Platelet-driven monocyte activation promotes hypoxic thromboinflammation through the HIF-1α-NLRP3-EGR-1 axis

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SUPPLEMENTAL MATERIALS

Detailed Methods

Culturing, Maintaining Cell lines and their treatments

THP-1, the human leukemia monocytic cell line; K-562, the human myelogenous leukemia cell line was obtained from the National Centre for Cell Science (NCCS, Pune, India). It was cultured, and maintained in RPMI-1640 media which was supplemented with Fetal bovine serum (FBS)-10%, and penicillin-streptomycin (PS)-1% antibiotic- at a cell density of 0.5×10^6/ml and 3×10^6/ml respectively in a 5% CO_2 incubator at 37°C.[1] THP-1 were differentiated to macrophages with 20ηM PMA for 12 h and then replaced with fresh growth media for another 12 h before treatment.[2] The megakaryocytic differentiation of K-562 into platelets was done using 50 ηM PMA for 24 h and then replaced with 12 h before treatment.[1,3] The human umbilical vein cell line, Ea. hy926 was a kind gift from Central Drug Research Institute (CDRI), Lucknow, India, which was cultured, in DMEM media supplemented with 15% FBS and 1%PS antibiotic at a cell density of 1×10^5/ml at 37°C in a 5% CO_2 incubator. Human umbilical vein endothelial cells (HUVECs) were a kind gift from Institute of Genomics and Integrative Biology (IGIB), Delhi, India. HUVECs were maintained in Human Large Vessel Endothelial Cell Baαsal Medium supplemented with Low Serum Growth Supplement (LSGS). For isolation of hPBMCs, peripheral venous blood was collected from donors/patients at ESIC Medical College with the written informed consent and approval from the Institutional Ethics Committee of ESIC Medical College, Faridabad. Blood was drawn into sterile vacutainer tubes containing EDTA and processed within 2h of collection. Briefly, whole blood was diluted 1:1 with PBS in a 15ml falcon. The diluted blood was carefully layered over Histopaque® 1077 (MP Biomedicals) in a fresh 15 mL tube by slowly dispensing the sample down the tube wall to avoid mixing of layers. Samples were centrifuged at 400xg for 30 min at RT with the brake off. Following centrifugation, the PBMC layer appearing as a whitish, cloudy band at the plasma–Ficoll interface was carefully aspirated and transferred to a new tube. The harvested hPBMCs were washed twice with sterile PBS (300xg 5 min at RT) to remove residual platelets and Ficoll. The cell pellet was resuspended in RPMI-1640 medium (Gibco) supplemented with 2% heat-inactivated fetal bovine serum (FBS; Gibco) for cell counting and viability assessment. Freshly isolated hPBMCs were used immediately for downstream experiments.

Monocyte endothelial Adhesion assay

After experimental treatment, THP-1 cells or hPBMCs were incubated with 2′,7′- bis-(Carboxyethyl)-5(6′)-carboxyfluorescein Acetoxymethyl Ester (BCECF-AM) dye.[4] After internalization, the acetoxymethyl ester hydrolyzed, resulting in BCECF labeled cells. After Phosphate-buffered saline (PBS) washed twice, the labeled cells were added to activated endothelial cells (Ea. hy926 or HUVECs), subjected to hypoxia for 8 h, and incubated for 30 min at 37°C. After PBS washed, the fluorescence intensity was measured. The adherence of monocytes was extrapolated using standard curves produced by serially diluting a known number of labeled cells.

Gene Expression/Transcriptional analysis

In accordance with the manufacturer's instructions, total RNA was isolated from the all the cells and thrombosed IVC/whole blood samples using TRIzol reagent (Sigma-Aldrich). Briefly, an iScript cDNA synthesis kit (BioRad) was utilized to reverse-transcribe 1 µg of total RNA. SyBR Green (Thermo Fisher) for quantification of mRNA was used in quantitative real-time PCR machine (StepOne Real-Time PCR). Each gene's C^t values were compared to the housekeeping 18sRNA for normalization, and the ΔΔCt method was utilized to determine the relative expression of each gene.

Histopathological analysis

Liver tissue samples from all the mentioned groups of animals were fixed in formalin solution and morphological assessment was done. These specimens underwent regular drying for the histological section, paraffin embedding, sectioning, and mounting on poly-lysine coated glass slides for the ensuing hematoxylin and eosin staining and TF immunohistochemistry analysis.

Immunocytochemistry

THP-1, the human monocytic cell line was stimulated to differentiate into macrophages induced with 20ηM PMA on coverslips coated with poly l-lysine (Sigma-Aldrich) at a cell density of $5×10^5$. After 12 h of PMA induction, following 12 h in fresh media incubation. The cells were stimulated with experimental conditions. After PBST (PBS having tween20) wash thrice, the cells with the help of 4% paraformaldehyde fixed at room temperature (RT) for 15 min (Sigma-Aldrich). Two

consecutive washes with ice-cold PBS were then performed. After permeabilization with PBST (PBS containing Triton X-100-0.2%) for 10 min, the cells were washed thrice with PBS. Subsequently, with 5% BSA (Himedia) the cells were blocked for 30 min and incubated with primary antibodies against NLRP3 (1:200 dilution), and TF (1:200 dilution) in a humidified chamber at 4°C with a gentle shaker. This was followed by PBS washing thrice for 5 min followed by incubation of the cells with Alexa Fluor 488, and Alexa Fluor 568 respectively tagged secondary antibody (Thermo Fisher Scientific, Inc.) for 1 hr at RT in the dark. Following a PBS wash, the cells were mounted on a glass slide with DAPI (ProLong™ Gold Antifade Mountant) on a glass slide and images were captured using fluorescence microscopy (ZOE Fluorescent Cell Imager, BioRad). The image J image analysis software was used to measure the intensity of each image, and the plot was made using Graphpad software.

Enzyme-linked Immunosorbant assay (ELISA)

To determine the activated HIF-1α, NLRP3 levels, cytokine IL-1β levels, Egr-1 and TF antigen in cell culture supernatants/lysates from leukocytes/monocytes (THP-1) and platelet-monocytes co-culture, after experimental treatment using an ELISA kit according to the manufacturer's instruction (Krishgen Biosystem/ElabBiosciences). Briefly, 100μl of cell culture supernatants/lysates was pipette into each sample well of a 96-well plate as required. After sealing the plate, it was incubated for 90 min at 37°C. Following the sample's disposal, diluted wash buffer was used four times to wash the plate's well. The plate's wells were then pipetted with diluted biotinylated antibody, and the mixture was incubated for 60 min at 37°C. The plate was again washed four more times, and conjugated HRP was pipetted to each. Subsequently, the plate was incubated for 30 min at 37°C. After final wash, TMB substrate was added to the wells and the plate was incubated for 10 min at 37°C. And, with the use of stop solution, the reaction was stopped and the absorbance (OD) was taken at 450ηm in a multimode microplate reader (Synergy HTX Multi-Mode Reader) within 10-15 min. The levels of protein were estimated from the standard curve.

Knockdown of HIF-1α, NLRP3, EGR-1 using siRNAs

In order to perform RNA interference (RNAi), THP-1 cells were maintained to 50–60% confluence in 6 well plates at a cell density of $0.5*10^6$/well. As per the manufacturer's instructions, the cells were transfected using Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA). Briefly, 242.5μl of Opti-

MEM (Invitrogen) was mixed with 1.5μl siRNA of HIF-1α, NLRP3, and EGR-1 gene solution (100μmol) separately and 6μl Lipofectamine™ 3000 and was prepared for each well that was going to be transfected. A gentle mix was made of the two solutions, and they were incubated at RT for 5 min. The solution was added to serum free media and subsequently added to each well. After 8 hrs of incubation in transfection media, the cells were further maintained for an additional 12 hours in fresh complete growth media lacking PS antibiotic at 37°C at 5% CO2.[5]

Chromatin Immunoprecipitation Assay

Following the experimental treatment, the cells were fixed in 1% paraformaldehyde, and a Fisher Scientific F550 microtip cell sonicator was used to shear the chromatin that was extracted from the separated nuclei. After centrifugation, the chromatin-sheared supernatants were incubated with either control IgG (Thermo Fisher) or an anti-HIF-1α antibody from Affinity Biosciences. After adding Protein G Sepharose beads and incubating for overnight at 40°C, the immune complexes were eluted. After being subjected to RNase and proteinase K treatment, complexes were extracted using phenol-chloroform method and subsequently use of isopropanol. After precipitating, cleaning, drying, and resuspending in water, DNA was examined using PCR. The primers employed in this analysis, sense (5′-ATTTGGAGTGGCCCGATATGG -3′) and antisense (5′-AGGAAGCCCTAATATGGCAGG -3′), spanned 271bp around the putative HIF-1-binding region within the Egr-1 promoter.

