NGAL contributes to hemostasis and thrombosis by potentiating or suppressing coagulant or anticoagulant factors

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Abstract

Coagulation is related to inflammation, but the key pathways, especially innate immunity inflammatory response-coagulation, hemostasis, and thrombus regulation is poorly understood and need to be further explored. In the current study, we showed that innate immunity inflammatory mediator neutrophil gelatinase-associated apolipoprotein (NGAL) interacted with and potentiated thrombin, kallikrein, and FXIa and suppressed the inactivation of thrombin by antithrombin to induce hypercoagulability. In multiple mice hemostasis and thrombus models, NGAL overexpression or intravenous administration promoted hemostasis and aggravated thrombotic tendency, whereas NGAL knockout or treatment with anti-NGAL monoclonal antibody significantly prolonged bleeding time and alleviated thrombus formation. Importantly, NGAL knockout prolonged mice tail bleeding time or artery occlusion time to over 40 min or 30 min, respectively. Furthermore, anti-NGAL monoclonal antibody treatment significantly alleviated thrombus formation in mice-tail thrombosis model induced by carrageenan, which is associated with inflammation. Collectively, these findings clarify NGAL is an important coagulation regulator and mediates the crosstalk between innate immunity inflammation and coagulation, hemostasis, and thrombus, and provide new targets and strategies for the development of safe and efficient innovative antithrombotic drugs.

Introduction

Inflammation and coagulation regulation are closely related(*1-4*). Studies have shown that tissue factor activates clotting factors, triggering pro-inflammatory responses, and initiating downstream cell signaling pathways(*1, 5-7*). Tissue factor is located at the intersection of coagulation and inflammation, serving as a trigger for host responses to injury or invasion by pathogens. Additionally, white blood cells, platelets, and endothelial cells release inflammatory cytokines and procoagulant microparticles (MPs), which sustain the exposure of tissue factor in the blood(*1, 3*). Thrombin plays a central role in controlling bleeding and thrombus formation, and exerts hemostatic and procoagulant effects through the coagulation-inflammation axis(*1, 6, 8, 9*). Thrombin can activate various types of cells by hydrolyzing protease-activated receptors (PARs)(*10-12*), which are expressed in multiple cells, including platelets, immune cells, endothelial cells, and neurons(*13*). After thrombin-mediated PAR activation, platelets degranulate, change shape, and aggregate. In terms of inflammation, thrombin activates PAR-1 in multiple cells, triggering the production of chemotactic protein-1, TNFα, and IL-6. PAR-1-dependent signaling also leads to endothelial cell activation, resulting in increased exposure of P-selectin and expression of monocyte chemotactic protein-1, plasminogen activator inhibitor-1, and IκB-β(*1*). These actions of endothelial cells facilitate the adherence and recruitment of platelets and leukocytes, and play an important role in the early stages of venous thrombus formation(*1*). Overall, these processes form a strong positive feedback loop, amplifying inflammation, and coagulation processes on the surface of blood vessels(*1*). Activated protein C (APC) inhibits the coagulation reaction by cleaving thrombin factors Va and VIIIa(*3, 5, 6, 9*). The APC-thrombomodulin-protein C receptor axis has significant vascular protective properties. APC induces PAR-mediated anti-inflammatory/cellular protective signals through endothelial protein C receptor, upregulates apoptosis inhibitors (IAP-1, A20) and sphingosine-1 phosphate (S1P) signaling pathways, and inhibits endothelial cell cytokine release. In addition, the contact activation of the coagulation system releases bradykinin (BK) (*14, 15*). BK binds to B2R receptors on neutrophils and endothelial cells triggers a

proinflammatory response.

Toll-like receptors and complement system of innate immunity related to inflammation play important roles in the pathogenesis of thrombosis(*1-3, 16*). Toll-like receptors are activated by lipopolysaccharide, DNA, heat shock proteins, etc., and produce inflammatory cytokines such as interferon, TNF-α, and IL-1β, thereby promoting tissue factor expression and coagulation. The complement system is an important inducer of inflammation and thrombosis(*17*), and the binding of complement factors C3a and C5a to receptors C3aR and C5aR can mediate the early stages of inflammation and thrombosis(*1, 18*). C3a and C5a can also induce endothelial cells to express IL-8. The exposure of P-selectin on endothelial cells can be triggered by C5a, which plays a crucial role in facilitating the adhesion of neutrophils to endothelial cells (*1, 6, 18*). Furthermore, C5a mediates tissue factor and hemophilia factor expression in neutrophils and endothelial cells. Monocyte tissue factor expression induced by C5b-7 and platelet activation mediated by C5b-8/C5b-9 can directly induce the formation of procoagulant phenotypes(*1, 6, 18*). The fibrinolytic system is essential for the breakdown of blood clots, but interestingly, it can also contribute to inflammation processes associated with thrombosis(*1, 6*). For example, plasmin can directly activate the production of inflammatory C5a and C5b. Plasminogen can cleave iC3b, leading to the disruption of its ability to inhibit IL-12 expression in macrophages. Neutrophil extracellular traps (NETs) are formed by neutrophils and can capture circulating cells. The cellular components associated with NETs, such as histones, DNA, and proteases, have been implicated in inducing inflammation and potentially promoting the formation of blood clots(*1, 2, 19*). Overall, our understanding of how the innate immune system regulates coagulation, hemostasis, and thrombosis is still limited, and research in this area is relatively weak, requiring further in-depth study(*1*). Furthermore, an optimal therapy for the prevention or treatment of thromboembolism would aim to suppress excessive inflammation and coagulation without interfering with normal hemostasis or compromising the immune system (*20*).

