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Deep vein thrombosis (DVT), caused by alterations in venous homeostasis is the third most common cause of cardiovascular mortality; however, key molecular determinants in venous thrombosis have not been fully elucidated. Several lines of evidence indicate that DVT occurs at the intersection of dysregulated inflammation and coagulation. The enzyme ectonucleoside tri(di)phosphohydrolase (*ENTPD1*, also known as CD39) is a vascular ecto-apyrase on the surface of leukocytes and the endothelium that inhibits intravascular inflammation and thrombosis by hydrolysis of phosphodiester bonds from nucleotides released by activated cells. Here, we evaluated the contribution of CD39 to venous thrombosis in a restricted-flow model of murine inferior vena cava stenosis. CD39-deficiency conferred a >2-fold increase in venous thrombogenesis, characterized by increased leukocyte engagement, neutrophil extracellular trap formation, fibrin, and local activation of tissue factor in the thrombotic milieu. This was orchestrated by increased phosphorylation of the p65 subunit of NFκB, activation of the NLRP3 inflammasome, and interleukin-1β (IL-1β) release in CD39-deficient mice. Substantiating these findings, an IL-1β-neutralizing antibody attenuated the thrombosis risk in CD39-deficient mice. These data demonstrate that IL-1β is a key accelerant of venous thrombo-inflammation, which can be suppressed by CD39. CD39 inhibits in vivo crosstalk between inflammation and coagulation pathways, and is a critical vascular checkpoint in venous thrombosis.



# Ectonucleotidase tri(di)phosphohydrolase-1 (ENTPD-1) disrupts inflammasome/interleukin 1beta-driven venous thrombosis

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**Abstract:**

Deep vein thrombosis (DVT), caused by alterations in venous homeostasis is the third most common cause of cardiovascular mortality; however, key molecular determinants in venous thrombosis have not been fully elucidated. Several lines of evidence indicate that DVT occurs at the intersection of dysregulated inflammation and coagulation. The enzyme ectonucleoside tri(di)phosphohydrolase (*ENTPD1*, also known as CD39) is a vascular ecto-apyrase on the surface of leukocytes and the endothelium that inhibits intravascular inflammation and thrombosis by hydrolysis of phosphodiester bonds from nucleotides released by activated cells. Here, we evaluated the contribution of CD39 to venous thrombosis in a restricted-flow model of murine inferior vena cava stenosis. CD39-deficiency conferred a >2-fold increase in venous thrombogenesis, characterized by increased leukocyte engagement, neutrophil extracellular trap formation, fibrin and local activation of tissue factor in the thrombotic milieu. This was orchestrated by increased phosphorylation of the p65 subunit of NFκB, activation of the NLRP3 inflammasome, and interleukin-1β (IL-1β) release in CD39-deficient mice. Substantiating these findings, an IL-1β-neutralizing antibody attenuated the thrombosis risk in CD39-deficient mice. These data demonstrate that IL-1β is a key accelerant of venous thrombo-inflammation, which can be suppressed by CD39. CD39 inhibits in vivo crosstalk between inflammation and coagulation pathways, and is a critical vascular checkpoint in venous thrombosis.

**Introduction:**

The cell surface enzyme ectonucleoside tri(di)phosphohydrolase-1 (ENTPD1, also known as CD39), is a key molecular factor protecting blood fluidity at the vessel-wall interface by catalyzing the hydrolytic cleavage of phosphodiester bonds, thereby dissipating the extracellular purinergic nucleotides ATP and ADP. These extracellular purines function as intravascular autocrine and paracrine "danger" signals to a variety of cells including innate immune cells, platelets, and endothelium, activating inflammatory and thrombotic pathways.(1, 2) Our group has recently shown that CD39 haploinsuffficiency is a critical mediator of arterial leukocyte trafficking, atherosclerosis, and platelet hyperactivity,(3-5) but the role for CD39 in the venous milieu has not been fully elucidated; in contrast to arterial beds, veins are characterized by low shear, absence of atherosclerotic plaque, and an emphasis on tissue factor-driven thrombosis. Despite the substantial morbidity and mortality burden of deep venous thrombosis,(6) proximate mechanisms that trigger and propagate venous thrombosis are incompletely understood. As a window to understanding venous thrombosis pathogenesis, many acquired risk factors are associated with DVT, including trauma, infection, and chronic inflammatory diseases suggesting an integral association between inflammation and coagulation.(7) Although multiple, sometimes coalescing pathologic drivers can result in venous thrombosis, current studies have largely focused on coagulation, while the precise mechanisms underlying inflammatory triggers and their endogenous checkpoints remain unknown. We hypothesized that CD39 haploinsufficient mice would have impaired protection against DVT, enhanced leukocyte recruitment and activation, and unrestrained inflammatory signaling.