Microparticles bound tissue factor activity (modified plasma recalcification assay)

Preparation and storage of microparticles

The THP-1 human monocytic cell lines were seeded in six-well plates and differentiated to macrophages with 20 nM PMA for 12 h and kept for 12 h in fresh RPMI-1640 media. After PBS wash, the cells were treated with hypoxia for 8h, DMOG-1000μM for 12 h, DIM-10μM, MCC950-10μM, and SML0499- 10μM for 4 h each. The total microparticles (MPs) were isolated from culture media/cell supernatants immediately after stimulating the cells and pelleting out. Microparticles were sedimented at $2*10^4*g$ at 4°C for 30 min and resuspended at tris-buffered saline (TBS containing- 100M NaCl, 50mM tris-HCl, pH 7.4) containing 1% BSA (TBS/BSA). Immediately after isolation, 0.5 ml aliquots of MPs were frozen in liquid nitrogen in 1.5 ml

eppendorf and stored at -80^0 C for not more than a month, but it was preferred to use it immediately.[6]

Platelet-rich plasma/platelet-poor plasma isolation

The whole blood of 3-4 healthy individuals was used to isolate plasma. The whole blood of an individual was collected using sodium citrated tube in a proportion of 1:9. The whole blood were mixed and centrifuged at 350*g for 20 min for the production of platelet-rich plasma (PRP) where the supernatant was collected and again centrifuged for 15 min at 2000*g for finally production of platelet-poor plasma (PPP).

Microparticles bound tissue factor activity (TFU)

After the isolation of microparticles, it was subjected to tissue factor activity. Platelet-poor plasma (PPP) was stored at 37°C of which 100µl was added in a glass tube containing 100µl cell suspension/thawed MPs which was incubated at 37°C for atleast 2-5 min. In this100µl of 25mM $CaCl_2$ was added and the time for the clot formation was measured by slowly swirling the tube. A standard curve was made using rabbit brain thromboplastin (Sigma) which was suspended saline solution. A serial dilution of thromboplastin was prepared which was added to 100µl of 25 mM $CaCl_2$ and 100µl PPP. By comparing the data to a standard curve, the procoagulant activity is given in arbitrary units (AU) of the results.[6,7]

In vitro Platelet Aggregation assay

K-562 cells were differentiated to platelets with 20 nM PMA and treated with 1000µM DMOG, 10µM DIM, 10µM MCC950, and 10µM SML0499. 100µl (2×10^4) differentiated K-562 cell suspension was added to a pre-prepared 96-well immuno-plate with agonist ADP-10µM. The absorbance and the kinetics of the aggregation for 10 min were recorded on a multimode plate reader (Synergy HTX., Multimode reader) at 575 nm, 600 nm, and 650 nm. The reading was extrapolated with the standard curve obtained from PPP and PRP based on light transmission aggregometry.[8] The % aggregation can be extrapolated against PRP showing 0% aggregation and the transmission of light through PPP as being 100%, hence maximum.

In vitro Platelet Adhesion assay

After experimental treatment to the monocytes and endothelial cells, $0.3*10^6$ differentiated platelets were added to each well of the 12-well plates pre-coated with 10μM collagen solution. The cells were stained with calcein dye at 10μM for 60 min.[9] The cells were kept for 60 min at $37°C$ to adhere to the coated plates in a CO_2 incubator. The loosely attached or non-adherent cells were removed followed by a gentle wash with PBS. After 60 min of incubation in the dark, calcein absorbance was recorded on a multimode plate reader (Synergy HTX, Multimode reader).[10] A standard plot between absorbance and stained cell count (from 0.5×10^4 to 1×10^6 cell numbers) was performed before adhesion assays were started. After staining for 60 min with calcein andmild washing, three times with PBS, stained cells were suspended at a series of concentrations to determine their fluorescence intensity.[3,4]

Calcium Mobilization

The effect of hypoxia-mediated calcium mobilization was studied according to Ishii et al. with slight modifications.[52] Briefly, 2μM Fluo-4 AM was incubated with a differentiated megakaryocytic cell line for 1 hour at 37°C. The cells were collected by centrifuging for 5 min at 1500*g. Subsequently the cell pellet at cell density of $5x10^6$ cells/ml in serum-free media. For extracellular calcium, 1 mM $CaC1_2$ was added prior to incubation for 5 min at 37°C. Later the cells were stimulated with thrombin, and the fluorescent intensity was recorded at an excitation and emission of 485/20ηm and 528/20ηm wavelengths respectively using Synergy HTX Multimode Microplate Reader (BioTek). To obtain the fluorescence signals at maximal Ca^{2+} saturation of the dye. Further, the platelets were lysed by the addition of 0.1% Triton X-100 and 8mM EGTA added to obtain the maximum and minimum fluorescent signals respectively. The Ca^{2+} levels were calculated as previously described by Gee KR.[11]

Animal Sample collection and storage

The thrombus was separated from IVC and was measured for length and weight after that it was immediately snap-frozen and stored for further experiments. The blood samples were collected by retro-orbital vein in 3.2% trisodium citrate in a volume ratio of 9:1. The sample tubes were centrifuged at 2000*g for 20 min at RT to separate and collect plasma. Fresh plasma was used for

activated partial thromboplastin time (aPTT), prothrombin time (PT), platelet adhesion, and aggregation whereas the rest was deep-frozen (–80°C) for future investigation.

FeCl₃ induced thrombosis model

To model the thrombosis, FeCl₃ tissue injury model was used.[12] Animals were given free access to food and water before surgery. Intraperitoneal injections of ketamine and xylazine (ketamine 50mg/kg and xylazine 10 mg/kg) were used to anesthetize rats. While performing median laparotomy the animals were placed in a supine position. The abdominal organs were exteriorized and placed on sterile impregnated gauze to prevent drying. For the FeCl₃-induced model, the inferior vena cava (IVC) having fat deposits was carefully cleaned and filter paper (1mm×1mm×1mm) with 10% FeCl₃ was placed superficially on the top of the IVC. After 4 min of induction, the filter paper was removed and the abdominal organs were placed back carefully in the abdomen and sterile saline was applied during the entire procedure to prevent drying of organs. The peritoneum and skin were closed with interrupted suture. The rats were kept back in cages without having husks. Only one rat was kept per cage to avoid disturbance by the other. Rats were euthanized after the designated time point and gross thrombus was inspected *in situ*. The thrombus is harvested by excising IVC from the confluence of the iliac veins. The attached muscular tissue, artery, and ligatures were removed by microdissection to have only an IVC-containing thrombus.

Thrombus weight, length determination

The measurement of thrombus size and weight provides an indirect way of accessing thrombus formation and resolution. The harvested IVC having thrombus was split longitudinally, and the thrombus was removed. According to the previously reported method by Myers et al., 2002, the thrombus was weighed (mg) and length was measured (mm).[13]

NO concentration in plasma

The Griess reagent (Sigma-Aldrich) technique was used to detect the amount of nitric oxide (NO) in plasma. This technique detects nitrite (NO2-), one of the two main, stable, and nonvolatile breakdown products of NO. It is based on a chemical phenomenon that use sulfanilamide and N-1-naphthyl ethylenediamine dihydrochloride in the presence of phosphoric acid (acidic

environment), the Griess Reagent System is a method for analyzing chemical reactions. Following the manufacturer's recommendations, a 96-well microliter plate was used to measure the NO concentration. After 10 min, the absorbance was recorded in OD using a multimode plate reader at a wavelength 540 ηm. The samples, standards, and controls were all measured and expressed in μM.

eNOS activity

In platelets, eNOS enzyme activity was quantified using a fluorometric detection system (BioTek, USA) and expressed in Units of Fluorescence (UF) per min (m) /$1*10^6$ platelet cells.[9,14] Triazolofluoresceine, which is produced from 4, 5-diaminofluoresceine diacetate by intracellular esterase, is known to fluoresces when NO interacts with it. The wavelengths for excitation and emission were 485ηm and 515ηm, respectively. Since endothelial NOS is only activated in platelets, the activity of this specific isoform was only measured and expressed as UF/mn.

Platelets adhesion

Platelets from all the treated animals were isolated which was fluorescently labeled and allowed to adhere to collagen (10uM) coated wells for up to 60 min at 37°C. Quantification of platelet adhesion was done with the fluorescence intensity at 485/20, and 528/20 on a multimode plate reader (Synergy HTX., Multimode reader). The result was extrapolated with the standard curve with a known number of platelets.

Platelets aggregation

For platelets aggregation, PRP was separated from the whole blood of all the treated groups, incubated for 5 min at 37°C. It was induced with agonist like Adenosine diphosphate (ADP). The rate and the extent of ADP-induced platelet aggregation were recorded with rotational shaking initially.

aPTT, PT

A semi-automated coagulation analyzer (Labitec) was used to do aPTT and PT assays as per the manufacturer's instructions. For conducting aPTT assay 50µl plasma was incubated with 50µl of

aPTT reagent (C.K. PREST® 5, Stago) in a cuvette for 3 min placed at 37°C, followed by recalcification with 50µl of 25 mM $CaCl_2$ clotting time was recorded in sec.