In the current study, we documented that the innate immunity inflammatory

mediator neutrophil gelatinase-associated apolipoprotein (NGAL) plays a significant role in the modulation of coagulation, hemostasis, and thrombosis. It achieves this by interacting with and enhancing the activity of thrombin, kallikrein, and FXIa, while concurrently inhibiting the inactivation of thrombin by antithrombin.

Results

NGAL interacts and potentiates thrombin, kallikrein, and FXIa while inhibiting thrombin-antithrombin complex formation in vitro

Label-free quantitative proteomics showed NGAL was increased in plasma of deep vein thrombosis patients (Data not shown). In order to investigate the potential mechanism underlying NGAL-induced hypercoagulability, various tests were conducted to assess the impact of NGAL on coagulation pathway and platelet aggregation. As depicted in Figure 1A and B, NGAL was found to promote coagulation by shortening the recalcification time without affecting platelet aggregation that was stimulated by ADP or collagen. Furthermore, the enzymatic activities of various coagulation factors and inhibitors were tested to assess the effects of NGAL on coagulation. Results showed that NGAL enhanced the activity of thrombin, kallikrein, and FXIa, while it had no impact on FXIIa, FIXa, and plasmin (Figure 1C-F). It was observed that NGAL blocked the formation of thrombin-antithrombin (TAT) complexes in vitro (Figure 1G). Additionally, NGAL enhanced the ability of thrombin to hydrolyze fibrinogen, resulting in the production of Fibrinopeptide A (FbpA) and FbpB (Figure 1H). Confocal analysis revealed the physiological interaction between NGAL and thrombin/kallikrein (Figure 1I). The presence of NGAL-thrombin-/kallikrein-positive deposits indicated the formation of NGAL-thrombin/kallikrein complexes in thrombus (Figure 1I). These findings suggest that NGAL can induce hypercoagulability by interacting and enhancing multiple coagulation factors and neutralizing the inhibitory effects of antithrombin on thrombin.

NGAL overexpression promotes hypercoagulability and hemostasis that are

inhibited due to NGAL deficiency

To investigate the function of NGAL in coagulation, we conducted experiments to evaluate the effects of NGAL overexpression, intravenous administration, NGAL monoclonal antibody interference, and NGAL knock out on various indexes such as thrombin generation, activated partial thromboplastin time (APTT), and prothrombin time (PT). Results showed that thrombin generation was significantly increased in plasma from NGAL overexpression mice (AAV9-NGAL) and mice with intravenous NGAL administration, APTT and PT was shortened (Figure 2A-C). Conversely, NGAL monoclonal antibody-treated mice (NGAL mAb) showed significantly reduced thrombin generation, prolonged APTT and PT (Figure 2A-C). As expected, the blank virus controls (with empty AAV9 vector) and control IgG showed no effects on thrombin generation, APTT and PT (Figure 2A-C).

The role of NGAL in hemostasis was further investigated using mice tail-bleeding and saphenous vein bleeding models. Significantly shorten tail-bleeding and saphenous vein bleeding time was found in AAV9-NGAL and intravenous NGAL administration mice, with AAV9 control mice showing no effects (Figure 2D and E). Conversely, NGAL monoclonal antibody-treated mice (Figure 2D and E) and NGAL knock out mice (Figure 2F) showed significantly prolonged tail-bleeding or saphenous vein bleeding time, whereas the control IgG showed no effect on the prolongation of bleeding time (Figure 2D and E). Significantly, tail-bleeding time in mice which treated with anti-NGAL antibody or NGAL was knocked out was over 15 min and 40 min (Figure 2D and F), respectively. These results suggest that NGAL plays a crucial role in coagulation and hemostasis.

Thrombus tendency is alleviated by NGAL deficiency, whereas NGAL overexpression has the opposite effect

The role of NGAL in promoting thrombus was further investigated using mice thrombosis model induced by $FeCl_3$ and deep venous thrombosis (DVT) model. Decreased blood flow (Figure 3A and B) and aggravated thrombus formation (Figure 3C and D) were observed in the carotid arteries and deep vein of NGAL

overexpression and intravenous NGAL administration mice, whereas NGAL monoclonal antibody-treated mice showed significant increased blood flow and alleviated venous thrombus formation (Figure 3A-D), with with AAV9 control and IgG showing no effects (Figure 3A-D). To further investigate the role of NGAL in thrombus, mice of NGAL knock out mice was exerted in thrombus models described above. As illustrated in Figure 4A and B, artery occlusion time in NGAL knock out mice was over 30 min, whereas about 7 min in wild type mice. Furthermore, NGAL knock out can significantly inhibit venous thrombus formation (Figure 4C). These results suggest that NGAL plays a key role in regulating thrombus pathogenesis.

