Supplementary Materials

Gab2-MALT1 Axis Regulates Thromboinflammation and Deep Vein Thrombosis

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Materials and Methods

Materials

Recombinant TNFα, IL-1β, and goat polyclonal antibodies against human/mouse myeloperoxidase (MPO) were purchased from R&D Systems (Minneapolis, MN). Antibodies against phospho-serine, fibrin, and VWF, and the PKH67 labeling kit were from Sigma Aldrich (St. Louis, MO). Antibodies against phospho-specific or total p65, PKCα/β/δ, TRAF6, TAK1, and VCAM1 were obtained from Cell Signaling Technology (Danvers, MA). Monoclonal antibodies against P-selectin, Gab2, PLCγ2, BCL-10, and CARMA3 were procured from Santa Cruz Biotechnology (Dallas, TX). Rho activation assay kit was purchased from Cytoskeleton, Inc (Denver, CO). Rat anti-mouse Ly-6G and rat anti-mouse F4/80 antibodies were from BioLegend (San Diego, CA). Mepazine (pecazine) was purchased from MedChemExpress (NJ). Rabbit polyclonal antibodies against citrullinated histone H3 antibody (Cit-Arg17, Arg2- Arg8, CitH3) was from Novus Biologicals (Centennial, CO).

Isolation of human neutrophils

Neutrophils from the human blood were isolated based on the protocol described previously.[1] Briefly, heparinized blood was diluted (1:1) with Ca^{2+} and Mg^{2+} free Hank's Balanced Salt Solution (HBSS), and 30 ml of diluted blood was carefully layered on 15 ml of Ficoll-Histopaque solution and centrifuged at 400 x g for 30 min at room temperature. The upper layer of platelet-rich plasma and buffy coat containing PBMC were removed, and the sedimented layer of RBC and neutrophils were collected and diluted (1:1) with Ca^{2+} and Mg^{2+} free HBSS. Then, 20 ml of 3% dextran T500 was added to the suspension of RBC and neutrophils, inverted several times gently, and allowed to stand for 20 min to sediment RBC. The upper colorless layer containing neutrophils was collected and centrifuged at 400 x g for 10 min at room temperature. The pellet was collected, washed, and the residual RBC was lysed by adding 10 ml of 33 mM NaCl for 30 sec. Thereafter, 10 ml of 267 mM NaCl was added to the sample to equilibrate it, followed by 20 ml of HBSS. The suspension was centrifuged at 400 x g for 10 min at room temperature to sediment neutrophils. The neutrophil pellet was resuspended in 3 ml of RPMI medium, and the cell number was counted using an automatic cell counter (BioRad, TC20).

Gene silencing, cell treatments, immunoprecipitation, and immunoblotting

HUVEC were transfected with scrambled RNA (scRNA), siRNA specific for Gab2, CARMA3, BCL10, or MALT1 siRNA (200nM) using Lipofectamine RNA1MAX transfection reagent (Invitrogen). After culturing cells for 48 h and washing them with serum-free medium, they were stimulated with TNFα, IL-1β, or TNFα/IL-1β

as indicated in Results and Figure Legends. The cells were lysed in the SDS-PAGE sample buffer, and an equal amount of protein or volume was subjected to SDS-PAGE, followed by immunoblot analysis to probe for specific signaling proteins or prothrombotic mediators. The immunoblots were developed with chemiluminescence using Western Lightning Plus HRP-substrate (Millipore). Densitometric analysis was performed using the Bio-Rad Chemi XRS system and Image J software.

For immunoprecipitation studies, the cells were lysed in ice-cold cell lysis buffer (50 mM HEPES, 2 mM EDTA, 150 mM NaCl, 1% Triton X-100) containing protease and phosphatase inhibitors single-use cocktail (Thermo Fisher). Cell lysates were incubated with the Gab2 antibodies (Sigma) or other antibodies as specified in Figure Legends overnight at 4°C. The immunocomplexes were pulled down by adding 20 µl of protein A/G agarose beads to the reaction mixture, incubating them at room temperature for 2 to 3 h with a constant rotation, and sedimenting beads by centrifugation at 150 x g for 8 min at 4°C. The pelleted agarose beads were washed thrice with the cell lysis buffer to remove the unbound material. The bound material was eluted by adding 25 µl of SDS-PAGE buffer to the beads and heating the sample at 95°C for 15 min.

Rho activation assay

Rho activation was determined using the Rho activation assay kit according to the manufacturer's instructions by pull-down assay using the GST-Rhotekin-Rho-binding domain (GST-RBD).[2] HUVEC were serum-starved for 6h and then stimulated with IL-1β as indicated, washed, and lysed using 0.5 ml of lysis buffer. The cell lysates were centrifuged at 10,000 x g, 4°C for 5 min. The supernatants were incubated with 30 µg of rhotekin-RBD beads for 1h at 4°C. The beads were centrifuged at 5000 x g at 4°C for 3 min, washed, and eluted with 2x SDS sample buffer. The samples were analyzed by western blotting.