For PT assay, 50µl of plasma was pipetted in a cuvette and incubated for 2 min at 37°C. Later 100µl PT reagent was added to it (NÉOPLASTINE® CI PLUS, STAGO), a recombinant tissue factor. The clotting time was recorded in sec.

Tail-bleeding assay

The tail bleeding assay was done by giving anaesthesia to the animal and transacting the tail 0.5 cm from the tail tip using a fresh disposable surgical blade. Immediately after being cut tail was vertically placed in 10 ml isotonic saline at RT and bleeding time was recorded until the bleeding had ceased completely.

Results
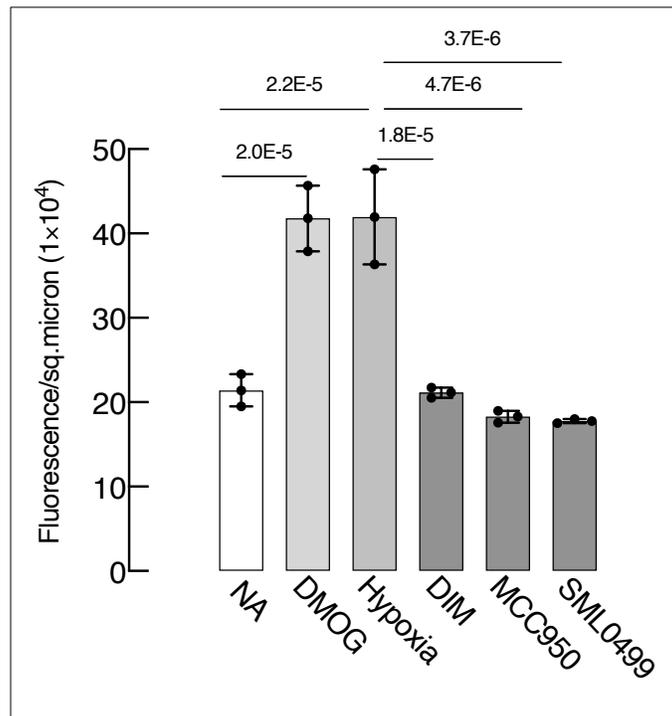
Figure S1: Adherence of monocytes to endothelial cells. HUVECs were exposed to hypoxia for 8h for all experimental conditions. Purified monocytes (hPBMCs) isolated from whole blood and treated to various experimental conditions and added to ECs for 20min to adhere to it. Statistical

analysis was performed using the One-way ANOVA with the Tukey multiple comparison test. Data is represented as SEM ±.
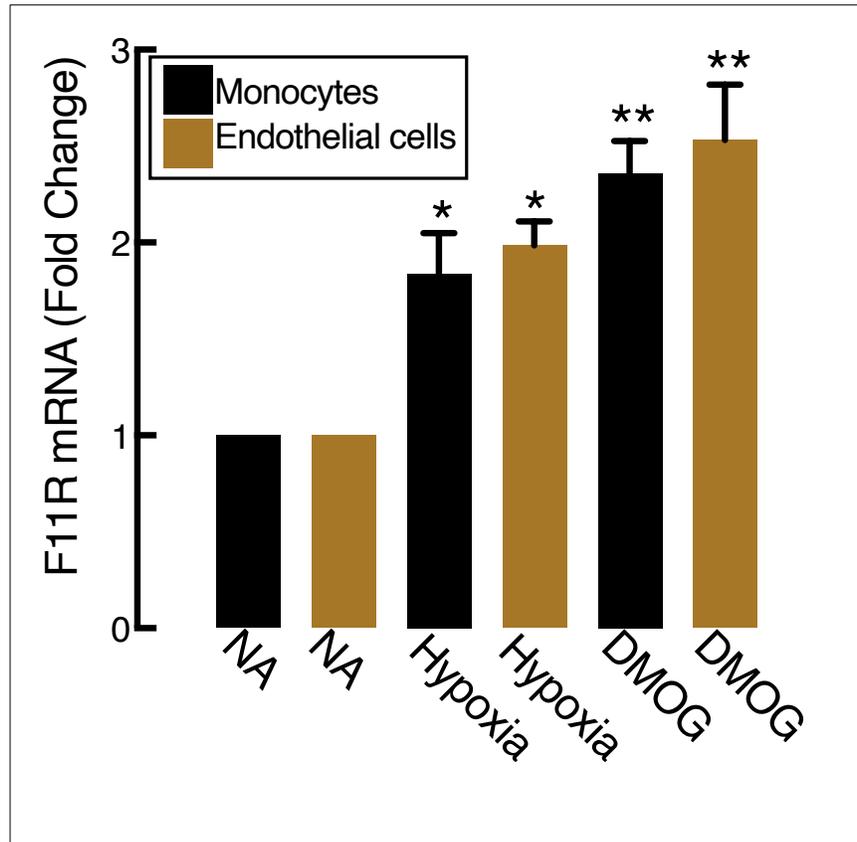