Here, we show that CD39 plays a critical role in protection from venous thrombogenesis in restricted venous flow conditions by checking leukocyte engagement and inflammasome activation. Our data show that loss of a single *Cd39* allele increases venous thrombosis and

propagation, with enhanced tissue factor and fibrin deposition, neutrophil extracellular trap (NET) formation, and canonical inflammasome activation, under control of NFκB, the NOD-like receptor pyrin domain containing 3 protein (NLRP3) inflammasome, and interleukin-1β (IL-1β) expression. Taken together, these data provide evidence that CD39 serves as an indispensable checkpoint of venous inflammation and coagulation at the blood-vessel interface by restraining innate immune activation.

**Results and Discussion**:

**CD39 protects against venous thrombosis following inferior vena cava (IVC) flow restriction.**

Our group and others have previously shown that CD39 has a potent anti-thrombotic and anti-inflammatory effect in the context of arterial flow conditions.(3, 4, 8) We first validated our model of flow-restricted venous thrombosis in mice with a normal complement of CD39 (WT), with thrombus occurring in 23% of mice (**Figure 1A**), consistent with prior reports in this model.(9-11) These thrombi contained regions that are RBC-rich and RBC-poor, similar to the morphology seen in human venous thrombi.(12) As CD39 expression can be dynamically downregulated by systemic cytotoxic stress, or local turbulent blood flow patterns, and is being investigated as an inhibition target in cancer, we examined whether partial deficiency of CD39 could contribute to venous thrombosis under flow-restricted conditions. CD39 haploinsufficient mice were used to test the effect of CD39-deficiency on venous thrombosis. *Cd39*$^{+/-}$ mice have similar circulating leukocyte, RBC, and platelet counts when compared with genotype controls (WT) containing the non-excised LoxP sites used to generate the *Cd39*$^{+/-}$ mice.(13) When subjected to IVC flow-restriction, *Cd39*$^{+/-}$ mice developed significantly more venous thrombosis compared with WT controls (**Figure 1A)**, coupled with more thrombus propagation, sometimes extending into the iliac veins (**Figure 1B, C**). As activation of the coagulation cascade results in

thrombin-induced cleavage of fibrinogen to fibrin, we examined the effect of CD39 haploinsufficiency on fibrin formation in the venous thrombus milieu. Using a fibrin-specific antibody, we determined that *Cd39*$^{+/-}$ mice had greater fibrin accumulation than WT mice, suggesting an increase in the insoluble fibrin networks in the vein lumen lending to heightened thrombus stabilization in *Cd39*$^{+/-}$ mice (**Figure 1D**). Whether the increased fibrin content in venous thrombi from *Cd39*$^{+/-}$ mice reflects greater fibrin deposition or deficient fibrinolysis is unknown and remains to be elucidated. Tissue factor, a significant determinant of thrombus initiation and propagation is released by hematopoietic cells under venous flow-restricted conditions, and complexes with factor VII/VIIa in blood to activate the tissue factor (extrinsic) coagulation pathway.(14) Consistent with our findings of increased venous thrombogenesis, thrombus lysate from *Cd39*$^{+/-}$ mice contained more tissue factor when compared with WT controls (**Figure 1E**).

**CD39 haploinsufficiency increases neutrophil extracellular trap formation in venous thrombosis.**

We next examined whether the increased thrombogenesis in *Cd39*$^{+/-}$ mice could in part be explained by leukocyte engagement. Venous thrombi in *Cd39*$^{+/-}$ mice revealed enhanced leukocyte recruitment, driven primarily by neutrophils (**Figure 2A,B)**. Neutrophil activation with chromatin decondensation and release of web-like traps facilitate heterotypic cell interactions in venous thrombosis under flow-restriction, serving as a scaffold for platelet aggregation, neutrophil and monocyte recruitment, and fibrin deposition.(14, 15) During thrombosis, NETs also construct a pro-coagulant milieu, sequestering von Willebrand factor, activating factor XII in the intrinsic coagulation cascade, and inactivating the tissue factor pathway inhibitor.(14, 16, 17) The prominent leukocyte recruitment and fibrin deposition observed in the developing thrombus in *Cd39*$^{+/-}$ mice led us to next examine whether CD39-deficiency would be sufficient