Cell surface ELISA and Immunofluorescence microscopy

HUVECs cultured in a 96-well plate were transfected with 200 nM scRNA or siRNA specific for Gab2, MALT1, or TAK1 or treated with specific inhibitors of NF-κB and Rho proteins. The cells were treated with IL-1β for 1h, and then the cells were fixed with 4% paraformaldehyde and blocked with 1% bovine serum albumin (BSA). The fixed cells were incubated with P-selectin (5 µg/mL) monoclonal antibody or control IgG for 3 h at room temperature. After removing the unbound antibody, the cells were washed 3 times with phosphate-buffered saline (PBS) containing 0.1% BSA and then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG, followed by the substrate tetramethylbenzidine for 30 min. The amount of P-selectin antibody bound to the cell surface was determined spectrophotometrically by measuring the absorbance at 650 nm.

The translocation of P-selectin to the cell surface was analyzed by immunofluorescence microscopy. HUVEC cultured on cover glass was transfected with 200 nM scRNA or Gab2-specific siRNA for 48 h. The cells were treated with IL-1β for 1 h, and then the cells were fixed with 4% paraformaldehyde and blocked with 10 % goat serum in PBS. The cells were incubated with a P-selectin monoclonal antibody (5 μg/mL) or control IgG overnight. The cells were washed and incubated with AF488-labelled donkey anti-mouse IgG along with DAPI for 90 min. The cells were mounted in a Fluoro-gel mounting medium (Electron Microscopy Sciences), visualized, and imaged using LSM510 Zeiss confocal microscope.

NETosis assay

The NET formation in the isolated human neutrophils was analyzed by immunofluorescence microscopy. Briefly, neutrophils (5 x 10^5) were plated on glass cover slips and treated with PMA (100 nM) or IL-1β/TNFα/IL (10 ng/ml) for 3 h. After the treatment, the cells were fixed with 4% formaldehyde for 15 min, washed, and blocked with 10% goat serum for 1 h at room temperature. The fixed cells were incubated with control IgG or goat anti-MPO antibody (5 μg/ml) and rabbit anti-CitH3 antibody (5 μg/ml) overnight at 4°C. The cells were then incubated with Hoechst dye, AF488-donkey anti-goat IgG, and AF647-donkey anti-rabbit IgG antibodies. The cells were mounted in a Fluoro-gel mounting medium (Electron Microscopy Sciences), visualized, and imaged using LSM510 Zeiss confocal microscope. The % of NETosis was calculated using the formula, NET [%] = 100 × number of objected stained positive with citH3 antibody/number of objects stained with Hoechst dye.[3]

Deep vein thrombosis in mice

C57BL/6J male and female mice (10-12 weeks old) were subjected to well-established murine models of inferior vena cava (IVC) ligation-induced flow restriction (stenosis) or stasis to induce DVT.[4,5] Briefly, to induce stenosis, mice were anesthetized using a continuous flow of isoflurane gas. A midline laparotomy was performed to expose the IVC. The IVC, below the renal vein, was ligated using a polypropylene suture (7.0) after placing a 30-gauge needle next to the IVC as the ligature is tied and then removing the needle. All side branches were either cauterized or fully ligated. This procedure results in 80 to 90% closure of the lumen of the IVC without endothelial damage. The median laparotomy was sutured, and the skin was closed by stapling. Mice were allowed to recover from the surgery and sacrificed at 48 h following the IVC ligation. For stasis, the procedure was essentially the same as that used for the stenosis, except that the IVC was fully ligated (no spacer), and mice were sacrificed at 24 h following the IVC ligation. The thrombus in the IVC, if it developed, was excised and collected for analysis. The length and weight of thrombi were measured. After that, they were processed for immunohistochemistry (IHC) staining of monocytes and

neutrophils. In stenosis experiments, the mice were administered with the MALT1 specific inhibitor, mepazine (12.5 mg/kg, Pecazine, MedChemExpress) via the tail vein injection 2 h prior and 24 h after the IVC ligation. In stasis experiments, the mice were given mepazine at a single dose of 12.5 mg/kg, either 2 h prior or 4 h post the IVC ligation.