Figure S2: Regulation of F11R transcripts. The F11R gene is expressed by monocytes and endothelial cells including others. To overrule its expression in the different cells, we determine the interaction of monocytes with endothelial cells. F11R expression was quantified independently in different cells under hypoxia and DMOG. Statistical analysis was performed using the One-way ANOVA with the Turkey multiple comparison test. Data is represented as SEM ±.
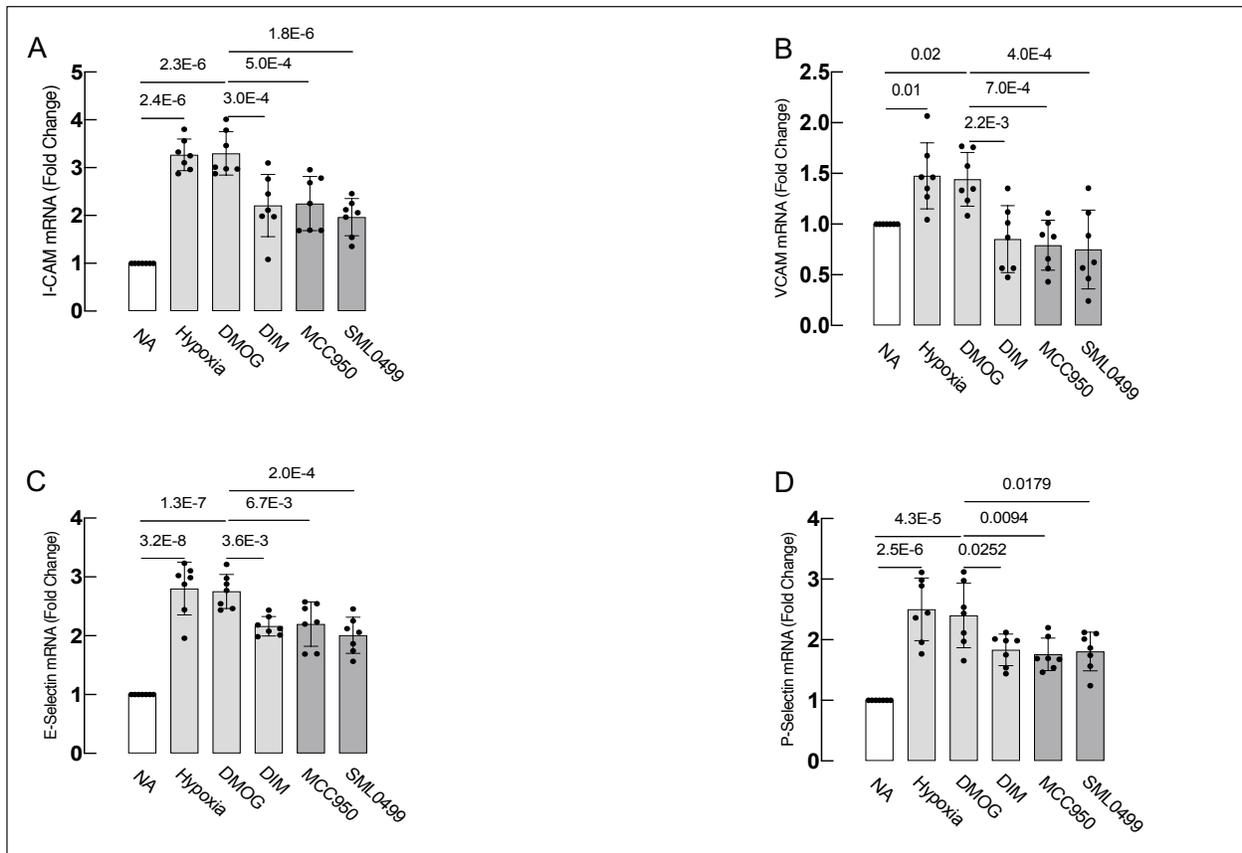
**Figure S3: Recruitment of endothelial adhesion molecules leads to endothelial dysfunction.**
Endothelial cells were treated with hypoxia (1% O$_2$) for 8 h, HIF-1α activator, Dimethyloxalylglycine (DMOG) for 12 h, DMOG+HIF-1α inhibitor-DIM, NLRP3 inhibitor-MCC950+DMOG and the inhibitor of catalytic activity of caspase-1-SML0499+DMOG. All the inhibitors were used for 4 h each: **A,** RNA expression of I-CAM; **B,** VCAM; **C,** E-Selectin; **D,** P-selectin. Statistical analysis was performed using the One-way ANOVA with the Tukey multiple comparison test. Data is represented as SEM ±.
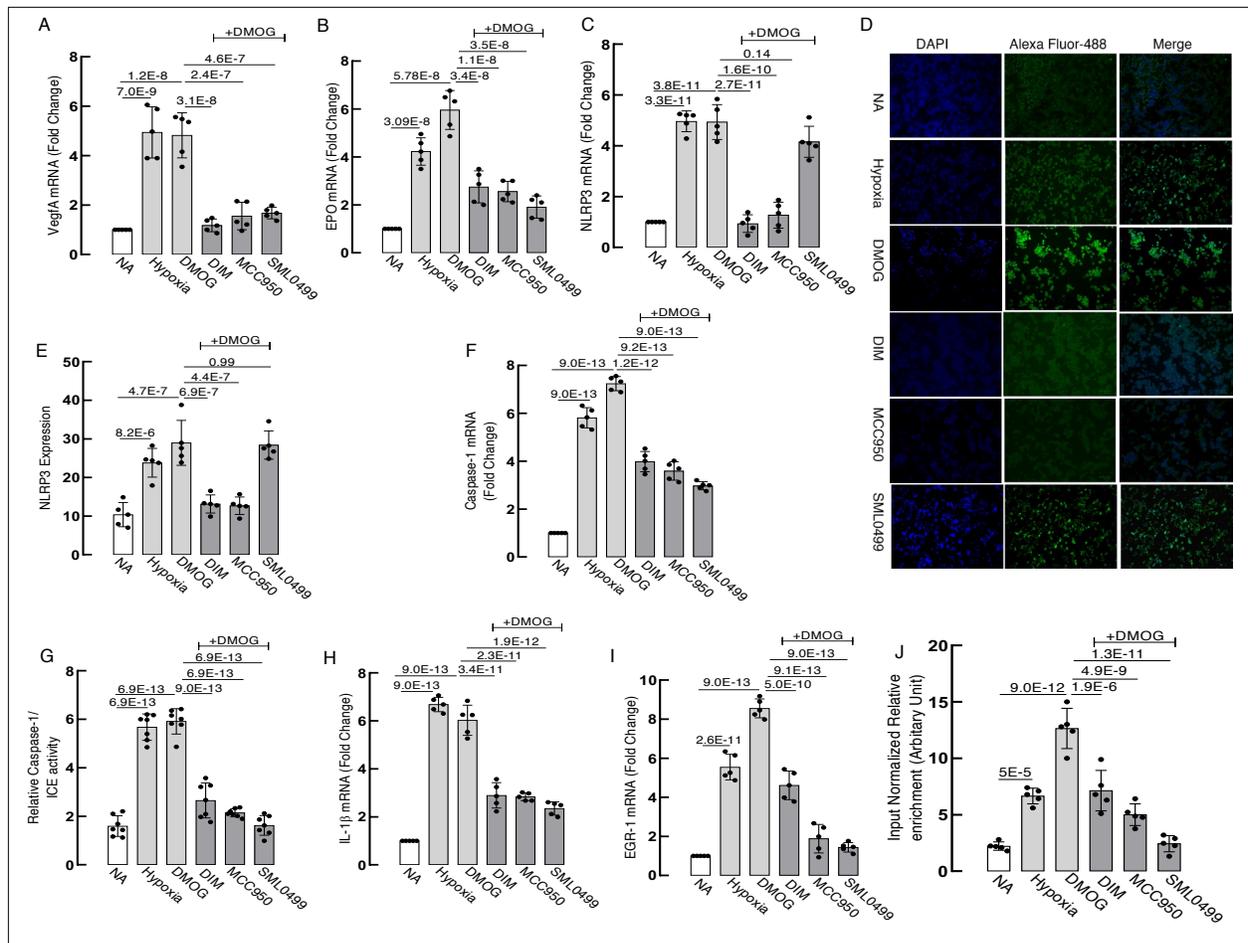
Figure S4: Implication of hypoxia in regulation of NLRP3 inflammasome axis. Monocytes were treated with experimental conditions. We determined mRNA expression of **A,** VEGF-A; **B,** EPO. Hypoxia exposure results in a pro-inflammatory state. **C,** NLPR3 mRNA transcripts, **D,** Representation of NLRP3 immunocytochemistry staining with Alexa Fluor 488; **E,** Relative NLRP3 fluorescence signal revealed hypoxia and DMOG induced NLRP3 protein; **F,** Caspase-1 mRNA transcript; **G,** Relative Caspase-1/Interleukin-1 (IL-1) converting enzyme (ICE) activity; and **H,** Protein expression of cytokine IL-1β; **I,** Relative mRNA expression of Egr-1 under different experimental conditions; The expression of the gene was normalized with 18sRNA, as an internal control to that of the NA group; J, The enrichment of the Egr-1 promoter region in CHIP experiments was quantified and the bar graph was plotted. Statistical analysis was performed using the One-way ANOVA with the Tukey multiple comparison test. Data is represented as SEM ±.
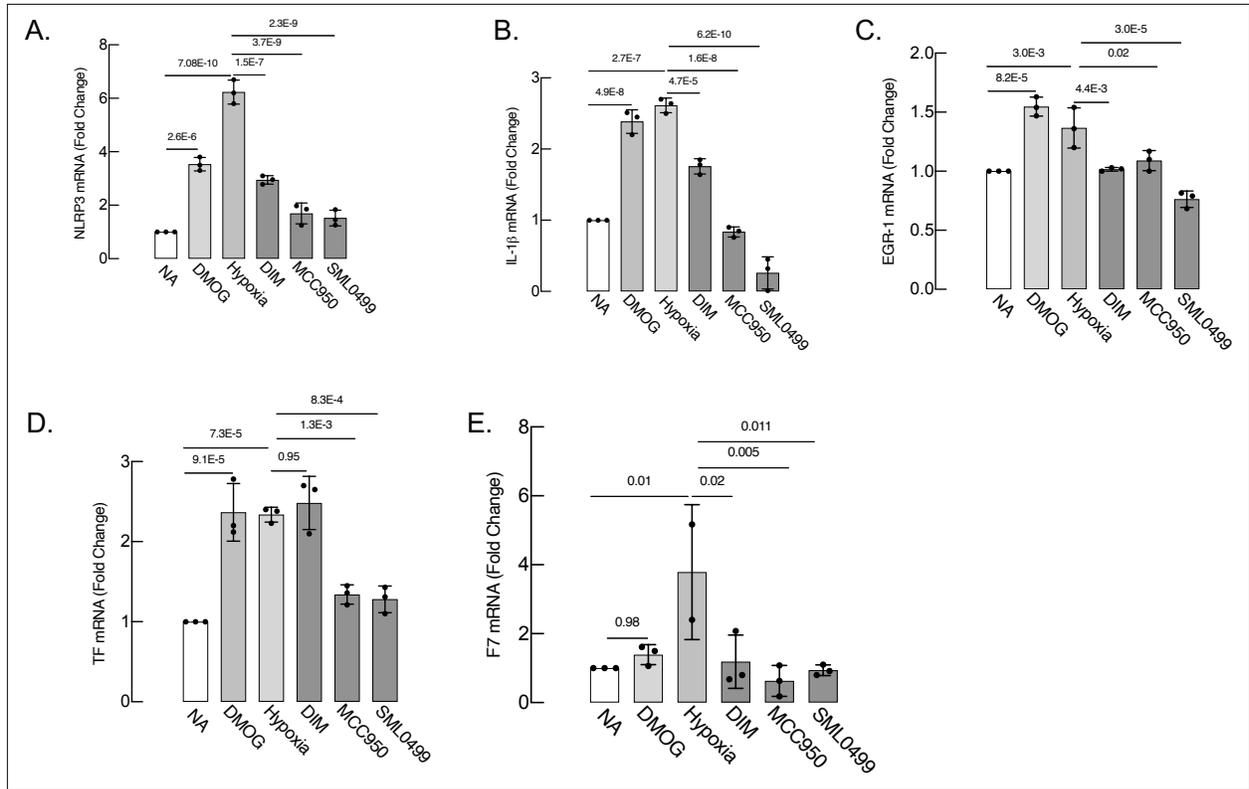
Figure S4A: Hypoxia exposure results in a pro-inflammatory and prothrombotic state. hPBMCs were treated to all the experimental conditions. RNA was isolated and mRNA expression was analyzed. A. NLRP3; B. IL-1β; C. Egr-1; D. Tissue factor; E. F7 expression under different experimental conditions. The expression of the gene was normalized with 18sRNA, as an internal control to that of the NA group. Statistical analysis was performed using the One-way ANOVA with the Tukey multiple comparison test. Data is represented as SEM ±.
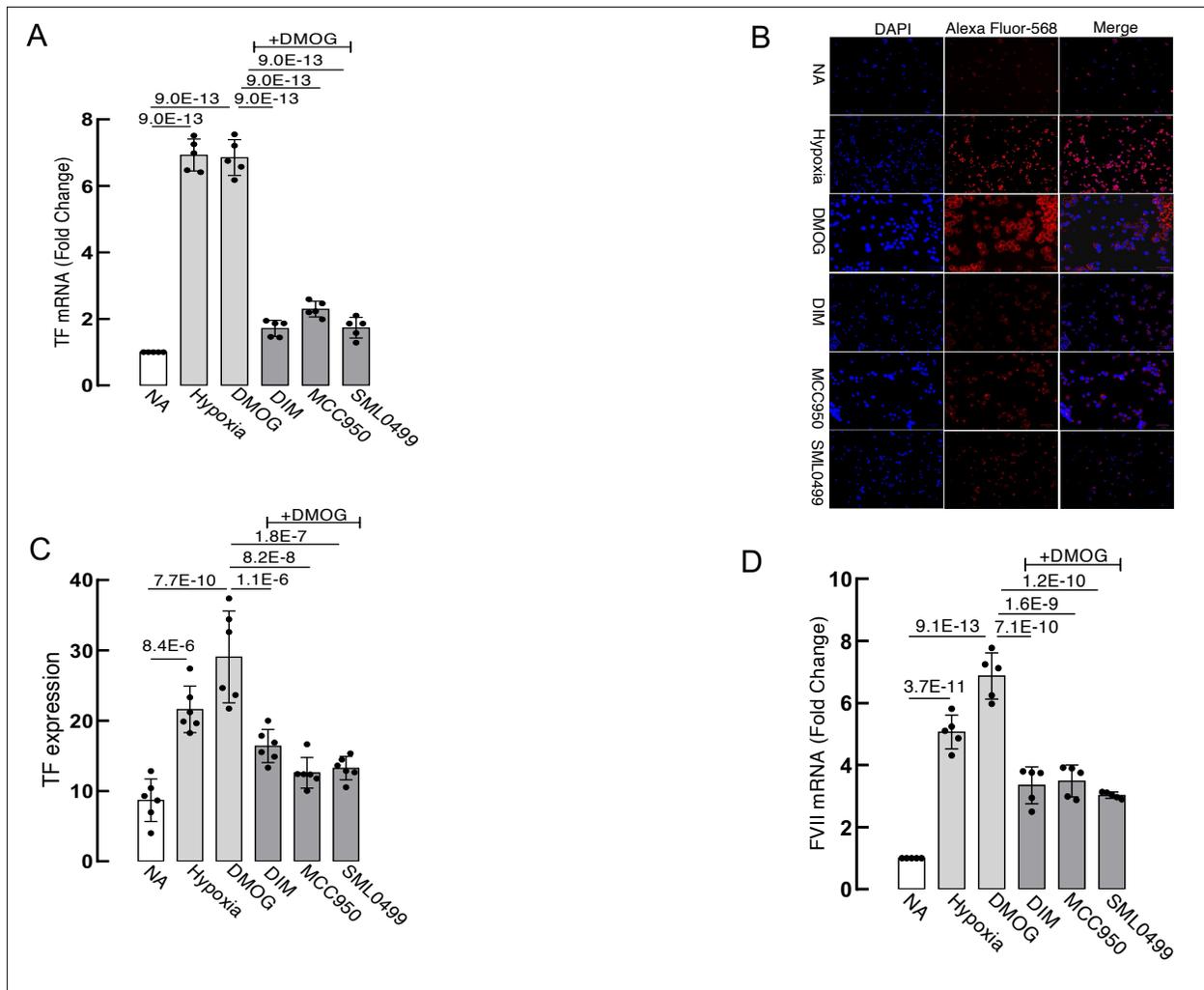
Figure S5: Hypoxia induced prothrombotic state. Expression of tissue factor (TF), a initiator of coagulation. **A,** Relative mRNA expression of TF; **B,** Represenation of TF immunocytochemistry staining with Alexa Fluor 568; **C,** Relative fluorescence signal revealed hypoxia and DMOG induced TF protein levels while its suppressed by inhibiting HIF-1α, NLRP3 and catalytic activity of caspase-1. **D,** Relative mRNA levels of FVII transcripts in all experimental conditions. Statistical analysis was performed using the One-way ANOVA with the Tukey multiple comparison test. Data is represented as SEM ±.