to trigger an increase in neutrophil activation and NET formation. Neutrophils from *Cd39*$^{+/-}$ mice had reduced expression of CD39 compared with WT neutrophils (**Supplemental Figure 1A**). Following in vitro stimulation with phorbol 12-myristate 13-acetate (PMA), a potent inducer of NADPH oxidase-dependent NET formation, mature neutrophils from naïve *Cd39*$^{+/-}$ mice, as compared with genotype controls, demonstrated a marked >2.5-fold increase in expression of a citrullinated form of histone H3 (H3cit), which is a NET-associated histone modification that results in loosened chromatin structures which can be extruded as NETs (**Figure 2C**). Consistent with these in vitro findings, immunoblots of venous thrombus lysate from *Cd39*$^{+/-}$ mice also showed significantly higher H3Cit expression than WT mice (**Figure 2D** and **Supplemental Figure 1B**). Confirming the role of neutrophil CD39 haploinsufficiency in promoting thrombosis, *Cd39*$^{+/-}$ neutrophils administered to WT mice increased thrombus size compared with WT neutrophils (**Figure 2E**). Taken together, these data suggest that during venous thrombogenesis CD39 is an obligatory restraint on H3cit-positive NET formation in vitro and in vivo.

**Exaggerated inflammasome activation in venous thrombosis in CD39-deficient mice.**

Extracellular release of ATP and ADP through cell death, injury, or activation is a potent stress response, altering the local microenvironment to activate paracrine and autocrine signaling pathways.(18, 19) Binding of extracellular ATP to the plasma membrane receptor ionophore P2X7 activates a potent stress response signaling pathway, characterized by potassium efflux, triggering assembly and activity of the inflammasome, a multiprotein oligomer that activates highly pro-inflammatory cytokines including interleukin-1β.(20) Gupta et al. recently reported increased NLRP3 inflammasome assembly in patients at high altitude at risk for DVT.(21) Canonical inflammasome activation requires a “priming” step marked by NFkB activation and inflammasome component transcription.(20) A second signal initiates NLRP3-mediated

assembly and oligomerization of inflammasome component fibers, proteolytic cleavage of pro-caspase-1 to caspase-1 to generate mature IL-1β, and release of prothrombotic tissue factor-rich microparticles.(22, 23) As ATP, the substrate for CD39's phosphohydrolytic activity is the primary activating ligand for the vascular P2X7 receptor,(24) we hypothesized that CD39 could suppress inflammasome activation in venous thrombosis and thus haploinsufficiency would drive a pro-inflammatory state. We examined expression of NLRP3 inflammasome components in venous thrombi from *Cd39*$^{+/-}$ and WT mice and observed markedly enhanced phosphorylation of the transactivator serine$^{534}$ site (murine homolog of human serine$^{536}$) of the NFkB p65 subunit in *Cd39*$^{+/-}$ thrombi (**Supplemental Figure 2A**). This was accompanied by increased expression of NLRP3, apoptosis-associated speck-like protein containing a CARD (ASC), and active caspase-1 expression in *Cd39*$^{+/-}$ mice (**Figure 3A-C** and **Supplemental Figure 2B**), and exaggerated mature (processed) IL-1β content (**Figure 3D**) compared with WT thrombi. Concordant with these data, we observed elevated plasma levels of IL-1β in *Cd39*$^{+/-}$ compared with WT mice following venous thrombosis (**Figure 3E**). A recent large clinical trial demonstrated reduced arterial cardiovascular events in high-risk patients following treatment with a monoclonal antibody targeting IL-1β.(25) To determine if the exaggerated venous thrombosis conferred by CD39 haploinsufficiency could be rescued by reduced IL-1β bioavailability, we next performed IVC flow-restriction in WT and *Cd39*$^{+/-}$ mice treated with an IL-1β neutralizing antibody (**Figure 4A, B**). IL-1β blockade resulted in a striking decrease in venous thrombogenesis in CD39-deficient mice; specifically, *Cd39*$^{+/-}$ mice treated with a neutralizing IL-1β antibody developed smaller and fewer venous thrombi than isotype IgG-injected *Cd39*$^{+/-}$ mice under flow-restricted conditions, while WT mice treated with neutralizing IL-1β antibody had similar rates of thrombogenesis as IgG controls and a non-significant decrease in thrombus size. IL-1β blockade did not prolong tail bleeding time (data not shown). To exclude an IgG-dependent effect of IL-1β blockade on *Cd39*$^{+/-}$ venous thrombosis, *Cd39*$^{+/-}$ mice were next treated with