Analysis of prothrombotic mediators in thrombus

Thrombus was collected and homogenized in 0.5 ml of PBS, and the homogenate was centrifuged at 10,000xg at 4^{o}C for 15 min. The supernatants were collected, and IL-1β levels in the supernatants were measured by ELISA using a kit (Invitrogen, MA). An equal amount of protein was subjected to SDS-PAGE and immunoblotted for the analysis of VWF, fibrin, platelets (CD41), VCAM1, and TF. The immunoblots were developed with chemiluminescence using Western Lightning Plus HRP-substrate (Millipore). Densitometric analysis was performed using the Bio-Rad Chemi XRS system and Image J software.

Immunohistochemistry

For immunohistochemistry, the antigen retrieval was done by boiling thrombi tissue sections for 15 min in sodium citrate buffer (10 mM, pH 6.0). The sections were incubated in 3% hydrogen peroxide for 20 min to quench endogenous peroxide activity. After blocking the tissue sections with an antibody diluent containing background reducing components (Agilent, Santa Clara, CA), they were incubated with control IgG, or primary antibodies against F4/80 or Ly-6G (5 µg/ml) overnight at 4°C. The sections were then incubated with biotin-labeled goat anti-rat IgG (1:500) and ultrasensitive streptavidin-HRP (1:500) (Sigma, St. Louis, MO, USA) and developed using an AEC-hydrogen peroxide substrate solution. The sections were counterstained with hematoxylin, mounted, and visualized with an Olympus BX41 microscope.

For the analysis of NETs in the thrombus by immunofluorescence microscopy, thrombus sections were incubated with control IgG or rat anti-mouse Ly-6G antibody (5 µg/ml) and rabbit anti-CitH3 antibody (5 µg/ml) overnight at 4°C. The sections were then incubated with Hoechst dye, AF488-donkey anti-rabbit IgG, and AF546-donkey anti-rat IgG antibodies. The sections were mounted in a Fluoro-gel mounting medium, visualized, and imaged using LSM510 Zeiss confocal microscope. The number of NETs was calculated by counting individual structures stained positively for both CitH3 and Ly6G antigens.

Data analysis

All experiments were repeated three or more times independently. Data shown were either representative images or the mean ± SD. In animal studies, mice were randomly assigned to each group. We have compared male and female animal data separately and found no noticeable differences between

the two groups. Therefore, the data from males and females were pooled in a group for statistical analysis. The normal distribution of the data was analyzed by Shapiro-Wilk or Kolmogorov-Smirnov test. Since the data passed the normality test, we have analyzed the statistical significance using one-way ANOVA or Student's t-test, as appropriate. GraphPad Prism version 8.4.1 (676) software was used for data analysis.

References

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Supplementary Figure Legends

Supplementary Figure S1. MALT1 inhibition protects against venous thrombosis induced by stasis. C57/BL 6J wild-type mice were administered with a single dose of MALT1 inhibitor, mepazine (12.5 mg/kg body weight, i.p.), 2 h prior (pre) or 4 h after (post) ligating the IVC fully to induce stasis. Control mice were administered with a control vehicle (DMSO) at 2 h prior to the IVC ligation. Twenty-four hours following the IVC ligation, mice were killed, and thrombus formation in the ligated vein was evaluated. (A) Representative images of thrombus; (B) thrombus length; (C) thrombus weight. Data are mean ± SD of two independent experiments (a total of 7 animals/group). **, $p<0.01$, ***, $p<0.001$.

Supplementary Figure S2. MALT1 inhibition attenuates elaboration of thrombotic and inflammatory mediators at the local milieu in a murine model of venous thrombosis. (A) C57/BL 6J wild-type mice were administered with a single dose of MALT1 inhibitor, mepazine (12.5 mg/kg body weight, i.p.), 2 h prior to IVC ligation-induced stasis. Control mice were administered with a control vehicle (DMSO). Twenty-four hours following the IVC ligation, mice were killed, and thrombi were collected. TF, VCAM1, CD41 (platelet marker), and fibrin content were analyzed by immunoblotting. Immunoblots represent samples of 3 different mice in each group. Bar graphs in the bottom panel represent data from densitometric analysis of immunostained bands. **(B)** The IL-1β levels in thrombi were analyzed by ELISA. Data are the mean ± SD of seven animals/group. ***, $p<0.001$.