Correlation
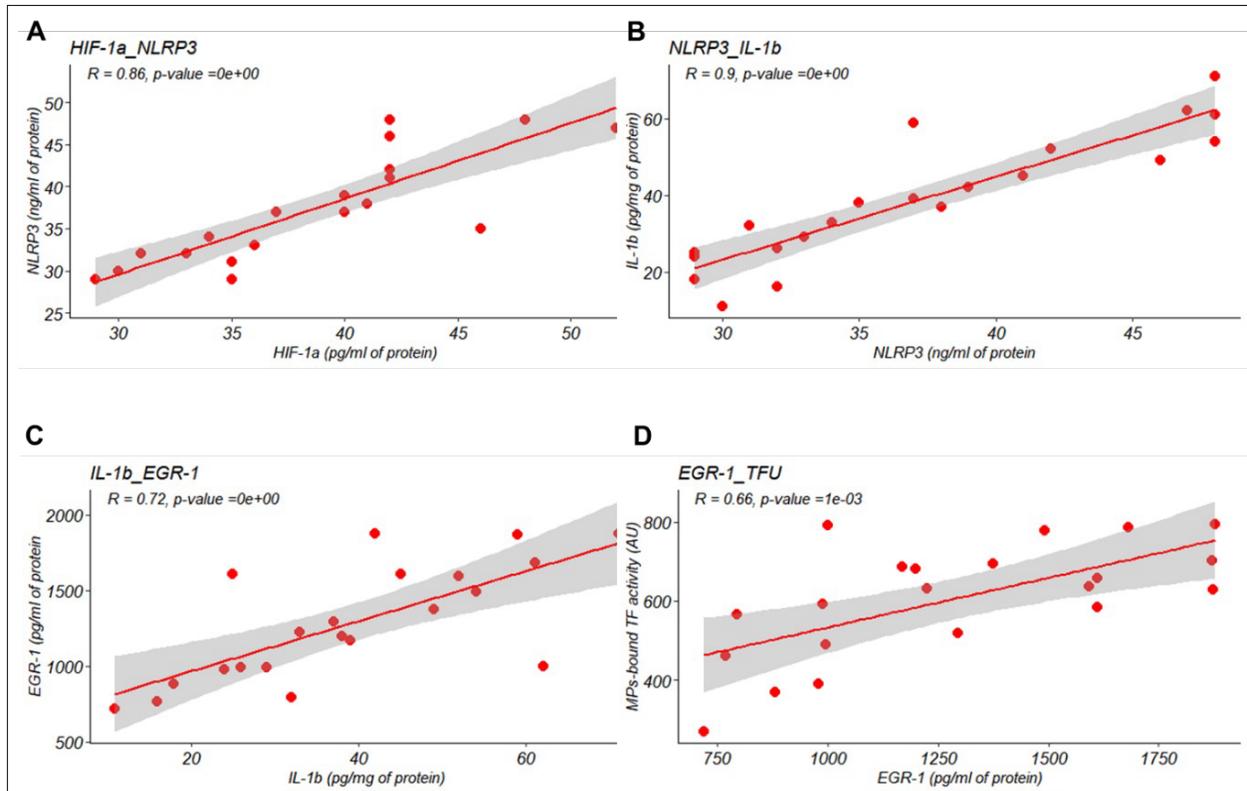
Figure S6: Egr-1 protein contributed to the hypoxia-induced tissue factor activity (Tfu). **A,** The protein concentration of HIF-1α (pg/ml) was significantly correlated with the concentrations of NLRP3 (ng/ml). **B,** The protein concentration of NLRP3 (ng/ml) was significantly correlated with the concentrations of IL-1 β (pg/ml). **C,** The protein concentration of IL-1β (pg/ml) was significantly correlated with the concentrations of Egr-1 (pg/ml). **D,** The protein expression of Egr-1 (pg/ml) was significantly correlated with MPs-bound tissue factor activity (TFU/AU). Pearson Correlation analysis was used for correlation analysis as data met the criteria of normal distribution and collinearity.
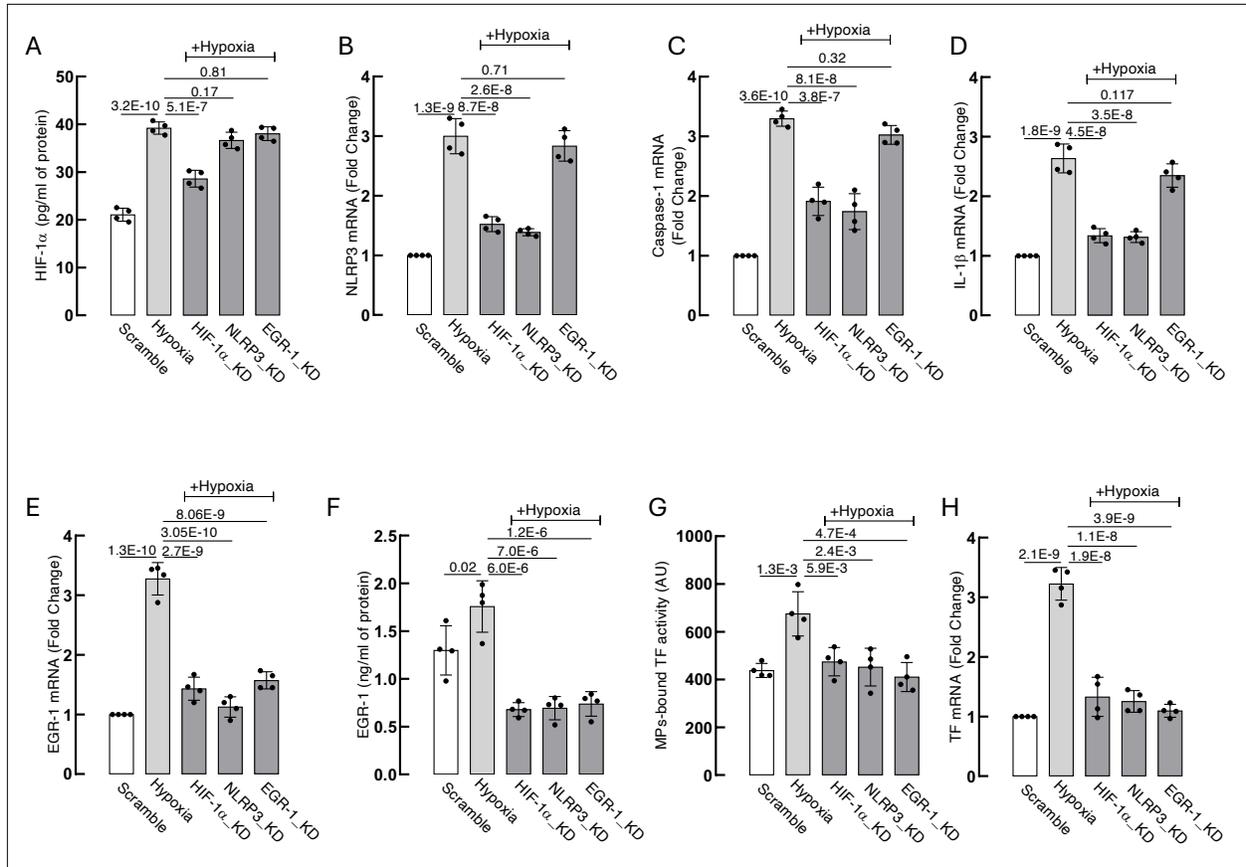
Figure S7: Egr-1 promotes thrombogenesis through tissue factor expression via HIF-1a-NLRP3 inflammasome axis: THP-1 cells were cultured and treated with siRNA of HIF-1α, NLRP3 and Egr-1 for 6 hrs and then exposure to hypoxia; **A,** Protein levels of HIF-1α under different experimental conditions; **B,** Relative levels of NLRP3; **C,** Caspase-1; **D,** IL-1β; **E,** Egr-1 transcripts, and **F,** Egr-1 protein level; **G,** Estimation of microparticles bound tissue factor activity, and **H,** Relative expression of F3 in all the experimental conditions. (Comparison was done Scramble v/s Hypoxia; Hypoxia versus HIF-1α, NLRP3, and EGR-1 knockdown). 18sRNA was used as an internal control and expression was normalized to that of Scramble; Statistical analysis was performed using the One way ANOVA with the Tukey multiple comparison test. Data is represented as SEM ±.
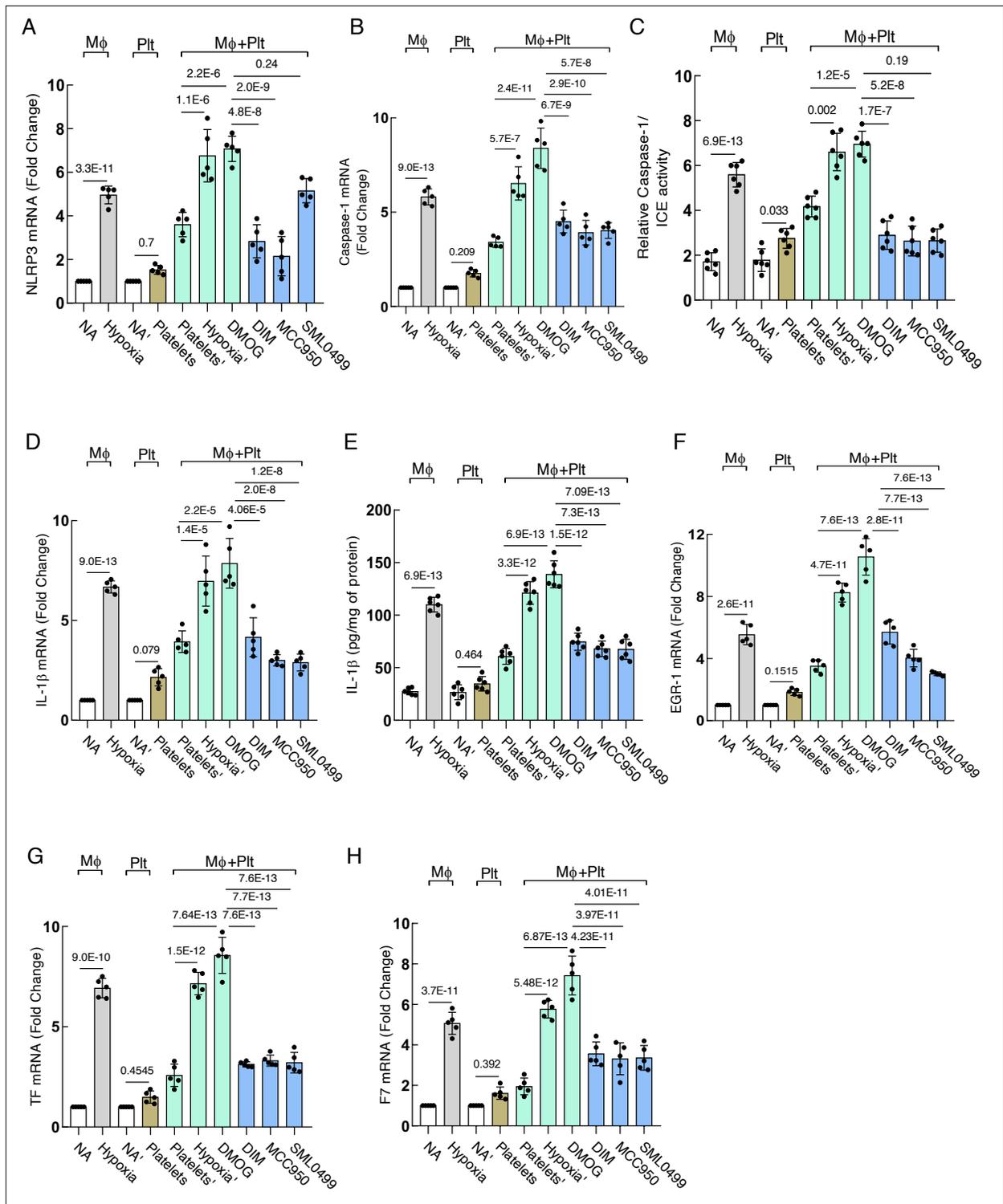
Figure S8: Platelets strengthen the pro-inflammatory and pro-thrombotic milieu under hypoxia. **A,** Relative expression of NLRP3 transcripts; **B-C**, Caspase-1 transcript and Caspase-1/Interleukin-1 (IL)-1 converting enzyme (ICE) activity respectively; **D-E,** IL-1β transcripts and

