell cortex that increases KRAS/RAF1 activity. RAF1 in turn activates ERK1/2. ERK1/2 promotes STIM1/ORAI1 interaction. The resulting calcium influx results in increased cytokine expression and necroptosis. ***$p<0.005$, ****$p<0.001$.

Methods:

Cloning:

Generation of *IL8-GFP* reporter construct

pCDH-CMV-Nluc (a gift from Kazuhiro Oka, Addgene 73038) was cut with ClaI and BamHI to remove CMV enhancer and promoter. Human IL8 (hIL8) promoter was PCR cloned from genomic DNA using primers CTCATCGAT GTAACGTGATCACTGCCATGT and CTCGGATCC AGTGCTCCGGTGGCTTTTTA and ligated into the ClaI and BamHI sites of the pCDH backbone. *Enhanced GFP (EGFP)* was PCR cloned from pLenti CMV GFP Puro (Addgene 658-5) with primers CTCGTCGACCATGGTGAGCAAGGG and CTCGGATCC CGTTACTTGTACAGCTCGT and cloned into *hIL8*-pCHD at the SalI and BamHI restriction sites.

Cloning for bimolecular fluorescence complementation (BiFC) of store operated calcium entry (SOCE) genes lentivirus constructs:

N-terminal Venus fragment fused to full-length ORAI1 and C-terminal Venus fused to full-length STIM1 were cut out and amplified from plasmids pcDNA3-Venus-173-N-ORAI1 (a gift from Jin Zhang; Addgene plasmid #87618) and pcDNA3.1-STIM1-Venus-173-C (a gift from Jin Zhang; Addgene plasmid #87619), respectively. These two inserts were separately cloned into the pCDH-CMV-Nluc plasmid (a gift from Kazuhiro Oka; Addgene plasmid #73038) in place of the Nanoluciferase (Nluc) reporter using BamH1 and EcoR1 restriction sites to yield two distinct plasmids, each containing a unique fragment for BiFC. These plasmids were used for lentiviral transfection in 293T cells, and viral supernatant containing the lenti-plasmids with V173N-ORAI1 and STIM1-V173C fragments were transduced into one sample of THP-1 cells at multiplicity of infection of 10.

Cell Culture and viral transfection:

Human monocytic cells THP-1 (ATCC) were cultured in RPMI-1640 media supplemented with 10% FBS (Gibco), 50 U/mL penicillin, and 50 µg/mL streptomycin (Gibco) in 5% CO2 at 37°C. Human embryonic kidney cells Lenti-X 293T (Clontech) were cultured in DMEM media supplemented with 10% FBS (Gibco), 50 U/mL penicillin, and 50 µg/mL streptomycin (Gibco) in 5% CO2 at 37°C. pCDH-hIL8-EGFP plasmid was transfected into 293T cells using TransIT-Lenti (Mirus Bio). Viral supernatants were collected at 48 hours after transfection and transduced in THP-1 cells.

Exposure to hemodynamic stress.

For the *in vitro* hemodynamic stress model, monocytes at a density of 2 million cells/mL were sheared for either 2hrs or 30min in a 10ft-long Masterflex® Tygon E-3603 L/S13 pump tubing (Cole-Parmer), using the Masterflex® miniflex pump model 115/230 VAC 07525-20 (Cole-Parmer) at 10 mL/min. More than three-quarters of the tubing was submerged in the water bath for temperature control at 30°C.

CRISPR Screen and *in vitro* CPB shear treatment:

Wild-type and *IL8-GFP* THP-1 cells were transduced with a lentiviral preparation of the Brunello gRNA pooled library in lentiCRISPRv2 (a gift from David Root and John Doench, Addgene #73179-LV) at multiplicity of infection (MOI) of 1. Cells were

puromycin selected after 4 days post transduction and recovered for another 2 days. Then, sheared in an *in vitro* CPB system (ref) at 2 million cells/mL at a rate of 10mL/min for 2 hours. After shear, cells were prepared for flow cytometry and fluorescence-activated cell sorting (FACS).