Supplementary Figure S3. MALT1 inhibition reduces NET formation *in vivo* in a murine model of venous thrombosis and human neutrophils *in vitro*. (A) C57/BL 6J wild-type mice were administered with 2 doses of MALT1 inhibitor, mepazine (12. 5 mg/kg body weight each dose, i.p.), the first dose at 2 h prior to the surgery and a second dose 24 h after the IVC ligation. Control mice were administered with a control vehicle (DMSO). Mice were subjected to the IVC ligation-induced stenosis. Forty-eight hours following the IVC ligation, mice were killed, and thrombi were collected and processed for tissue sectioning. Thrombus tissue sections were immunostained for Ly-6G (red) or citH3 (green) and DNA was stained with Hoechst dye. The sections were visualized under 63X magnification. Number of NETs (LY-6G/citH3-dual positive cells) were counted at 10 randomly chosen fields covering the entire section and averaged/field. Data are the mean ± SD of three animals/group. **(B, C)** Human neutrophils (5×10^5) plated on cover glass were treated with mepazine (20 μM) or vehicle for 2 h. The cells were stimulated with PMA (100 nM) or TNF/IL-1β (10 ng/ml) for 3 h. The cells were fixed, blocked, and incubated with primary antibodies against citH3 and MPO overnight at 4°C. The cells were washed and incubated with Hoechst stain and AF647 or AF488 labeled secondary antibodies for 90 min. The cells were washed and mounted with antifade Fluro gel. The

cells were visualized under a confocal microscope. Images represent data from three independent experiments. Histogram presented at the right side represents % of NETosis. The % of NETosis was calculated using the formula, NET [%] = 100 × number of objected stained positive with citH3 antibody/number of objects stained with Hoechst dye. ** p<0.01, *** p<0.001.

Supplementary Figure S4. Gab2$^{-/-}$ mice are protected against venous thrombosis in a murine model of stasis-induced thrombosis. Gab2$^{-/-}$ mice or littermate controls (Gab2$^{+/+}$) were subjected to the IVC ligation-induced stasis. Twenty-four hours following the IVC ligation, mice were killed, and thrombus formation in the ligated vein was evaluated. (A) Representative images of thrombus; (B) thrombus length; (C) thrombus weight. (D, E) Thrombi collected were processed for tissue sectioning, and sections were stained with antibodies against Ly-6G (D) or F4/80 (E) antigens. The sections were visualized under a bright field microscope and analyzed as described in Figure 7 of the main article. Data are mean ± SD of two independent experiments (a total of 6 animals/group). **, p<0.01.