protein levels of Cytokine IL-1β; **F,** Egr1 mRNA transcripts; **G-H,** mRNA transcripts of Tissue factor and Factor 7 in all the experimental conditions. The expression of the gene was normalized with 18sRNA, as an internal control to that of the NA group. Statistical analysis was performed using the One-way ANOVA with the Tukey multiple comparison test. Data is represented as SEM ±.
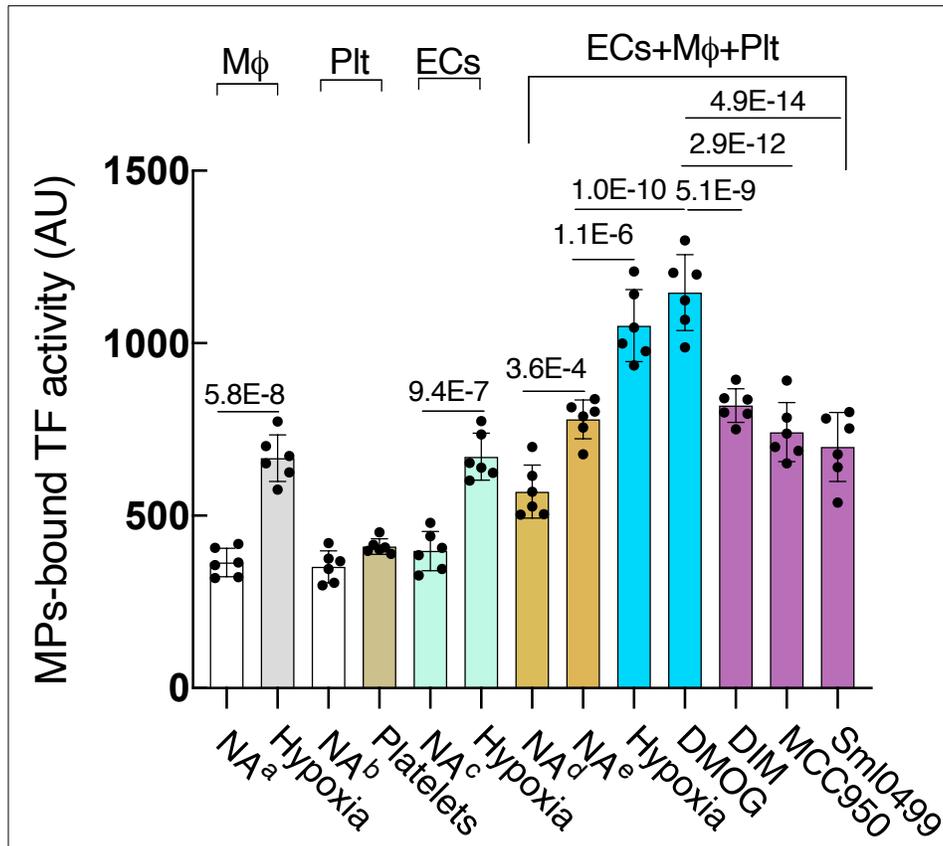
Figure S9: Platelets strengthen pro-thrombotic milieu under hypoxia. Endothelial cells (ECs) were pre-treated with 1% O_2 (hypoxia) for 8h for all the experimental condition of monocytes; Monocytes were treated with hypoxia, DMOG, DIM, MCC950, and SML0499. Platelets were pre-activated with ADP and washed before adding to co-culture of ECs and Monocytes. **A.** Microparticles bound tissue factor activity in different experimental conditions. No addition (NA[a]) of monocytes; No addition (NA[b]) of platelets; NA[c] of ECs; NA[d] of ECs, monocytes and Platelets; NA[e] of monocytes, Platelets and activated ECs. Statistical analysis was performed using the One-way ANOVA with the Tukey multiple comparison test. Data is represented as SEM ±.
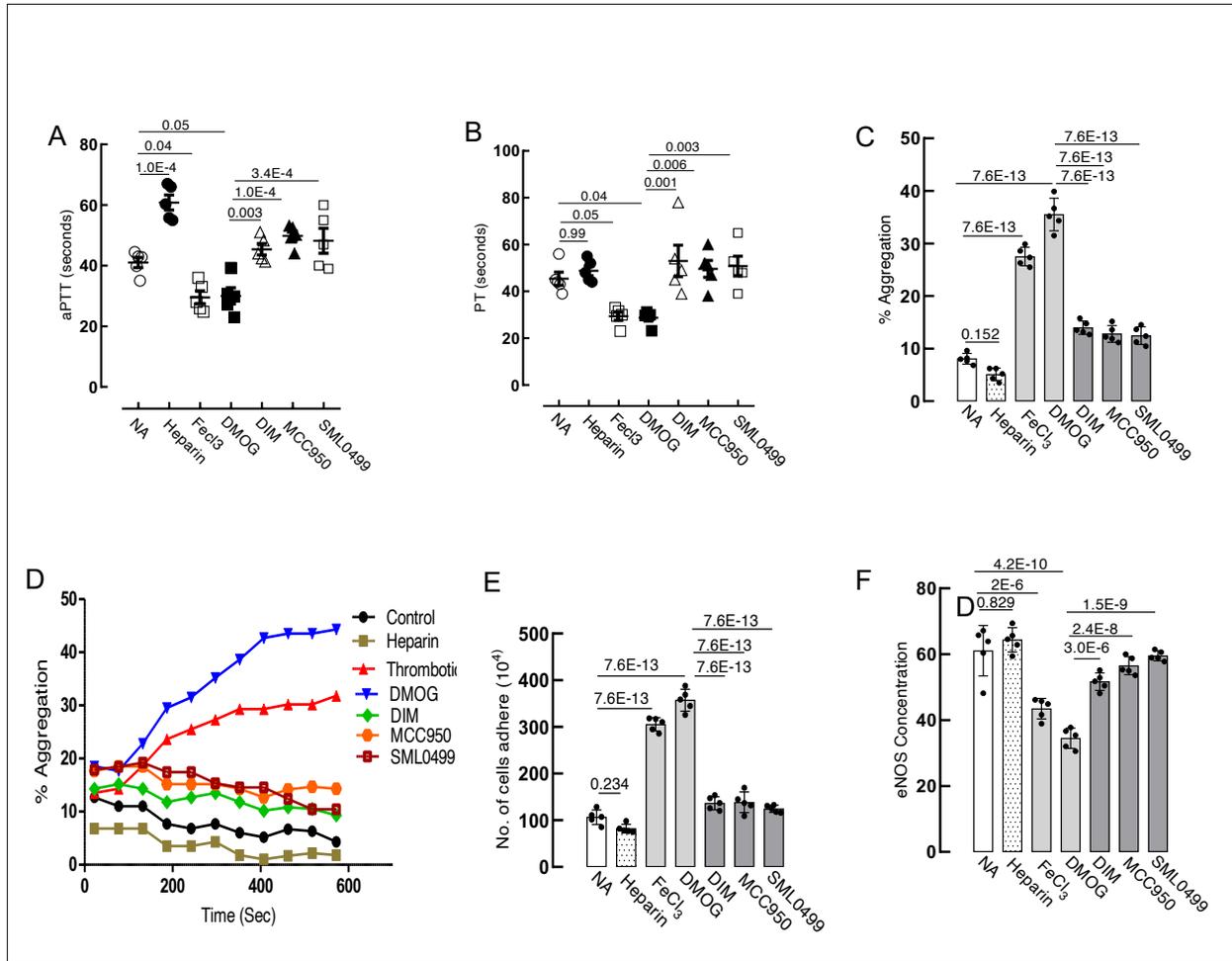
Figure S10: Acceleration of venous thromboembolism through HIF-1α-NLRP3-Egr-1 axis.
A, aPTT (Activated Partial Thromboplastin Time); **B,** PT (Prothrombin Time); **C,** Platelets percent aggregation under the influence of hypoxia through HIF-1a-NLRP3; **D,** Kinetics of platelets aggregation at different time point; **E,** Platelets adhesion to collagen; **F,** eNOS concentration under all the experimental condition in-vivo. Statistical analysis was performed using the One-way ANOVA with the Tukey multiple comparison test. Data is represented as SEM ±.
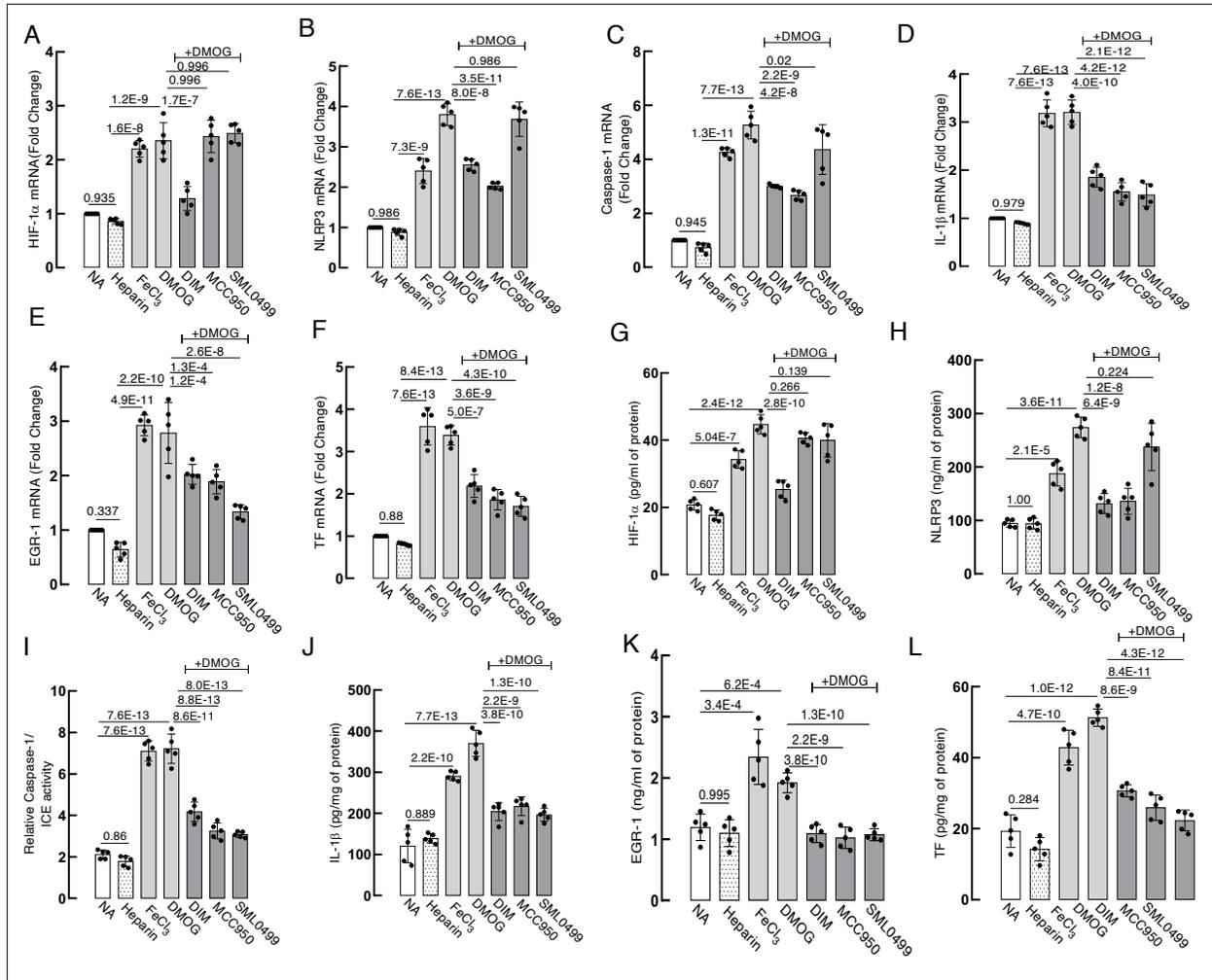
Figure S11. Hypoxia induced pro-inflammatory and pro-thrombotic state through HIF-1a-NLRP3-Egr-1 axis. A-F, Relative levels of **A**, HIF-1α; **B**, NLRP3; **C**, Caspase-1; **D**, IL-1β; **E**, Egr-1, and **F**, F3 transcripts in all experimental condition as described; G-L, Estimation of protein level of **G**, HIF-1α; **H**, NLRP3, **I**, Caspase-1/Interleukin converting enzyme-1 activity; **J**, IL-1β, **K**, Egr-1, and **L**, F3 by ELISA in all the experimental conditions. β-actin was used as an internal control and expression was normalized to that of NA group; Statistical analysis was performed using the One way ANOVA with the Tukey multiple comparison test. Data is represented as SEM ±.