anakinra, a synthetic inhibitor of the IL-1 receptor, or saline vehicle control. Concordant with IL-1β neutralization experiments, IL-1 receptor inhibition resulted in lower thrombotic burden than saline-treated *Cd39*$^{+/-}$ mice in contemporaneous experiments (**Figure 4C**). Taken together, these experiments show a pivotal role for CD39, the dominant vascular ectonucleotidase, in inhibiting NLRP3 inflammasome activation and IL-1β signaling during venous thrombogenesis.

In summary, our findings demonstrate for the first time, that inflammasome-mediated IL-1β is essential for venous thrombogenesis under vascular conditions which include limited flow. These data also reveal an indispensable role for vascular CD39 in protecting against venous thrombogenesis, altering multiple aspects of venous clot formation. A functional paradigm exists wherein venous thrombosis develops as a convergence of multiple inputs from several cell lineages, including neutrophils, monocytes, platelets and endothelium. In addition to pro-thrombotic and pro-inflammatory mediators, these vascular and innate immune effector cells possess the machinery for inflammasome activation and IL-1β processing.(14, 20, 26) Our data show that CD39 is vital for reducing intravascular fibrin deposition, which otherwise provides mechanical and structural support to nascent thrombi; for restraining neutrophil extracellular trap formation; for NLRP3 inflammasome activation and critically, IL-1β accessibility during venous thrombosis. IL-1β may facilitate venous thrombosis through several effector mechanisms in the thrombotic milieu including leukocyte recruitment,(27, 28) remote signaling via thrombogenic microparticles,(23) and platelet integrin activation.(29) Indeed, platelets in *Cd39*$^{+/-}$ mice are also hyperactive,(4) suggesting CD39-based targeted therapeutics may be effective in interrupting a positive feedback loop of thrombo-inflammation.(5, 30) Taken together, these data present a compelling role for CD39 as a vascular checkpoint in impeding venous thrombus accretion. Treatments for venous thrombosis have largely targeted the coagulation pathway, leading to incomplete protection from deep vein thrombosis. Strategies aimed at the nexus of inflammation

and coagulation will be critical for progress in the development of effective therapeutics in venous thrombosis.

Methods:

Details are provided in the **Supplemental Methods**.

**Statistics.** Data analysis was performed using GraphPad Prism 8 software. Normally distributed data were analyzed by unpaired two-tailed Student's *t* test and skewed data were analyzed with Welch's correction. Thrombus frequency was analyzed by Chi squared analysis. Statistical significance was defined as $P<0.05$.

**Animal housing, surgery, and treatments.** All experiments were conducted in accordance with NIH guidelines for use of live animals, and were approved by the University of Michigan Institutional Animal Care and Use Committee.

Author contributions:

Study concept and design (YK, DJP), data acquisition (YK, VY, LC, AB, RZ, HL, BNJ, SY, BT), data analysis (YK, VY, LC, AB, RZ, HL, BT), data interpretation (YK, BT, DJP, JSK, JMK, SHV, MAH, ACA), mouse strain generation and colony management: (SK, HL), study supervision, obtaining funding, drafting manuscript: (YK, DJP).