NGAL deficiency alleviates carrageenan-induced mice tail thrombus

Thrombosis and inflammation are closely interconnected, and the administration of carrageenan in mice induced systemic inflammation, which subsequently led to the development of thrombosis in the tail vein(*1, 21, 22*). Subsequently, we examined the impact of NGAL on carrageenan-induced tail thrombosis, which is associated with inflammation. After a 24 h induction, a substantial thrombus formation was observed in the carrageen-induced mice (Figure 5A-C). NGAL overexpression and intravenous administration increased tail thrombus, whereas NGAL monoclonal antibody-treatment can significantly reverse this tendency (Figure 5A-C). These results suggest that NGAL mediates the crosstalk between inflammation and coagulation and thrombus.

Discussion

Innate immunity is an important regulatory system for maintaining the internal environment stability of organisms, and excessive or insufficient strength can cause functional disorders in the body(*23, 24*). Neutrophils account for over 40% of all white blood cells and are the most abundant type of granulocyte, constituting the first line of defense in innate immunity and combating infections(*25, 26*). They are closely related to inflammation, infection, trauma, and cancer, and are natural candidates for performing important medical tasks in the human body(*25*). Blood clots are rich in

white blood cells, red blood cells, platelets, and fibrin. During the process of clot formation and hemostasis, neutrophils are recruited to the site of clot formation and vascular damage by locally exposed and released von Willebrand factor, P-selectin, chemokines, and other factors, and may be involved in the pathological process of thrombosis and vascular injury(*1, 26, 27*). Inflammatory reactions in neutrophils exhibit significant polarization, and platelets not only trigger the generation of NETs by physical contact but also affect the migration characteristics of neutrophils. In the pathological processes of sepsis and malignant tumors, NETs may be a major inducing factor for immune thrombosis(*1, 2, 28*). It should be noted that the inflammatory response of neutrophils in the process of clot formation and vascular damage is a progressive process, including the release of various enzymes and active substances such as neutrophil elastase, suggesting the existence of important factors related to coagulation, hemostasis, and thrombus regulation that have not been identified(*1, 2*). The current study clarify innate immunity inflammatory factor-NGAL plays critical roles in coagulation, hemostasis, and thrombus by potentiating multiple coagulation factors and inhibiting TAT complex formation and mediates the crosstalk between innate immunity and coagulation.

Previous reports have shown that NGAL is diagnosed urgently one of the most effective biological markers of sexual kidney injury and early diabetic nephropathy (*29, 30*). NGAL is found from activated neutrophils in response to inflammation related to a relatively small molecular weight secretory protein(*30*). As the important member of apolipoprotein family, NGAL is associated with inflammation, embryonic development, angiogenesis, diabetes, tumorigenesis, and atherosclerotic plaque instability(*30-32*). The bacteriostatic properties of NGAL play a vital role in the antibacterial innate immune response by sequestering bacterial ferric siderophores, which results in iron depletion(*33, 34*). In particular, NGAL has been reported to be associated with cardiovascular disease, with studies focusing on the correlation between NGAL and atherosclerotic plaque instability, myocardial damage, and myocardial fibrosis(*30, 35*). It has been demonstrated NGAL has the ability to bind to proinflammatory ligands, including bacterial formyl peptides, leukotriene B4, and

platelet-activating factor *(36)*. The role of NGAL in platelet activity, especially the thrombin induced platelet aggregation, will be further investigated.

This discovery collectively elucidates the significance of NGAL as a crucial regulator of coagulation, hemostasis, and thrombus. It also highlights its role in facilitating communication between innate immunity, inflammation, hemostasis, and thrombus. These findings offer novel targets and approaches for effective and safe antithrombotic medications.

Materials and Method

Animals and ethics statement

Animal experiments conducted in this study were approved by the Animal Care and Use Committee at Qingdao University (QDU-AEC-2023364) and were conducted in accordance with Guide for the Care and Use of Laboratory Animals. $NGAL^{-/-}$ mice (#024630, 8 weeks of age, C57BL/6J background) were obtained from Jackson Laboratory while BALB/c mice (8 weeks old) were from Vitalriver Experiment Animal Company (Beijing, China). Housing for both strains was maintained in pathogen-free environments.