IL8 Flow Cytometry:

On the day of shear treatment, transduced THP-1 cells were pre-treated with eBioscience™ Protein Transport Inhibitor Cocktail 500x (Thermo 00-4980-93) at 10µL/mL for 30 minutes. Sheared Brunello library transduced and untransduced THP-1 along with static control THP-1 cells were washed in 1.5mL PBS, fixed in 1mL IC Fixation Buffer (Thermo 00-8222-49) and incubated at 20 minutes in the dark at room temperature, with gentle agitation. Cells were permeabilized in 1.5mL 1x Permeabilization Buffer (00-8333-56), incubated at room temperature for 10 minutes and spun at 500 x g for 5 minutes. Directly conjugated IL8 antibody (Invitrogen, 12808842) was incubated with THP-1 cells in 1x Permeabilization Buffer for 45 minutes in the dark. Cells were then washed in more 1x Permeabilization Buffer and resuspended in Flow Cytometry Staining Buffer (Thermo 00-4222-26) at 10 million cells/mL for flow cytometry analysis.

IL8-GFP FACS:
Sheared Brunello library transduced THP-1 cells were sorted using FACS analysis (BD FACSAria) for GFP intensity. Cells were sorted into two groups, the top 10% intensity and the next or subsequent 25% intensity. Transduced but non-sheared cells were fixed and stained using the same protocol to serve as the baseline.

Genomic DNA Extraction and Next-Generation Sequencing:
Sorted and fixed cells were spun at 300g for 3 mins. Proteinase K (Zymo) was added at 5uL and incubated at 55C for 2 hours. Samples were then de-crosslinked by incubation at 94C for 20 mins and subsequently lysed and extracted for genomic DNA (gDNA) using the Quick-DNA/RNA Miniprep Kit (Zymo D7005T). gDNA samples of both samples were sent to Cellecta Inc. for next-generation sequencing, using Illumina NextSeq.

Bioinformatic analysis.
MAGeCKFlute was used to analyze the sequencing data for gRNAs that were enriched. Data from the 10% brightest cell population were compared to the data from the next 25% brightest and unsheared cell populations.

The candidate genes underrepresented in the 10% brightest pool with an adjusted p-value<0.05 were inputted into Metascape to identify enriched GO-terms.

For CRISPR/Cas9 mediated-gene knockdowns of STIM1, ORAI1, SPTAN1 and Raf1.
Lentiviral vectors together with helper plasmids encoding gag, pol and rev, were co-transfected in 293T cells using TransIT-Lenti (Mirus Bio). Viral supernatants were

collected at 48 hrs and 72 hrs after transfection and centrifuged at 1,000 g for 10 mins. THP-1 cells were then transduced at the multiplicity of infection of 10 using the viral supernatants supplemented with 5 µg/mL polybrene for 6 hrs. Three days after transduction, positive cells were selected in 1 µg/mL puromycin for 72 hrs, if necessary. Gene knockdowns for *SPTAN1* and *Raf1* were also transduced into THP-1 cell lines containing the Venus BiFC plasmids and studied for shear stress experiments. Raf1 gRNA sequence derived from a 3rd generation lentiviral gRNA plasmid targeting human Raf1 (A gift from John Doench, David Root, Addgene plasmid #76708).
Specifically, for CRISPR/Cas9-mediated gene knockout, we first generated a doxycycline-inducible Cas9-expressing THP-1 cell line (iCas9-expressing cells) by transducing THP-1 cells with lentiviruses carrying the Lenti-iCas9-neo plasmid (a gift from Qin Yan; Addgene plasmid #85400). Multiple guide RNAs targeting a gene were cloned into the Lenti-multi-Guide plasmid (a gift from Qin Yan; Addgene plasmid #85401) and delivered to iCas9-expressing THP-1 cells by the lentiviral system. gRNA sequences can be found in Table S2. Cells were then treated with 1 µg/mL of doxycycline for 48 hrs to induce editing at the targeted loci.
For *IL8* gene expression analysis by qPCR and pERK/ERK western blotting, THP-1 knockdown samples were sheared for 2hrs and collected immediately for RNA and total lysate. For cell death assaying, THP-1 knockdown samples were sheared for 2 hrs and then rested in 37°C for 24 hrs, and then prepped for flow cytometry analysis. For ICC/IF and PLA analysis, wild type THP-1 cells were sheared for 2 hrs and then prepped onto microscope slides. For Venus BiFC analysis, THP-1 cells containing Venus BiFC of ORAI1 and STIM1 were sheared for 30 mins, rested at 37°C for 24 hrs, and then prepped for confocal microscopy imaging and flow cytometry analysis. Venus BiFC THP-1 cell lines with SPTAN1 and Raf1 knockdowns were sheared for 2 hrs, , rested at 37°C for 24 hrs, and then prepped for flow cytometry analysis.