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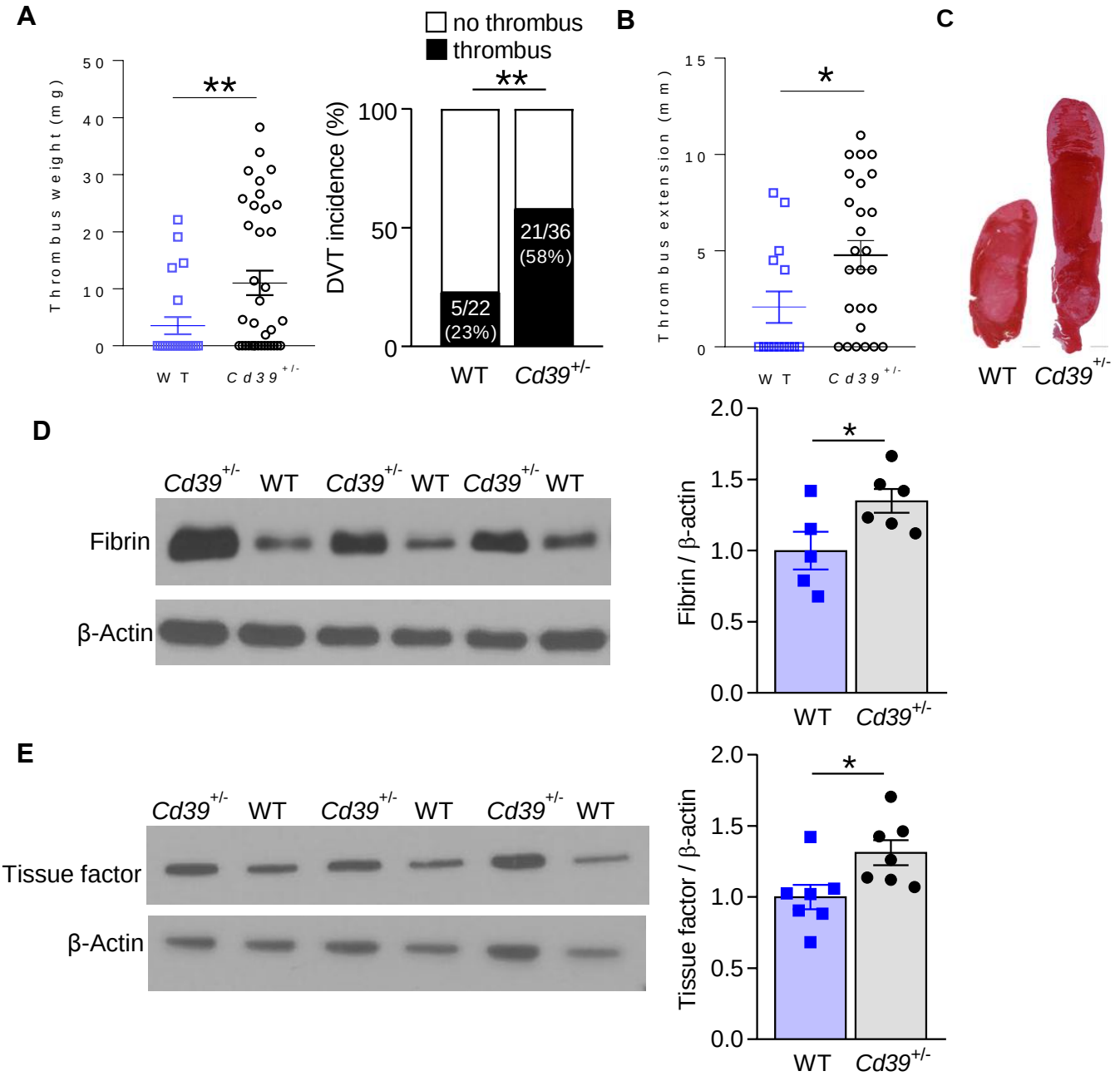


**Figure 1. Increased venous thrombogenesis, clot extension and fibrin content in *Cd39*$^{+/-}$ mice compared with genotype controls.** (**A**) Thrombus weights, and thrombus frequency two days after IVC flow-restriction (stenosis) (WT n=22, *Cd39*$^{+/-}$ n=36). (**B**) Thrombus extension (WT n=14, *Cd39*$^{+/-}$ n=26). (**C**) Representative H&E sections of thrombi (n=5, each). (**D**) Immunoblots of fibrin content of thrombus lysates (WT n=5, *Cd39*$^{+/-}$ n=6), and (**E**) tissue factor (n=7, each). Data represent mean ± SEM. **P*<0.05; **<0.01 using two-tailed Student's *t* test (**D**, **E**) with Welch's correction (**A**, **B**). (**A**) right panel analyzed using Chi squared method. Scale bar, 1 mm.

