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RESEARCH ARTICLE

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Didang decoction attenuates cancer-associated thrombosis by inhibiting PAD4-dependent NET formation in lung cancer

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Abstract

This research aims to investigate the impact of Didang decoction (DD) on the formation of neutrophil extracellular traps (NETs) and cancer-associated thrombosis in lung cancer. BALB/c nude mice were used to establish xenograft models for inducing deep vein thrombosis. Tumor growth and thrombus length were assessed. The impact of DD on NET generation was analyzed using enzyme-linked immunosorbent assay, immunofluorescence staining, quantitative real-time PCR, and western blot analysis, both in vivo and in vitro. CI-amidine, a PAD4 inhibitor, was employed to evaluate the role of PAD4 in the generation of NETs. In vivo studies demonstrated that treatment with DD reduced tumor growth, inhibited thrombus formation, and decreased the levels of NET markers in the serum, tumor tissues, neutrophils, and thrombus tissues of mice. Additional data indicated that DD could suppress neutrophil counts, the release of tissue factor (TF), and the activation of thrombin-activated platelets, all of which contributed to increased formation of NETs in mouse models. In vitro, following incubation with conditioned medium (CM) derived from Lewis lung carcinoma cells, the expression of NET markers in neutrophils was significantly elevated, and an extracellular fibrous network structure was observed. Nevertheless, these NET-associated changes

Abbreviations: ANOVA, analysis of variance; BCA, bicinchoninic acid; citH3, citrullinated histone H3; CM, conditioned medium; DAPI, 4′,6-diamidino-2-phenylindole; DD, Didang decoction; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; LLC, Lewis lung carcinoma; MPO, myeloperoxidase; NE, neutrophil elastase; NET, neutrophil extracellular trap; PAD4, peptidylarginine deiminase 4; PMA, phorbol-12-myristate-13-acetate; SD, standard deviation; TAT, thrombin-antithrombin; TBST, Tris-buffered saline with 0.1% Tween 20; TF, tissue factor; VTE, venous thromboembolism.

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were partially counteracted by DD. Additionally, CI-amidine reduced the expression of NET markers in CM-treated neutrophils, consistent with the effects of DD. Collectively, DD inhibits cancer-associated thrombosis in lung cancer by decreasing PAD4-dependent NET formation through the regulation of TF-mediated thrombin-platelet activation. This presents a promising therapeutic strategy for preventing and treating venous thromboembolism in lung cancer.

KEYWORDS

Didang decoction, lung cancer, neutrophil extracellular traps, thrombosis

INTRODUCTION

Lung cancer is the second most prevalent malignant tumor globally and the leading cause of cancer-related mortality.[1,2] Venous thromboembolism (VTE), which encompasses both pulmonary thromboembolism and deep vein thrombosis, is a frequent complication in patients diagnosed with lung cancer.[3] Among solid tumors, lung cancer is recognized for its elevated risk of VTE complications.[4] Consequently, exploring effective therapeutic strategies to prevent lung cancer-related thrombosis may improve the clinical prognosis for patients with lung cancer.

Neutrophil extracellular traps (NETs) are web-like structures composed of filamentous DNA and histones.[5] Studies have demonstrated that NETs can trap platelets, red blood cells, and pro-coagulant molecules, induce thrombin production, and enhance the stability of thrombin scaffolds through their DNA mesh structure.[6,7] NETs were modified by antimicrobial protein particles and enzymes, including neutrophil elastase (NE) and myeloperoxidase (MPO).[8] Citrullinated histone H3 (citH3) is regarded as a biomarker indicative of NET formation.[9] NETs are associated with the progression of cancer-associated thrombosis, which includes the development of both arterial and venous thrombosis.[10] Studies conducted on mouse models have demonstrated a strong association between the formation of NETs and thrombosis in breast cancer.[11,12] Furthermore, it has been reported that cancer cells can induce neutrophils to produce NETs, which are web-like structures that capture platelets and enhance tissue factor (TF) activity, thereby contributing to cancer-associated thrombosis.[10,13,14] This evidence suggests a close relationship between NETs and cancer-associated thrombosis.