Flow Cytometry and cell death assays:
For the cell death assays, THP-1 cells were stained with Alexa Fluor 488® annexin V and propidium iodide according to the manufacturer's protocol for the Dead cell apoptosis kit (V13241, Thermo Fisher). Samples were immediately analyzed by flow cytometry.
For flow cytometry on samples containing Venus BiFC, samples were collected and fixed in 2% paraformaldehyde, and then washed and suspended in PBS for flow cytometry analysis using the BD® LSR II Flow Cytometer. An Alexa 488 laser was used for excitation of the cells, and the band-pass filter of 530/30nm was used to capture the emission of Venus fluorescence.
Quantitative analysis and gating were performed on all flow cytometry experiments using FlowJo software.

Immunocytochemistry/Immunofluorescence (ICC/IF):
THP-1 cells were fixed in 2% paraformaldehyde for 10 mins at room temperature, centrifuged at 300g for 5 mins and washed with 1 mL of PBS. Cells were suspended in 20 µL of PBS, spread on microscope slides, let air-dried and rinsed with water before being stored at 4°C. When ready for ICC/IF procedures, fixed cells were permeabilized with 0.1% Triton X-100 made in PBS solution. ICC/IF analyses were performed using

primary antibodies against SPTAN1 (MAB1662), k-Ras (AB108602), and Raf1 (AB137435) and secondary antibodies Alexa 488 (A32723 mouse, Life Technologies) and Alexa 594 (A32740 rabbit, Life Technologies). Images were taken using confocal microscopy techniques.

Duolink® proximity ligation assay (PLA):
Protein-protein interactions (PPI) of SPTAN1-kRas and SPTAN1-Raf1 were identified using the Duolink® proximity ligation assay (PLA) in situ red for mouse/rabbit antibodies (DUO92101, Sigma). THP-1 cells were fixed onto microscope slides and permeabilized as outlined in the ICC/IF section. Fixed THP-1 cells were incubated with two primary antibodies for each PPI, SPTAN1 (MAB1662 mouse, Sigma) and k-Ras (AB108602 rabbit, Abcam), and SPTAN1 (MAB1662 mouse, Sigma) and Raf1 (AB137435 rabbit, Abcam) in a dark box overnight in 4°C. After primary incubation and PBS wash, the samples were incubated with secondary antibodies conjugated with PLA probe oligonucleotides (PLA PLUS/MINUS). Subsequently, a ligation solution containing hybridizing connector oligos and ligase enzyme was added to join the two PLA probes in close proximity (<40nm). Lastly, an amplification solution was added containing a DNA polymerase and red fluorescently labeled complementary oligonucleotides to create the red fluorescent puncta signal detected and imaged by confocal microscopy.

Statistics:
All statistical analyses were performed using Prism 8 (GraphPad). Numeric differences between 2 groups were compared using a 2-tailed Student's t test. Comparisons of experimental groups against a control group (e.g., CTR Shear 2h) were performed using 1-way ANOVA and post hoc Dunnett's test. Other pairwise comparisons for more than 2 groups were performed using 1-way ANOVA and post hoc Tukey's test. $P < 0.05$ was considered significant. Data are represented as mean ± SEM.