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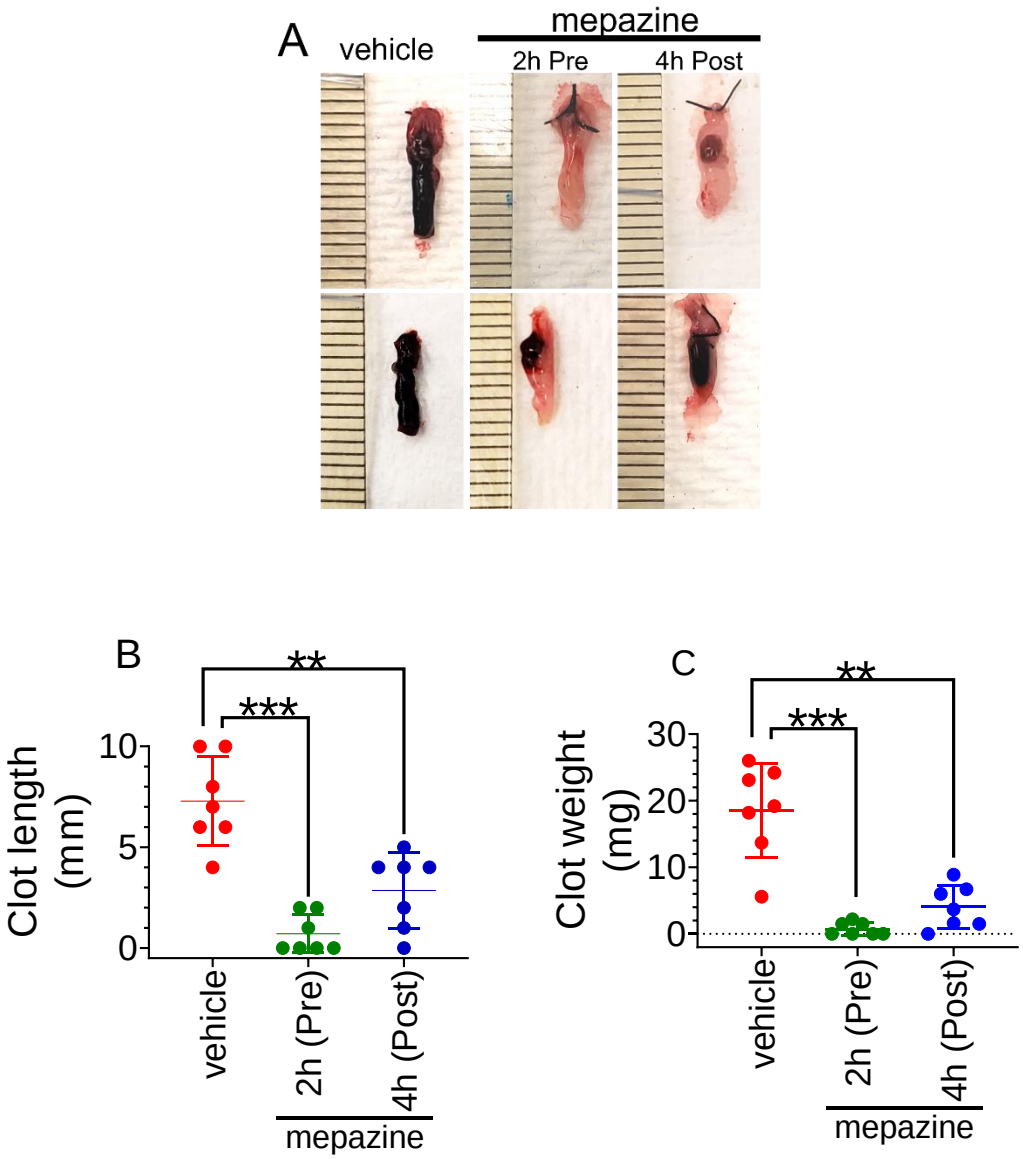