Effects of NGAL on coagulation and platelet aggregation

Plasma from healthy human donors was collected and prepared by mixing trisodium citrate (0.13 M) and blood in a 1:9 ratio. The mixture was then centrifuged at 3000 rpm/min for 30 minutes at 4 °C to obtain plasma. To study the effects of NGAL on plasma recalcification time, 20 μl of plasma was incubated with human NGAL (1 or 2 μg/ml, HY-P71156, MCE, USA) in 60 μl of HEPES buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) for 10 minutes at 37 °C. This was followed by the addition of 60 μl of 25 mM $CaCl_2$ preheated at 37 °C, and clotting was monitored at 650 nm using an enzyme-labeled instrument (Readmax 1500, Shanpu, Beijing, China). The time to half maximal increase in absorbance was measured to calculate clotting time.

Sodium citrate (1:9) was used as an anticoagulant agent during blood collection. Immediately after the blood draw, plasma of healthy was obtained by centrifugation at

150 g at room temperature for 10 minutes to separate platelet-rich plasma. NGAL (0.5, 1 or 2 μg/ml) was then added to the platelets, which were incubated at 37 °C for 5 minutes. Collagen (1 μg/mL) or ADP (10 μM) was used to elicit platelet aggregations, which were detected by a platelet aggregator (AG400, Techlink biomedical, China). The maximum platelet aggregation rate was counted and analyzed.

Effects of NGAL on activity of coagulation factors or inhibitors

The effect of NGAL on coagulation-related enzymes and inhibitors such as kallikrein, FXIIa, FXIa, FIXa, FVIIa, thrombin, plasmin, or antithrombin was evaluated using chromogenic substrates correspondingly. Specifically, the enzyme to be tested was incubated with NGAL (0.5, 1, or 2 μg/ml) in a 60 μL Tris-HCl buffer (50 mM, pH 7.4) for 5 minutes. Then, a particular concentration of chromogenic substrate was added according to the descriptions provided below. The absorbance at 405 nm was immediately measured using an enzyme-labeled instrument (Readmax 1500, Shanpu, China) and a kinetic curve recorded for 30 minutes. The relative enzyme activity was calculated by measuring the enzymatic hydrolysis velocity of its substrate. Human α-thrombin (20 nM, HT 1002a, Enzyme Research Laboratories, USA) and human plasmin (20 nM, HPlasmin, Enzyme Research Laboratories, USA) were incubated with 0.2 mM chromogenic substrate S-2238 (Chromogenix AB, Sweden). Human α-FXIIa (50 nM, HFXIIa 1212a, Enzyme Research Laboratories, USA) and kallikrein (20 nM, HPKa 1303, Enzyme Research Laboratory, USA) were incubated with 0.2 mM chromogenic substrates S-2302 (Chromogenix AB, Sweden). For FIXa (HFIXa 1080, Enzyme Research Laboratory, USA) and FVIIa (HFVIIa, Enzyme Research Laboratory, USA), a concentration of 20 nM and 100 nM, respectively, were used, with the corresponding chromogenic substrates being 0.2 mM S-2288 (Chromogenix AB, Sweden). Moreover, the concentration used for FXIa (HFVIIa, Enzyme Research Laboratory, USA) was 20 nM, with the chromogenic substrate being 0.2 mM S-2366 (Chromogenix AB, Sweden). For a brief overview of the experiment of NGAL on antithrombin activity, NGAL with a concentration range of 1 or 2 μg/ml, along with antithrombin (2 μM, HAT, Enzyme Research Laboratory, USA) and thrombin (10

nM), were incubated concurrently in 60 μl of Tris-HCl buffer (50 mM, pH 7.4) for 5 minutes at 37 °C. The thrombin activity was then evaluated using corresponding chromogenic substrates, similar to the approach used for thrombin alone or incubated with AT, also tested in the same buffer. The TAT complex was assessed using an ELISA kit (SEA831Hu 96T, Cloud-Clone Corp, China) per the manufacturer's instructions.

Furthermore, the effect of NGAL on the ability of thrombin to hydrolyze its natural substrate, fibrinogen, was evaluated. α-thrombin (20 nM) was incubated with fibrinogen (10 mg/ml) in Tris-HCl buffer (25 mM, pH 7.4), containing 0.15 M NaCl, and NGAL (0.5, 1, or 2 μg/ml) for 30 minutes at 37°C. The concentration of FbpA and FbpB in supernatant was analyzed by using FbpA and FbpB ELISA kits (CEB237Hu 96T and CEB307Hu 96T; Cloud-Clone Corp, China).

Confocal microscopy

To perform immunofluorescence detection of NGAL-thrombin/kallikrein complexes in the thrombus specimens of DVT, 8-μm sections were prepared by cutting the specimens using a freezing microtome. Next, the sections were incubated with anti-human NGAL (dilution 1:200, ab125075, abcam, USA), anti-thrombin (dilution 1:200, P00734, Abmart, China), or anti-human kallikrein (dilution 1:200, ab1006, abcam, USA) antibody overnight at 4 °C. Thereafter, the excess primary antibody was removed by washing the sections three times with PBS, followed by incubation with a fluorescently labeled secondary antibody for 1 h at 37 °C. The cell nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI, product number P36941 from Life technologies, USA). Finally, images of immunofluorescence were acquired using the Leica confocal microscope (STELLARIS 5), following the manufacturer's instructions.