Phospho-proteomics:
Sheared and static wild-type THP-1 cells were pelleted and resuspended in PBS with protease and phosphatase inhibitor.

Protein digestion and sample preparation for mass spectrometry
3 mls of 6 Molar Guanidine solution was added to cell pellet and mixed. The samples were then boiled for 5 minutes followed by 5 minutes cooling at room temperature. The boiling and cooling cycle was repeated a total of 3 cycles. The proteins were precipitated with addition of methanol to final volume of 90% followed by vortex and centrifugation at maximum speed on a benchtop microfuge (4000 rpm) for 20 minutes. The soluble fraction was removed by flipping the tube onto an absorbent surface and tapping to remove any liquid. The pellet was suspend in 4ml of 8 M Urea made in 100mM Ammonium Bicarbonate. TCEP was added to final concentration of 10 mM and Chloro-acetamide solution was added to final concentration of 40 mM and vortex for 5 minutes. 3 volumes of 50mM ammonium bicarbonate was added to the sample to reduce the final urea concentration to 2 M. Trypsin was in 1:50 ratio of trypsin and incubated at 37C for 48 hours. The solution was then acidified using TFA (0.5% TFA final concentration) and mixed. Samples were desalted using 100 mg C18-SPR (waters)

as described by the manufacturer protocol. The peptide concentration of sample was measured using BCA. 100 ug of each were then labeled using TMT10 (as suggested in manufacturer protocol, Thermo) for one hour and quenched using hydroxylamine. The samples were then pooled and dried.
High-Select Fe-NTA Phosphopeptide Enrichment (A32992 Thermo Scientific): 1 mg of total peptide is used following manufacturer's protocol.
LC-MS-MS: 1 ug of total enriched phospho-peptides were analyzed by ultra-high pressure liquid chromatography (UPLC) coupled with tandem mass spectroscopy (LC-MS/MS) using nano-spray ionization. The nanospray ionization experiments were performed using a Orbitrap fusion Lumos hybrid mass spectrometer (Thermo) interfaced with nano-scale reversed-phase UPLC (Thermo Dionex UltiMate™ 3000 RSLC nano System) using a 25 cm, 75-micron ID glass capillary packed with 1.7-µm C18 (130) BEH™ beads (Waters corporation). Peptides were eluted from the C18 column into the mass spectrometer using a linear gradient (5–80%) of ACN (Acetonitrile) at a flow rate of 395 µl/min for 3h. The buffers used to create the ACN gradient were: Buffer A (98% H_2O, 2% ACN, 0.1% formic acid) and Buffer B (100% ACN, 0.1% formic acid). Mass spectrometer parameters are as follows; an MS1 survey scan using the orbitrap detector (mass range (m/z): 400-1500 (using quadrupole isolation), 120000 resolution setting, spray voltage of 2200 V, Ion transfer tube temperature of 275 C, AGC target of 400000, and maximum injection time of 50 ms) was followed by data dependent scans (top speed for most intense ions, with charge state set to only include +2-5 ions, and 5 second exclusion time, while selecting ions with minimal intensities of 50000 at in which the collision event was carried out in the high energy collision cell (HCD Collision Energy of 33%), and the fragment masses where analyzed in the ion trap mass analyzer (With ion trap scan rate of turbo, first mass m/z was 100, AGC Target 5000 and maximum injection time of 35ms). Protein identification and label free quantification was carried out using Peaks Studio 8.5 (Bioinformatics solutions Inc.)

Cell culture and treatments:
Human monocytic cells THP-1 (ATCC) were cultured in RPMI-1640 media supplemented with 10% FBS (Gibco), 50 U/mL penicillin, and 50 µg/mL streptomycin (Gibco) in 5% CO_2 at 37°C. Human embryonic kidney cells Lenti-X 293T (Clontech) were cultured in DMEM media supplemented with 10% FBS (Gibco), 50 U/mL penicillin, and 50 µg/mL streptomycin (Gibco) in 5% CO_2 at 37°C.
For experiments studying the effect of src-selective tyrosine kinase inhibitor PP1 (5678091MG, MilliporeSigma), THP-1 cells were pre-incubated with 25µM PP1 inhibitor or vehicle (DMSO) for 30 minutes before shearing for 2 hrs.