Table S1: Demographic, and clinical, characteristics of patients with DVT

Characteristics		Patients (n=10)	Healthy (n=10)	P-value
Age, years Median (Range)		46 (27-65)	42 (27-60)	0.2303
BMI		24.52	22.93	0.2644
Gender (F/M)		3/7	4/6	-
Food habit (Veg/Both)		2/8	3/7	-
BP	SBP, mmHg	96-118	120	0.0051
	DBP, mmHg	60-95	80	0.0835
PR, rate/min		88-95	56-100	0.0698

F, Female; M, Male; BMI, Body mass index; n, number of subjects; SBP, systolic blood pressure; DBP, diastolic blood pressure; PR-Pulse Rate

Table S2: List of gene with its Real-time PCR primer sequence.

Oligonucleotides		
Gene symbol	Sequence	Company
CD18	5'-CTGGTAGCAAAGCCCCCACG-3' (Sense) and 5'-TGGGTTTCAGCGAGGCTTGTG-3'	Sigma-Aldrich
F11R	5'-CTGTCCTGGTAACACTGATTCTC-3'(Sense) and 5'-TCTGTTTGAATTCTCCCTCACTG-3' (antisense)	Sigma-Aldrich
HIF-1α	5'-GCTACTACATCACTTTCTTGG-3' (Sense) and 5'-TCTACATGCTAAATCAGAGGG-3' (antisense)	Sigma-Aldrich
NLRP3	5'-CATTGAGAACTGTCATCGGG-3' (sense) and 5'-GCTGTTCACCAATCCATGAG-3'(antisense)	Sigma-Aldrich
Casp-1	5'-ATGTTGAATACCAAGAACTGCC-3' (sense) and 5'-CCAAGAAACATTATCTGGTGTGG-3' (antisense)	Sigma-Aldrich
IL-1β	5'-ACAGATGAAGTGCTCCTTCC-3' (sense) and	Sigma-Aldrich

	5'-CACATAAGCCTCGTTATCCCA-3' (antisense)	
V-CAM	5'-GCAAGTCTACATATCACCCA-3' (sense) and 5'-AATCTTCCATCCTCATAGCA-3' (antisense)	Sigma-Aldrich
E-selectin	5'-CTGCCTGTACCAATACATCC-3' (sense) and 5'-CAGTTCACAATTTGCTCACAC-3' (antisense)	Sigma-Aldrich
P-selectin	5'-CAGAGTCACAGAGGAGATGG-3' (sense) and 5'-TTTGTTAGTTCAGAGATCAGGG-3' (antisense)	Sigma-Aldrich
VEGF-A	5'-CTCCGAAACCATGAACTTTCTG-3' (sense) and 5'-CATGAACTTCACCACTTCGT-3' (antisense)	Sigma-Aldrich
EPO	5'-GCATGTGGATAAAGCCGTCAGTG-3' (sense) and 5'-GAGTTTGCGGAAAGTGTCAGCAG-3' (antisense)	Sigma-Aldrich
TF	5'-CAAATAAGCACTAAGTCAGGAG-3' (sense) and 5'-ATCTTCTACGGTCACATTCAC-3' (antisense)	Sigma-Aldrich
FVII	5'-CACACCCACAGTTGAATATCC-3' (sense) and 5'-GCTCCATTCACCAACAACAG-3' (antisense)	Sigma-Aldrich
uPA	5'-CACTGCTTCATTGATTACCCA-3' (sense) and 5'-TTTCCACCTCAAACTTCATCTC-3' (antisense)	Sigma-Aldrich
tPA	5'-AGAGGAGCCAGATCTTACCA-3' (sense) and 5'-TGGCCCTGGTATCTATTTCAC-3' (antisense)	Sigma-Aldrich
PAI-1	5'-GAACTTCAGGATGCAGATGTC-3' (sense) and 5'-CCCTTGTCATCAATCTTGAATCC-3' (antisense)	Sigma-Aldrich
EGR-1	5'-ATTTGGAGTGGCCCGATATGG-3' (sense) and 5'-AGCAGGAAGCCCTAATATGGC-3' (antisense)	Sigma-Aldrich
18sRNA	5'-CTTAGAGGGACAAGTGGCG -3' (sense) and 5'-ACGCTGAGCCAGTCAGTGTA -3' (antisense)	Sigma-Aldrich
Rat		
HIF-1α	5'-CACCTTCTACCCAAGTACCT-3' (sense) and 5'-GTAACGTTCCAATTCCTGCT-3' (antisense)	Sigma-Aldrich
NLRP3	5'- ACATTCAGAGACTGTGGTTGG-3' (sense) and 5'- ATGCGAGATCCTGACAACAC-3' (antisense)	Sigma-Aldrich

Caspase-1	5'- GCTTCAGTCAGGTCCATCAG-3' (sense) and 5'- TTCTTTCCATAACTTCTGGGCT-3' (antisense)	Sigma-Aldrich
IL-1β	5'- CAAATCTCACAGCAGCATCTC-3' (sense) and 5'- GTCATCATCCCACGAGTCAC-3' (antisense)	Sigma-Aldrich
Egr-1	5'- CAAACTGGAGGAGATGATGCTG-3' (sense) and 5'- AAAGGACTCTGTGGTCAGGT-3' (antisense)	Sigma-Aldrich
TF	5'- ACCCACCAACTATACCTACAC-3' (sense) and 5'-TCACATCCTTCACAATCTCGT -3' (antisense)	Sigma-Aldrich
β-actin	5'- AGATGACCCAGATCATGTTTGAG-3' (sense) and 5'- TTAATGTCACGCACGATTTCC-3' (antisense)	Sigma-Aldrich

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