Didang decoction (DD) is a classical traditional prescription documented in the *Treatise on Cold Pathogenic Diseases*.[15] Its primary components include leech, gadfly, peach seed, and rhubarb. According to traditional Chinese medicine theory, DD promotes blood circulation and alleviates blood stasis through catharsis.[16] Leech and gadfly are utilized in traditional medicine to help alleviate blood stasis. Rhubarb possesses anticoagulant properties and promotes blood circulation. Additionally, the glycerol trioleate derived from peach seeds also exhibits anticoagulant effects. In Chinese medicine, the synergistic use of these substances is particularly effective in removing blood stasis and clearing heat, making it a representative prescription for treating conditions associated with blood stasis and heat. Studies have demonstrated the antithrombotic effects of DD on conditions such as atherosclerosis, acute cerebral hemorrhage, and pulmonary interstitial fibrosis.[17,18] Therefore, we hypothesize that DD may play a protective role in cancer-associated thrombosis.

Herein, we explored the effect of DD on the formation of NETs and cancer-associated thrombosis, as well as the potential mechanisms involved in lung cancer. The completion of this work may unveil novel discoveries and provide new insights into drug strategies targeting DD for the treatment of cancer-associated thrombosis in lung cancer.

MATERIALS AND METHODS

Cell culture and treatments

The murine lung cancer cell line Lewis lung carcinoma (LLC) cells were obtained from the Shanghai Institutes

for Biological Sciences. LLC cells were cultured in Dulbecco's modified eagle medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) under 5% CO_2 at 37°C. After 24 h of culture, the supernatants from the LLC cells were collected and centrifuged at 3000 rpm for 10 min at 4°C. The conditioned medium (CM) was then collected and stored at −80°C.

DD preparation

To obtain DD, 54 g of raw materials were utilized, comprising 18 g of rhubarb, 12 g of peach seed, 12 g of leech, and 12 g of gadfly for the preparation. These materials were boiled in water two times, filtered, and concentrated into 27 mL of liquid medicine with a concentration of 2 g/mL.

Animal models

BALB/c nude mice (4–6 weeks old) were purchased from SLAC Laboratory Animal Co., Ltd. The mice were maintained on a 12-h light-dark cycle with 50%–60% humidity at a temperature of 25 ± 2°C. The animal experiments were conducted 1 week after the mice had acclimated to their new environment. Eighteen mice were randomly assigned to four groups ($n = 5$ mice per group): normal group, tumor group, DD group, and DNase I group. To establish a xenograft mouse model, a 100 μL cell suspension containing 1×10^6 LLC cells was subcutaneously injected into the mice. Mice in the DD group received a daily gavage of DD at a dose of 2 g per 100 g of body weight (200 μL). Mice in the DNase I group were administered 100 U of DNase I (D8071, Solarbio) daily via intraperitoneal injection. Mice in the tumor group received 200 μL of normal saline daily through gavage. Mice in the normal group did not receive any treatment. After 28 days, the tumors were excised, photographed, and their weight and volume were measured.

After 26 days of modeling, both the model mice and the normal mice were prepared for the induction of deep vein thrombosis. The mice were anesthetized with an intraperitoneal injection of 30 mg/kg pentobarbital sodium (Merck). A 2-cm incision was made along the abdominal midline, allowing access to the inferior vena cava (IVC) through the abdominal cavity. The IVC was then carefully separated. The IVC on the caudal side of the left renal vein was stenosed by inserting an acupuncture pin outside the vein. A 5-0 (1 mm) suture was passed through the IVC, while a 3-0 (2 mm) suture was placed parallel to the IVC as a blocking line. IVC ligation and the removal of the 3-0 sutures were performed to reduce the vascular lumen by nearly 90%. The other branches of the IVC were overlapped horizontally with the iliac vein. Subsequently, the abdominal incision was sutured, and the mice were killed 48 h later to collect the thrombus that had formed in the IVC. The animal experiments were approved by the Institutional Animal Care and Use Committee of Hunan University of Chinese Medicine and conducted in accordance with established protocols.