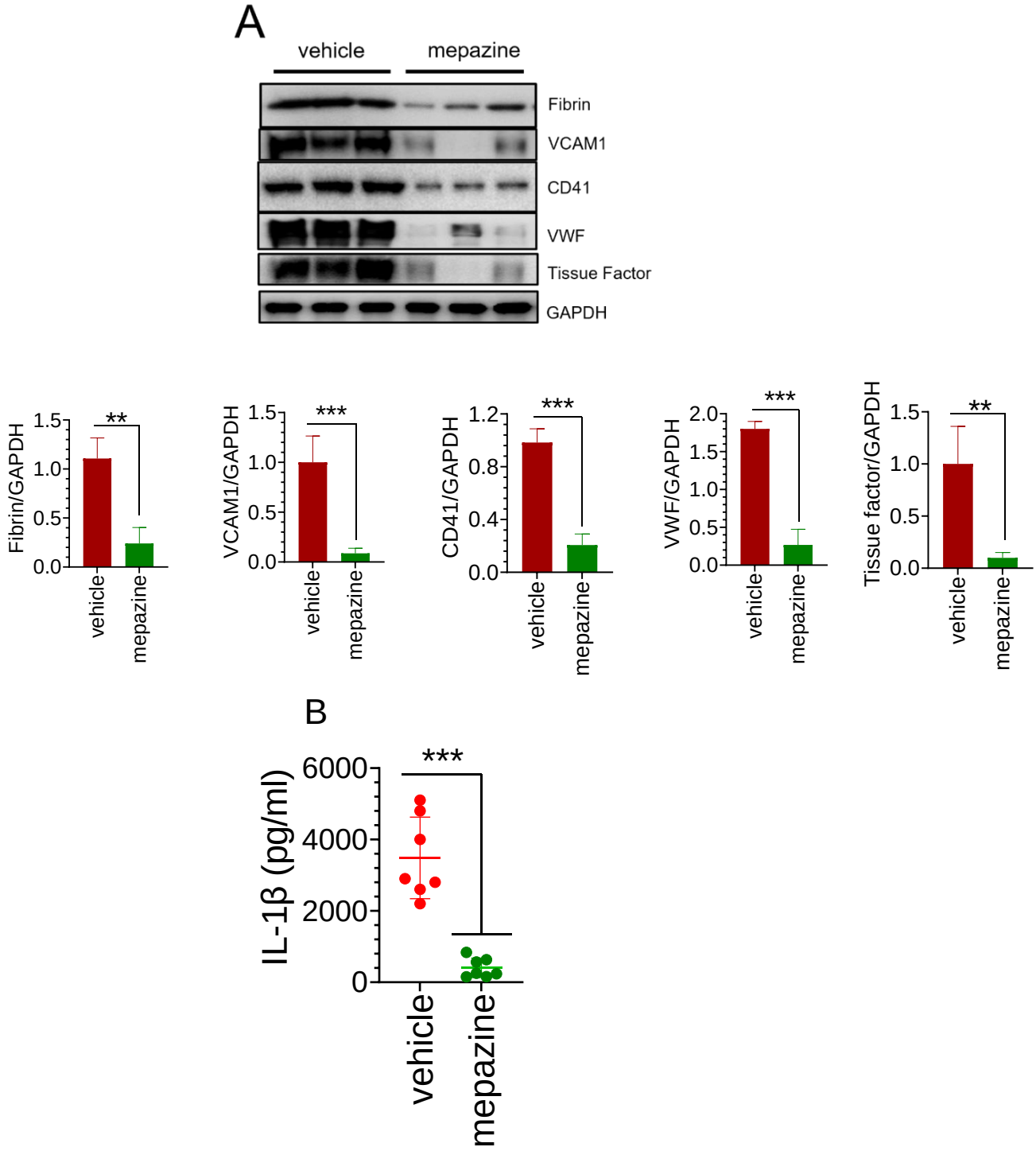
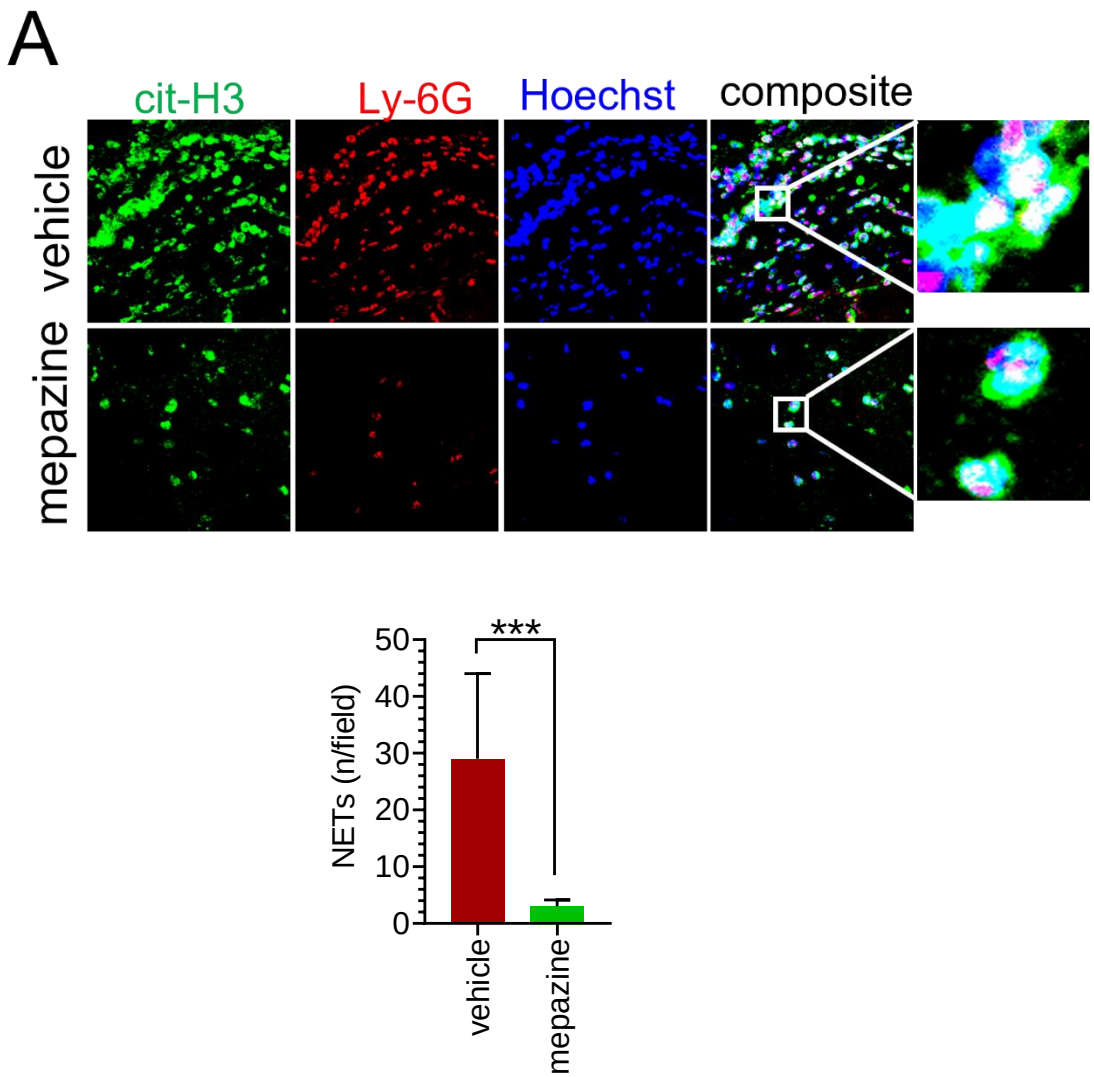
Supplementary Figure S1