Effects of NGAL deficiency and supplementation on coagulation, hemostasis, and thrombus

Recombinant adeno-associated virus serotype 9 (AAV9) expressing NGAL and

control virus were designed and manufactured by Jikai (Shanghai, China), and 10^{12} vg virus was injected into the tail vein of mice to induce NGAL overexpression. Effects of NGAL overexpression, mice NGAL intravenous injection (80 μg/kg, HY-P70658A, MCE, USA), NGAL mAb (80 μg/kg, 50060-R211, Sino biological, China), IgG control (80 μg/kg, ab199376, Abcam, USA) or NGAL knockout on APTT, PT, thrombus, and bleeding were tested.

APTT and PT assays

To test the APTT, a 100 μl sample of plasma was incubated with 100 μl of APTT reagent (R41056-50T, Yuanye, China) for 5 min at 37 °C, followed by the addition of 100 μl of $CaCl_2$ (25 mM) preheated at 37 °C to test clotting time. The PT assay kit (product number F007 from Nanjing Jiancheng Bioengineering Institute, China) was used, following our previous research(*37-39*).

Saphenous vein bleeding model

The saphenous vein bleeding model was carried out in accordance with previously published protocols(*39*). In brief, the saphenous vein of the mice was exposed and partially transected, and the time for the initial arrest of bleeding was recorded. The thrombus was then removed to elicit rebleeding, and this process was repeated over a duration of 30 minutes. The average hemostatic time within this 30-minute time frame was documented.

Bleeding time measurement

To measure the bleeding time, the tip of each mouse tail was precisely cut 4.5 mm away and submerged in 20 ml of 37 °C pre-warmed saline solution, following established protocols(*37-39*), with slight modifications. The bleeding time within 40-minute time was monitored and recorded until the flow of blood ceased.

$FeCl_3$-induced carotid artery thrombosis

To induce carotid artery thrombosis, $FeCl_3$ was utilized as previously outlined(*37-39*),

with slight modifications. The mice were put under anesthesia using isoflurane inhalation facilitated by an anesthesia respirator (R540IP, RWD Life Science, China. Thrombosis was induced in the carotid artery by applying a filter-paper disk soaked in 5 % $FeCl_3$. Carotid blood flow was continuously monitored for 60 minutes or until complete occlusion occurred using laser speckle perfusion imaging (RFLSI III, RWD Life Science, China) post $FeCl_3$ induction, with perfusion units utilized to quantify blood flow.

Mice DVT model

The mice DVT model was established by inducing stasis blood flow via infrarenal inferior vena cava (IVC) ligation(*39, 40*). In brief, the mice were anesthetized and midline laparotomy was performed. The venous side and dorsal branches of the IVC were obstructed, and stasis thrombosis was induced through ligation of the infrarenal IVC using a 7-0 Prolene suture (Ethicon Inc, Somerville, NJ). The peritoneum was closed using 5-0 Vicryl suture (Ethicon Inc, Somerville, NJ). The pathological changes were examined through HE staining, and the weight of the thrombus was calculated 24 hours later.

Mouse-tail thrombosis model induced by carrageen

This model was performed in accordance with our previously protocols(*41*). The mice received an intraperitoneal injection of 1% Type I Carrageenan (60 mg/kg, 9062-07-1, Coolaber, China), and were then maintained at 16 °C. The thrombosis region lengths in the tails were measured and photographed at 24 h after the carrageenan injection. The pathological changes of mice tail was examined through HE staining.

Statistical analysis

The mean ± SD was utilized to present the data obtained from independent experiments. All statistical analyses were two-tailed, with a 95% confidence interval (CI). The normal distribution of data was analyzed using the Kolmogorov-Smirnov test (K-S test), followed by one-way ANOVA or t-test. Post hoc Dunnett adjustments

were applied to p-values. The statistical analysis was performed using Prism 6.01 (Graphpad Software) and SPSS (SPSS Inc, USA). Differences were considered significant at $p < 0.05$.

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Author contributions

M.X., S.W., Y.W., M.L., D.X., and L.N. performed the experiments and data analyses; X.T., J.W., and C.S. conceived and supervised the project; X.T. prepared the manuscript. All authors contributed to the discussions.

Competing interests

The authors declare that they have no conflicts of interest.