Neutrophil isolation and treatments

The orbital blood collection was performed after the mice were anesthetized. A 15-mL centrifuge tube was prepared, and 4 mL of Reagent A from the Neutrophil Extraction Kit (P9201, Solarbio) was added according to the manufacturer's instructions. Subsequently, 2 mL of Reagent C was added to create a gradient interface. The blood samples were then layered onto Reagent C, and centrifuged at 1000*g* for 30 min. The middle layer, consisting of Reagent A and Reagent C, contained the neutrophils. The neutrophils were carefully aspirated into a separate 15 mL centrifuge tube, and 10 mL of phosphate-buffered saline was added to wash the cells. After centrifugation at 1000 g for 3 min, neutrophils were collected and resuspended in the RPMI-1640 medium (Gibco). The neutrophils were then seeded into a 48-well plate at a density of 5×10^5 cells per well. Polylysine-coated coverslips were placed in the wells to facilitate neutrophil adhesion. Neutrophils were stimulated with phorbol-12-myristate-13-acetate (PMA, 50 nM; MedChem Express) for 3 h to induce the formation of NETs.

In the neutrophil treatment, specific CMs were prepared for each group. The cells were divided into four groups: RPMI-1640, CM, CM + DD, and CM + DNase I. In the RPMI-1640 group, neutrophils were cultured with RPMI-1640. In the CM group, neutrophils were cultured with a 50% volume of RPMI-1640 and a 50% volume of CM. In the CM + DD group, neutrophils were cultured with a 50% volume of RPMI-1640 and a 50% volume of CM supplemented with 20 g/L of DD. In the CM + DNase I group, neutrophils were cultured with a 50% volume of RPMI-1640 and a 50% volume of CM containing 10 U/mL of DNase I. Cells in each group were cultured for 4 h. To inhibit the expression of peptidylarginine deiminase 4 (PAD4), neutrophils were pre-incubated with the PAD4 inhibitor Cl-amidine (100 μM; MedChem Express) for 30 min.

Enzyme-linked immunosorbent assay detection

The levels of MPO, NE, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating

factor (GM-CSF), TF, and thrombin-antithrombin (TAT) complex in mouse serum, as well as the MPO level in neutrophil supernatants, were measured using various murine enzyme-linked immunosorbent assay (ELISA) kits: the MPO ELISA kit (ab155458, Abcam), NE ELISA kit (E-EL-M3025, Elabscience), murine G-CSF ELISA kit (EMCSF3X5, Invitrogen), murine GM-CSF ELISA kit (BMS612, Invitrogen), murine TF ELISA kit (E-EL-M1163, Elabscience), murine TAT complex ELISA kit (ab108907, Abcam), and murine IL-8 ELISA kit (ml063162, mlbio), following the standard protocols.

Immunofluorescence staining

After specific treatment, neutrophils, tumor tissues, and thrombus tissues from mice were collected. All tissues and cells were fixed in 4% paraformaldehyde for 24 h, tissues were embedded in paraffin, and cut into 4-μm thick sections. The sections or cells were then incubated overnight at 4°C with primary antibodies: anti-MPO (1:100, 22225-1-AP, Proteintech), anti-citH3 (1:2000, ab281584, Abcam), and anti-NE (1:200, AF0010, Affinity Biosciences). Following this, the sections or cells were washed with Tris-buffered saline with 0.1% Tween 20 (TBST) and incubated with goat anti-rabbit secondary antibody (1:500, ab150077, Abcam) for 2 h. Next, the samples were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Solarbio) solution for 5 min in the dark. Finally, tissue and cell images were obtained using a confocal microscope (LSM 800, Zeiss) and analyzed with ImageJ software (NIH). The positive staining of MPO, citH3, and NE in neutrophils, tumor tissues, and thrombus tissues indicated the formation of NETs.

Blood cell analysis

The whole blood samples were collected from mice in each group and stored in ethylenediaminetetraacetic acid (EDTA)-coated tubes. The counts of red blood cells, monocytes, and neutrophils, along with the concentration of hemoglobin, were measured using an automated hematology analyzer (XN-1000; Sysmex).

Cell-free DNA measurement

Cell-free DNA in serum was quantified using the Quant-iT PicoGreen dsDNA kit (P11496, Invitrogen). Briefly, approximately 10 μL of mouse serum was diluted in 95 μL of Tris-EDTA buffer and subsequently incubated with 100 μL of PicoGreen. The incubation took place in a 96-well luminescence test plate. Finally, the concentration of DNA was determined by measuring the fluorescence intensity at 480 and 530 nm using a microplate reader.