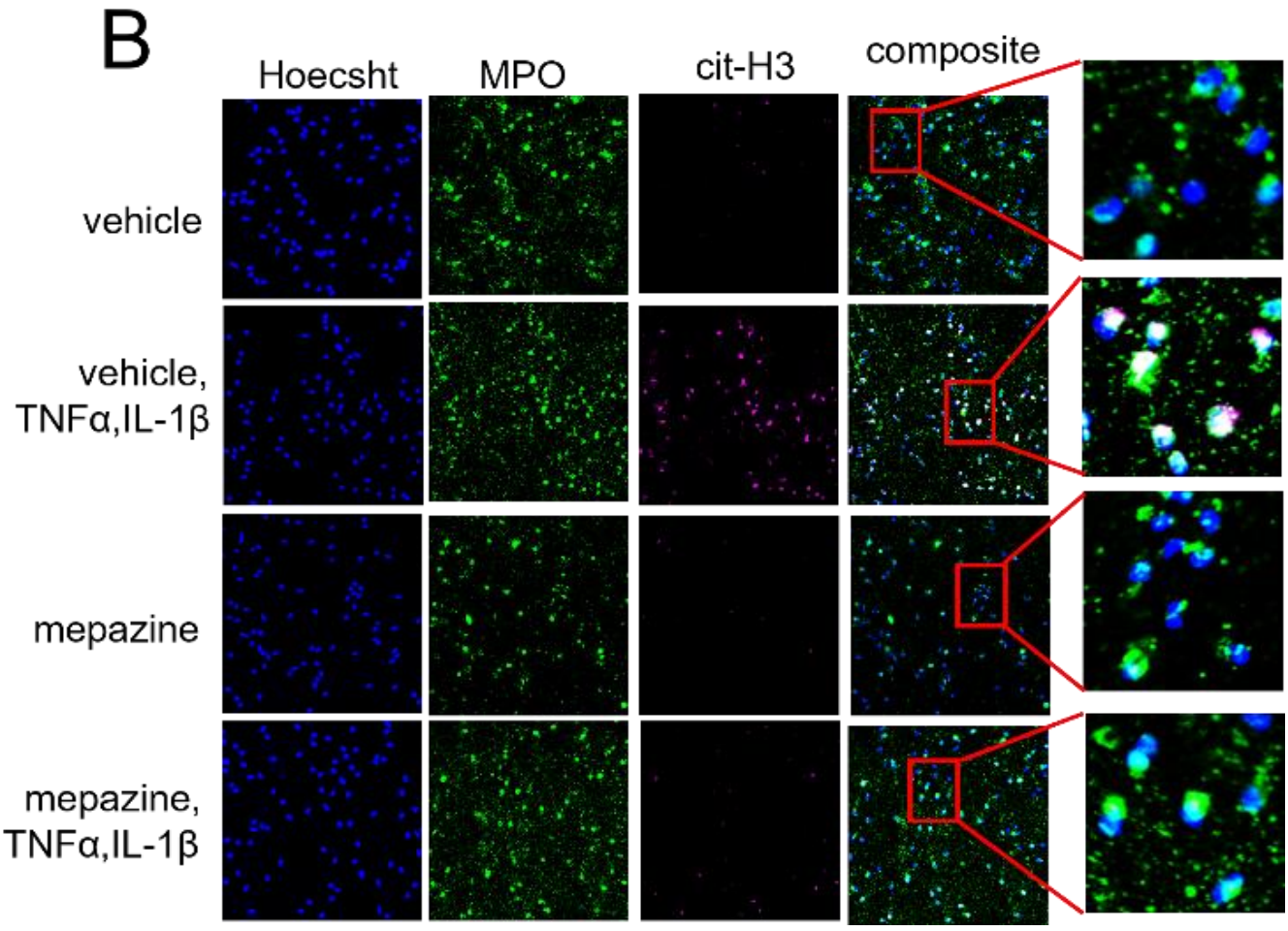


Supplementary Figure S2



Supplementary Figure S3




B
Hoecsht
MPO
cit-H3
composite
vehicle
vehicle,
TNFα,IL-1β
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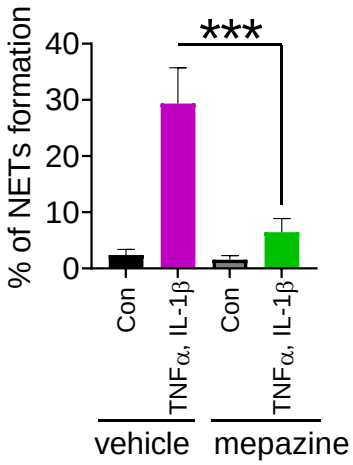
mepazine,
TNFα,IL-1β