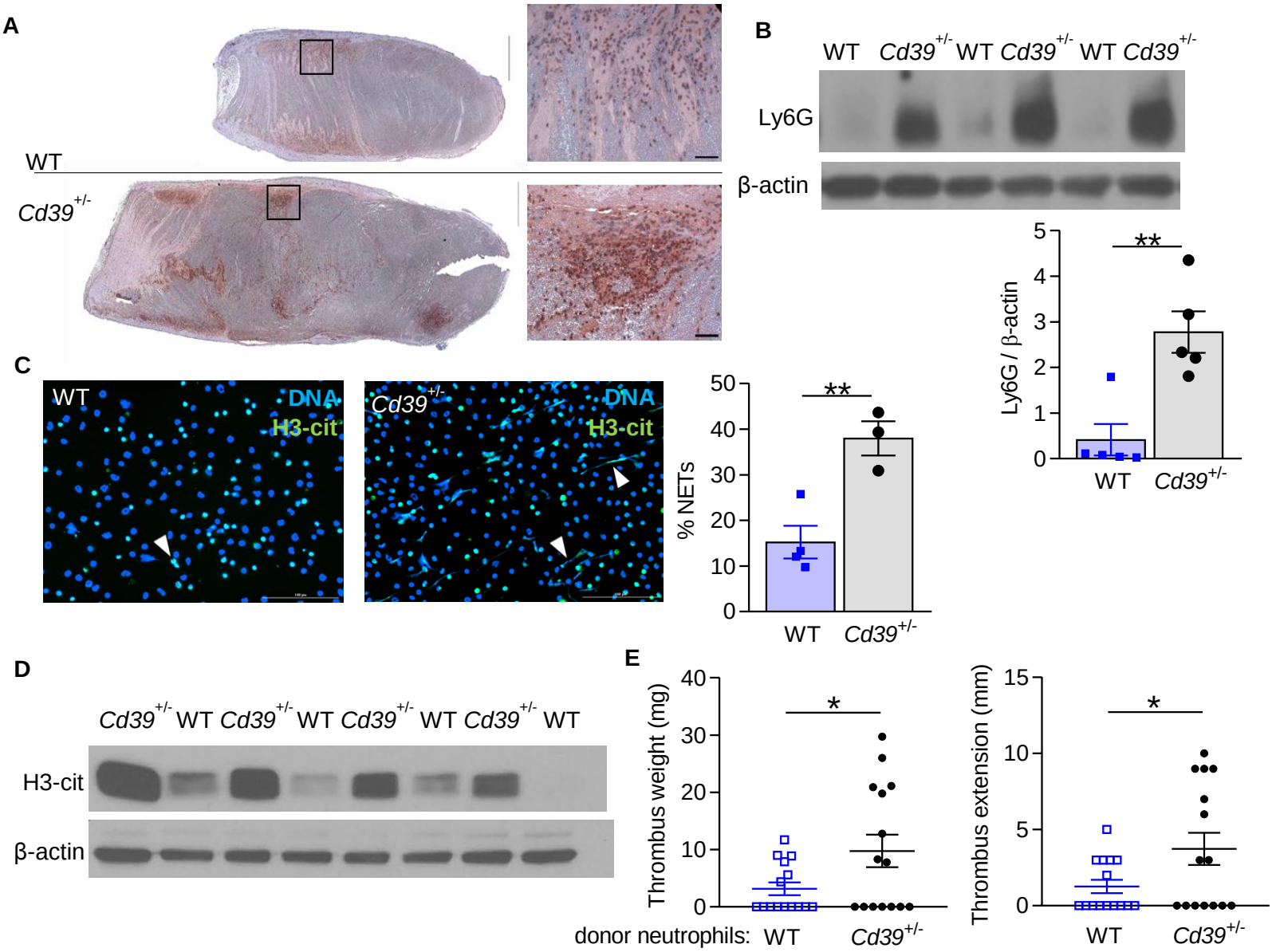


**Figure 2. CD39 tempers thrombus cellularity and leukocyte recruitment to growing thrombus.** (**A**) Neutrophil (Ly6G) stained venous thrombi (*brown*, Ly6G; n=5, each, scale bar 1 mm; inset (right) scale bar 50 μm). (**B**) Immunoblot for Ly6G in venous thrombi from WT and *Cd39*$^{+/-}$ mice (n=5, each). (**E**) Neutrophil extracellular trap (NET) formation assessed by histone 3 citrullination by immunofluorescence microscopy following *ex vivo* stimulation with PMA (100 nM) (*green*, H3cit; *blue*, DNA; *white arrowheads*, NETs; WT n=4, and *Cd39*$^{+/-}$ n=3. Scale bar, 100 μm). Representative images shown. (**E**) Citrullinated histone 3 content of venous thrombus lysates (n=4, each). (**E**) Venous thrombus weight and extension in WT mice following adoptive transfer of WT or *Cd39*$^{+/-}$ neutrophils (n=15, each). Data represent mean ± SEM. *$P$<0.05; **<0.01; ***<0.01 using two-tailed Student's *t* test.