Figures and Figure legends

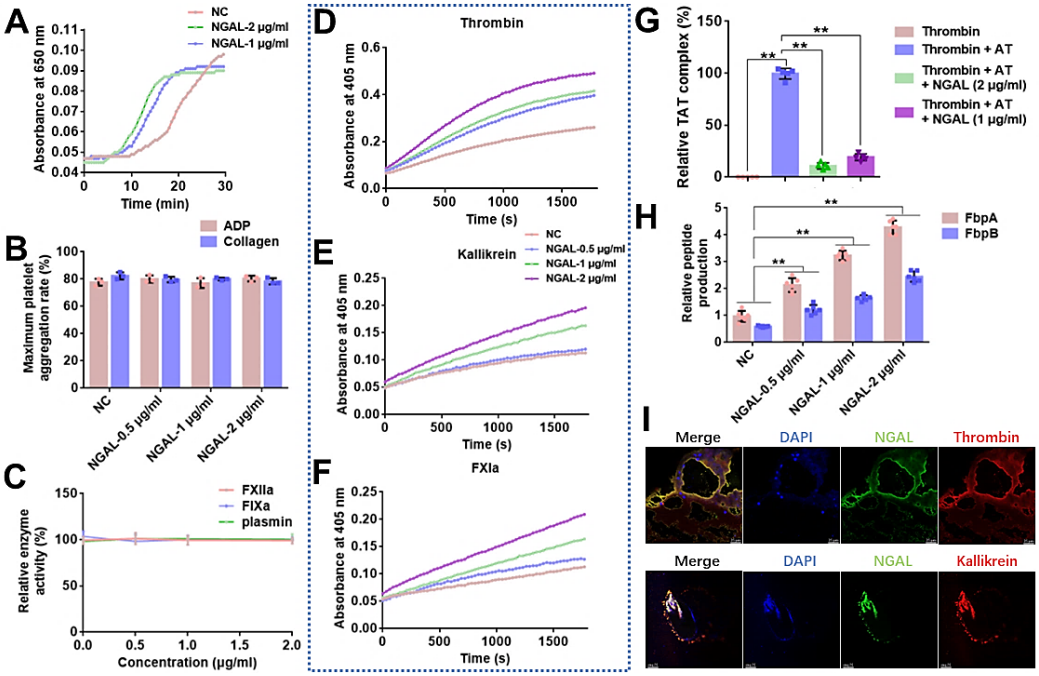

Figure 1. NGAL interacts and potentiates thrombin, kallikrein, or FXIa and inhibits thrombin-antithrombin complex formation. (A) Human plasma recalcification time was shortened by human NGAL. **(B)** NGAL showed no effect on ADP (10 μM) or collagen (1 μg/ml) induced platelet aggregation. **(C)** NGAL showed no influence on FXIIa, FIXa, and plasmin. Potentiating effects of NGAL on thrombin **(D)**, kallikrein **(E)**, and FXIa **(F)**. **(G)** NGAL inhibits thrombin–antithrombin complex (TAT) formation by ELISA analysis. **(H)** Representative ELISA quantification of fibrinopeptide A (FbpA) and fibrinopeptide B (FbpB) released from fibrinogen hydrolyzed by thrombin mixed with 0.5, 1, or 2 μg/ml NGAL, respectively. Data represent mean ± SD of 3-6 independent experiments, **$p < 0.01$ by one-way

ANOVA with Dunnett's post hoc test (G and H). **(I)** Confocal analysis of NGAL-thrombin/kallikrein-positive structures. Venous thrombus were labeled with anti-NGAL, anti-thrombin, or anti-kallikrein antibodies. Cell nuclei were labeled by DAPI. Scale bar represents 25 μm. Images are representative of at least three independent experiments.