RNA isolation and quantitative PCR:
Static and sheared cells were collected at a density of 2 million cells/mL, spun at 300g x 3mins, and lysed in 350µL RLT buffer from RNeasy® Mini Kit (74104, Qiagen). RNA was then isolated using the manufacturer's protocol for the RNeasy® Mini Kit column purification. cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (4368814, Thermo Fisher). Gene expression of IL8 was quantified using SYBR® Green detection method (primer sequence in Supplemental Table S1). All

expression data were normalized to internal control genes, 18S ribosomal RNA (RN18S1) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for human samples. Relative quantification of fold-change was performed using the 2-Δ/ΔCt method.

Western Blot:
Static and sheared cells were collected at a density of 2 million cells/mL, spun at 300g x 3mins, and lysed in 100uL of RIPA (89900, Thermo Fisher). Total protein concentrations in cell lysates were measured using the Pierce™ bicinchoninic acid (BCA) assay kit (23225, Thermo Fisher). 10-15 µg of proteins were separated by SDS-PAGE and immunoblotted using the following antibodies: p-ERK1/2 (9101, Cell Signaling), ERK1/2 (4695, Cell Signaling), SPTAN1 (MAB1622, Sigma), Raf1 (AB137435, Abcam), and the loading control β-actin ACTB (SC-47778, Santa Cruz). Co-detection was performed using IRDye 700 and 800-labeled secondary antibodies (926-68070 and 926-32211, Li-Cor) under the Odyssey infrared laser fluorescence scanner (Li-Cor) and quantified using ImageJ.

Table S1. List of SYBR primers

Primers	Species		Sequence
IL8	Human	F R	5' – CTTGGCAGCCTTCCTGATTT – 3' 5' – TTCTTTAGCACTCCTTGGCAAAA – 3'
GAPDH	Human	F R	5' – ACAACTTTGGTATCGTGGAAGG – 3' 5' – GCCATCACGCCACAGTTTC – 3'
RN18S1	Human	F R	5' – GGACATCTAAGGGCATCACAG – 3' 5' – GAGACTCTGGCATGCTAACTAG – 3'

Table S2. List of gRNAs used for CRISPR/Cas9-mediated gene knockdown:

Gene	Species	gRNA Sequence
STIM1	Human	5' – GGTAATTGAGGTCTTCCCTC – 3' 5' – GGATAAGCTCATCAGCGTGG – 3' 5' – CCGTAACATCCACAAACTGA – 3'
ORAI1	Human	5' – CGCTGACCACGACTACCCAC – 3' 5' – CCTCAACGAGCACTCCATGC – 3' 5' – TGGAGGCTTTAAGCTTGGCG – 3'
SPTAN1	Human	5' – AGTGGCAGCTCGTATGAATG – 3' 5' – GTGGAAGTGAACGATCGTCA – 3' 5' – CTTGGATCAATGGAATACGG – 3'
RAF1	Human	5' – GACCATGTGGACATTAGGTG – 3'

Legend for supplemental data.

Supplemental Table 1. MAGeCKFlute data from sheared THP-1 CRISPR screen.

Supplemental Table 2. Metascape analysis of the candidate genes from the CRISPR screen. The 1628 genes with an adjusted p-value <0.05 were inputted in to Metascape.

Supplemental Table 3. Phosphoproteomics data comparing static and sheared THP-1 cells.

Supplemental Figure 1. Src inhibitor PP1 does not reduce the shear activation of *IL8* expression.

Supplemental Figure 2. Western blots demonstrating knockdown of SPTAN1, RAF1, STIM1, and ORAI1 in THP-1 cells.