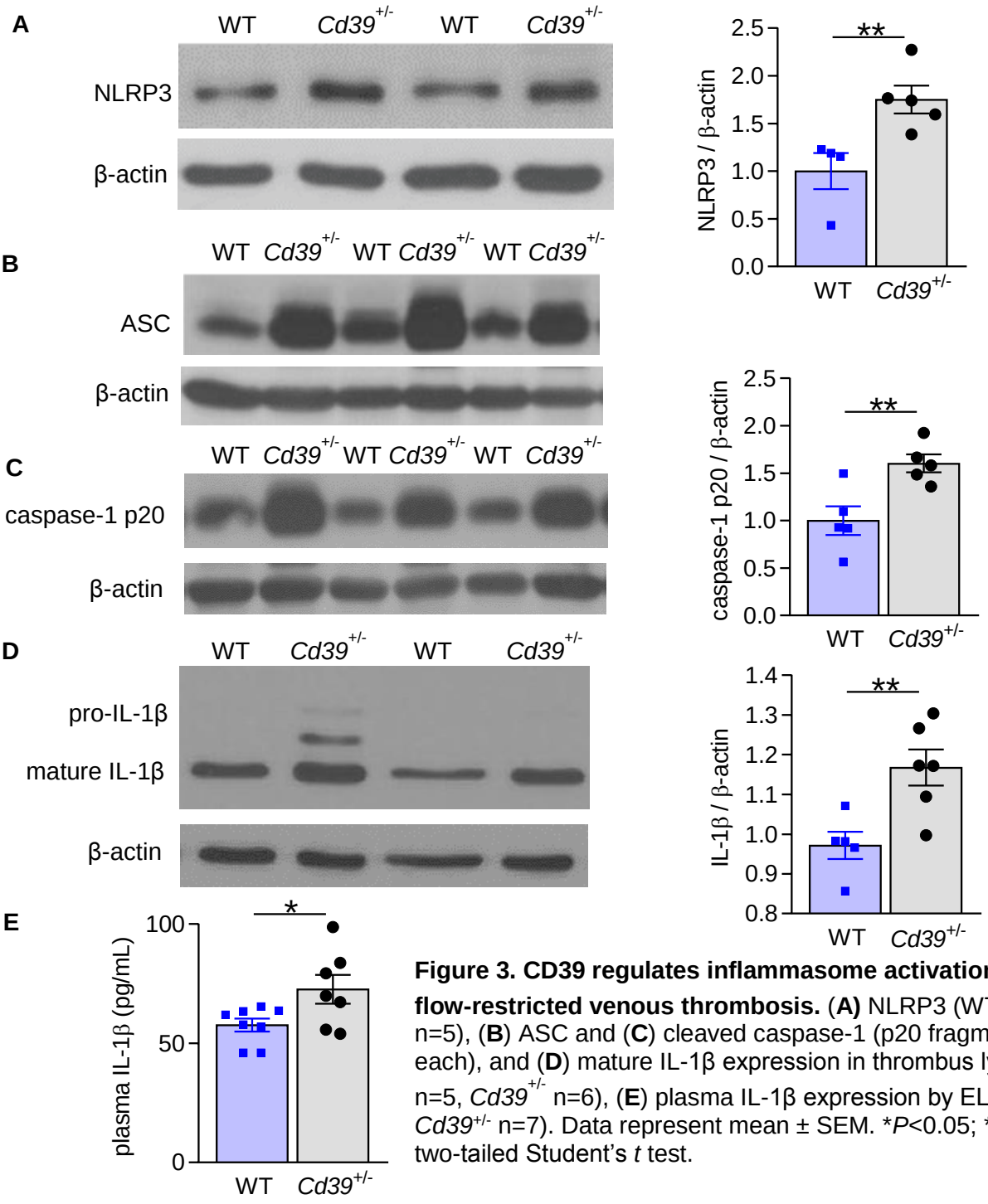


**Figure 3. CD39 regulates inflammasome activation in mice with flow-restricted venous thrombosis.** (**A)** NLRP3 (WT n=4, *Cd39*$^{+/-}$ n=5), (**B**) ASC and (**C**) cleaved caspase-1 (p20 fragment) (n=5, each), and (**D**) mature IL-1β expression in thrombus lysates (WT n=5, *Cd39*$^{+/-}$ n=6), (**E**) plasma IL-1β expression by ELISA (WT n=8, *Cd39*$^{+/-}$ n=7). Data represent mean ± SEM. **P*<0.05; **<0.01 using two-tailed Student's *t* test.