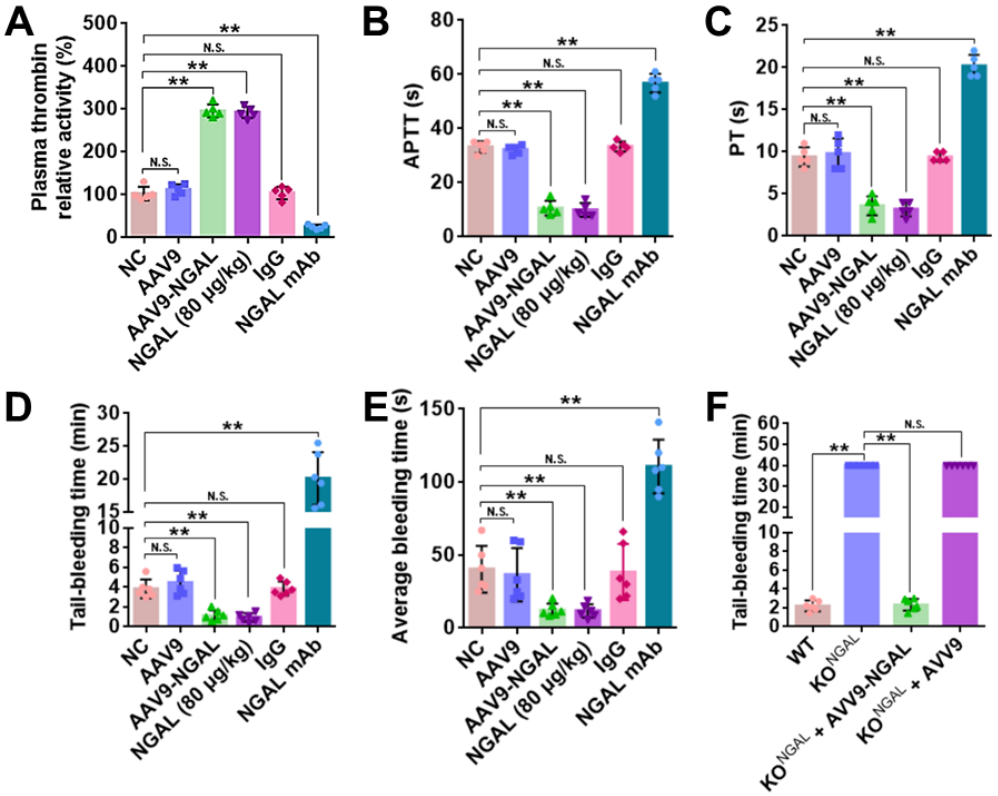

Figure 2. NGAL induces hypercoagulability and hemostasis which are reversed by NGAL deficiency. Effects of NGAL overexpression (AAV9-NGAL), blank virus (AAV9), intravenous injection of mice NGAL (80 μg/kg), anti-NGAL monoclonal antibody (NGAL mAB), or IgG control on thrombin generation **(A)**, APTT **(B)**, PT **(C)**, mice tail-bleeding time **(D)**, and mice saphenous vein bleeding time **(E)**. **(F)** Effects of NGAL knock out (KO^{NGAL}) on mice tail-bleeding time. Column 2 and 4 in panel F represent bleeding time that over 40 min. Data represent mean ± SD (n=5-6), **$p < 0.01$ by one-way ANOVA with Dunnett post hoc test. N.S.: no significance.

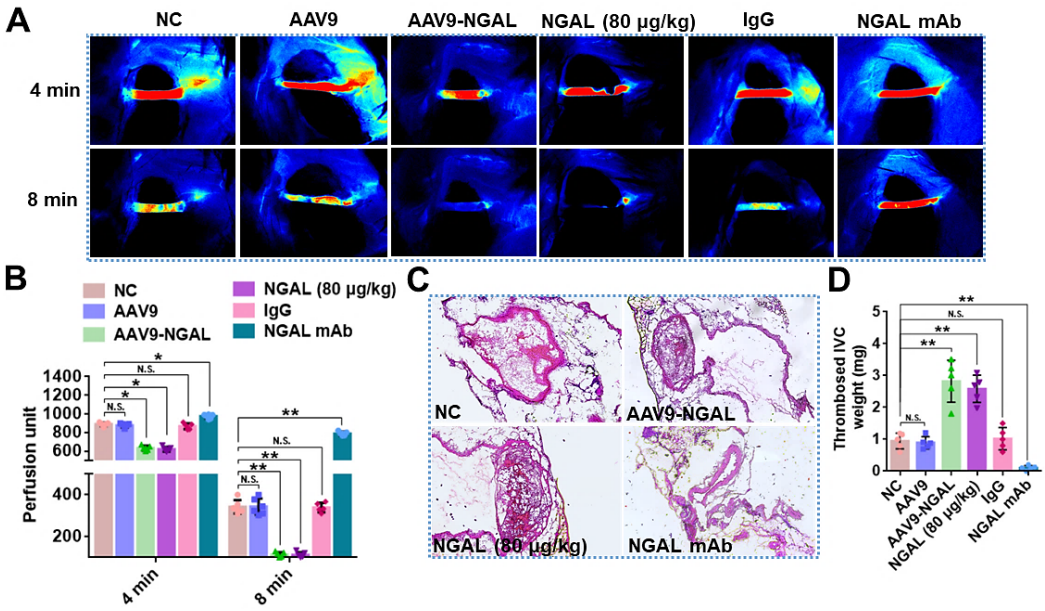

Figure 3. NGAL deficiency alleviates thrombus, which is aggravated by NGAL overexpression. Mice were received intravenous injection of blank virus (AAV9), NGAL overexpression virus (AAV9-NGAL), NGAL (80 μg/kg), anti-NGAL mAb, or IgG control. **(A)** Representative of carotid artery blood flow images in $FeCl_3$-treated mice by laser speckle perfusion imaging, with region of interest placed in carotid artery to quantify blood flow change. **(B)** Relative blood flow in the region of interest is shown by using perfusion unit. Mice were subject to inferior vena cava (IVC) stenosis for 24 hours to evaluate venous thrombogenesis. The pathological changes were observed through hematoxylin and eosin (HE) staining **(C)** and calculating thrombus weight **(D)**. Data represent mean ± SD (n=5-6), *p < .05, **p < .01 by one-way ANOVA with Dunnett post hoc test. N.S.: no significance.