% of NETs formation
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30
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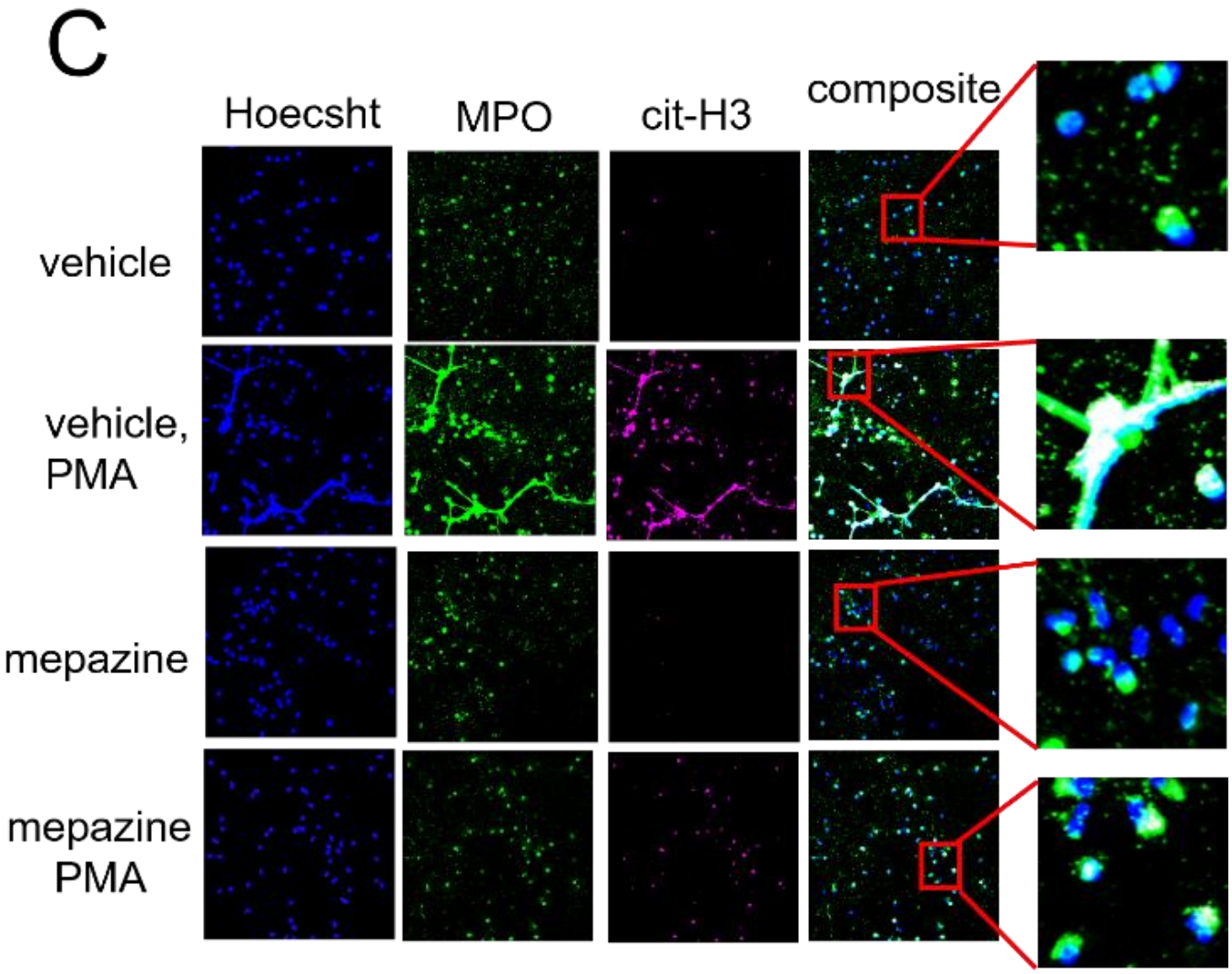
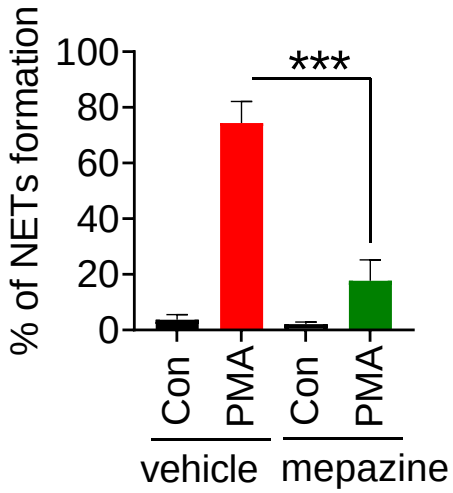
TNFα, IL-1β
vehicle
mepazine


C
Hoecsht
MPO
cit-H3
composite
vehicle
vehicle, PMA
mepazine
mepazine PMA


% of NETs formation
100
80
60
40
20
0
***
Con
PMA
Con
PMA
vehicle
mepazine

Supplementary Figure S4