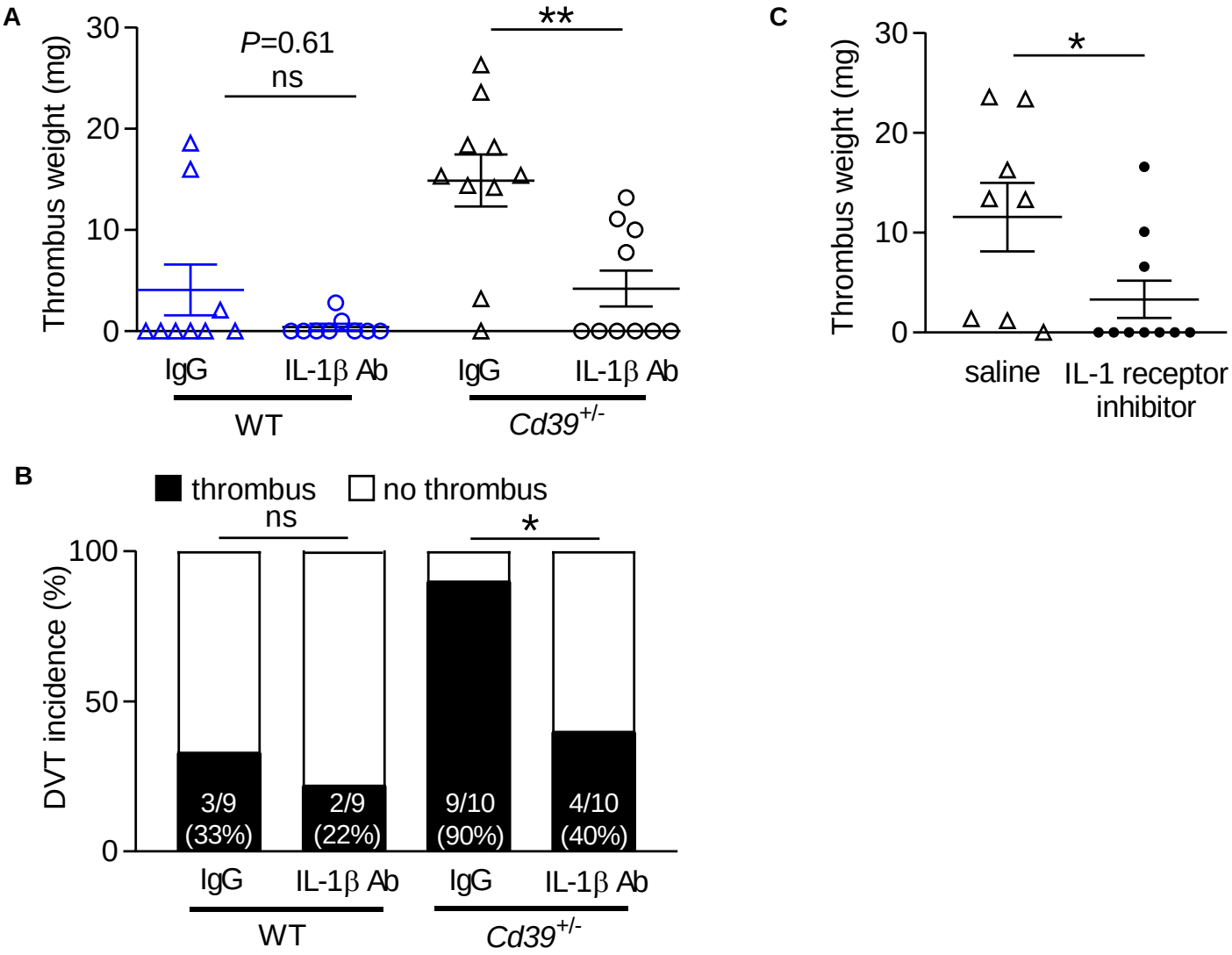


**Figure 4. Interleukin 1β inhibition reduces incidence of venous thrombosis in CD39-deficient mice.** (**A**) Thrombus size, and (**B**) frequency in WT and *Cd39*$^{+/-}$ mice administered IL-1β neutralizing or IgG isotype control antibody (200 μg IP 24 hours prior to surgery, n=9-10, each). (**C**) Thrombus size in *Cd39*$^{+/-}$ mice treated with vehicle control or IL-1 receptor inhibitor anakinra (100 mg/kg/day IP, n=8-10, each). Data represent mean ± SEM. **P*<0.05; **<0.01 using two-tailed Student's *t* test with Welch's correction (A, C) or using Chi squared method (B).