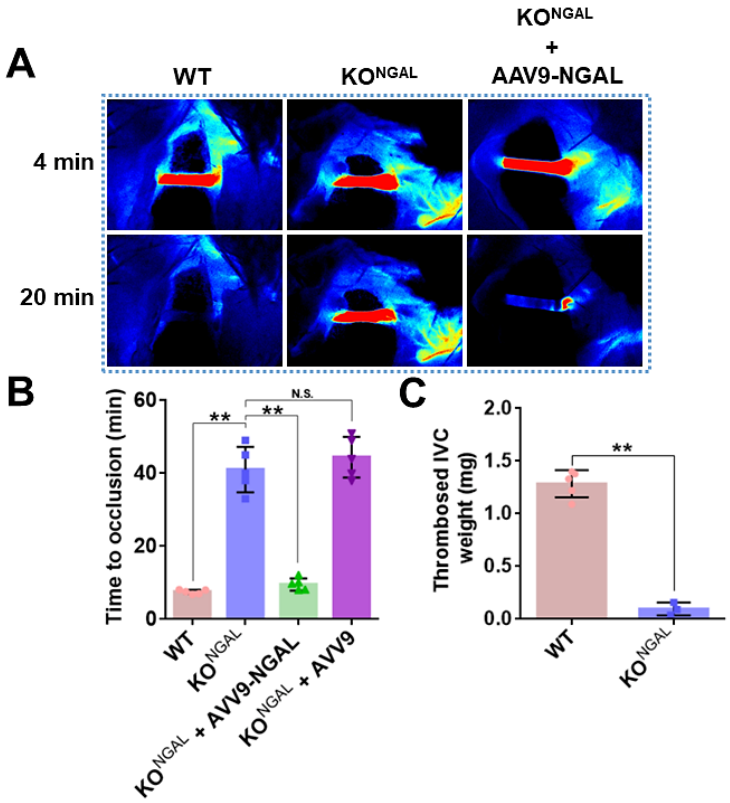

Figure 4. NGAL knockout significantly alleviates thrombus. Representative of carotid artery blood flow images in $FeCl_3$-treated mice by laser speckle perfusion imaging **(A)**, and time to occlusion was calculated **(B)**. **(C)** Thrombus inferior vena cava (IVC) weight of NGAL knockout mice was shown. Data represent mean ± SD (n=5), **$p < 0.01$ by one-way ANOVA with Dunnett post hoc test or t-test (C). N.S.: no significance.

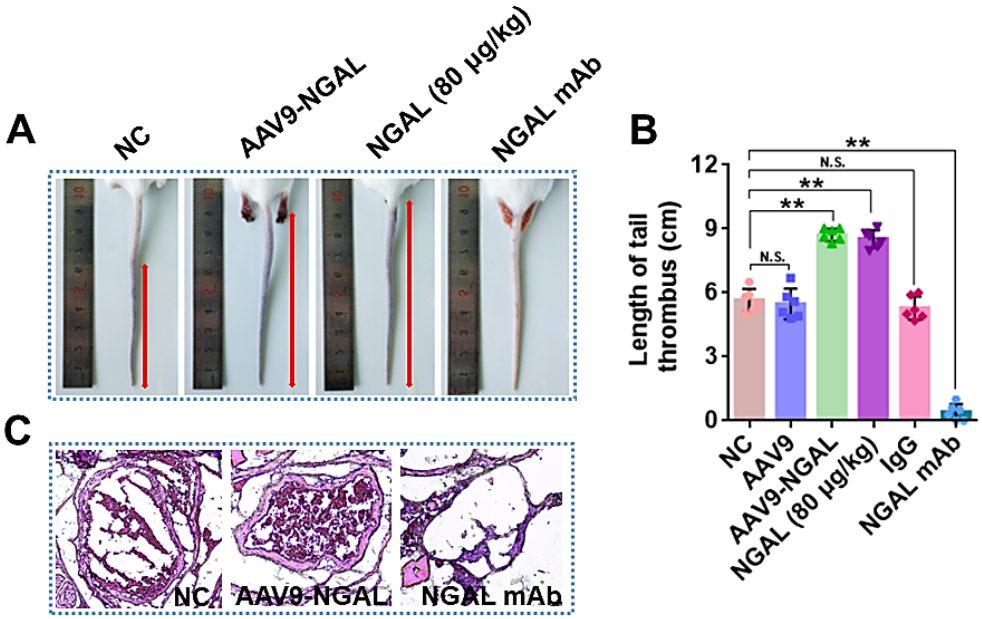

Figure 5. Carrageenan-induced mice tail thrombus is alleviated by NGAL deficiency, which is reversed by NGAL-supplementation. Mice were received intravenous injection of blank virus (AAV9), NGAL overexpression virus (AAV9-NGAL), NGAL (80 μg/kg), anti-NGAL mAb, and IgG control. **(A-B)** Thrombosis lengths in tails were measured and photographed at 24 h after carrageenan injection. Hematoxylin and eosin (HE) staining of tail at 6 cm from the tail tip was shown **(C)**. Data represent mean ± SD (n=6), **p < .01 by one-way ANOVA with Dunnett post hoc test. N.S.: no significance.