Quantitative real-time PCR

Total RNA was isolated from neutrophils using TRIzol reagent (Invitrogen). 1 μg of total RNA was reverse-transcribed into cDNA, and quantitative real-time PCR was conducted using the SYBR Green Mix (Solarbio). The expression levels of PAD4 mRNA were quantified using the $2^{-\Delta\Delta CT}$ method and normalized to β-actin. The primer sequences utilized in this study were as follows: PAD4 (forward: 5′-ACG CTG CCT GTG GTC TTT GAC T-3′, reverse: 5′-ACC TCC AGG TTC CCA AAG GCA T-3′) and β-actin (forward: 5′-CAT TGC TGA CAG GAT GCA GAA GG-3′, reverse: 5′-TGC TGG AAG GTG GAC AGT GAG G-3′).

Western blot analysis

Proteins from neutrophils or tumors were extracted using lysis buffer and quantified with a BCA Protein Assay kit (Beyotime). The protein samples were separated on sodium dodecyl-sulfate polyacrylamide gel electrophoresis gels and transferred onto a polyvinylidene fluoride membrane (Millipore). After blocking, the membranes were incubated overnight at 4°C with various antibodies, including anti-PAD4 (1:1000, ab214810, Abcam), anti-MPO (1:2000, 22225-1-AP, Proteintech), anti-citH3 (1:1000, ab281584, Abcam), anti-NE (1:1000, ab310335, Abcam), anti- NF-κB (1:2000, ab32536, Abcam), anti-p-NF-κB (1:1000, ab76302, Abcam), and anti-β-actin (1:2000, ab8227, Abcam). Following washing with TBST, an HRP-labeled anti-rabbit secondary antibody (1:5000, ab6721, Abcam) was incubated for 1.5 h at room temperature. β-actin served as an internal reference. The ECL reagent (Beyotime) was used to visualize the bands of target proteins.

Statistical analysis

Statistical data were expressed as the mean ± standard deviation (SD) and analyzed using GraphPad Prism 8.0 software (GraphPad Software, Inc.). Statistical differences among multiple groups were determined using one-way ANOVA followed by Tukey's post hoc test. A value of p less than 0.05 was considered statistically significant.

RESULTS

DD attenuated lung cancer-associated thrombosis in mice

To investigate whether DD influences the formation of lung cancer-associated thrombosis in vivo, xenograft mouse models were established by subcutaneously injecting LLC cells and administering DD through gavage, followed by the induction of venous embolization. The results indicated that the tumor weight and volume were significantly smaller in xenograft mice treated with DD or DNase I compared to those with tumors derived from untreated xenograft mice (Figure 1a). It was subsequently observed that both DD and DNase I significantly reduced the length of thrombus compared to that in xenograft mice (Figure 1b). Furthermore, DD or DNase I markedly decreased the protein expressions of NET markers MPO, citH3, and NE in the tumor tissues of xenograft mice when compared to the control group (Figure 1c). To assess the impact of DD on cancer-related inflammation, the expression of NF-κB signaling proteins and IL-8 was measured in xenograft mice. The results indicated that both DD and DNase I significantly inhibited the phosphorylation of NF-κB in tumor tissues and reduced serum IL-8 concentrations in the xenograft mice (Figures S1 and S2). These findings suggest that DD may inhibit thrombus formation.

DD reduced the formation of NETs in the thrombotic tissues of xenograft mice

The deposition of NETs promotes tumor-associated thrombosis, and the NETs inhibitor DNase I can reduce lung cancer-related thrombosis.[19] To assess the impact of DD on the formation of lung cancer-induced NETs in vivo, immunofluorescence staining was conducted on thrombosis in xenograft mouse models. The results indicated that the protein levels of NET markers MPO, citH3, and NE in thrombosis from xenograft mice were higher than those in normal mice. However, these effects were inhibited by treatment with DD or DNase I (Figure 2a–c). Therefore, DD may inhibit the accumulation of NETs in lung cancer-associated thrombosis.

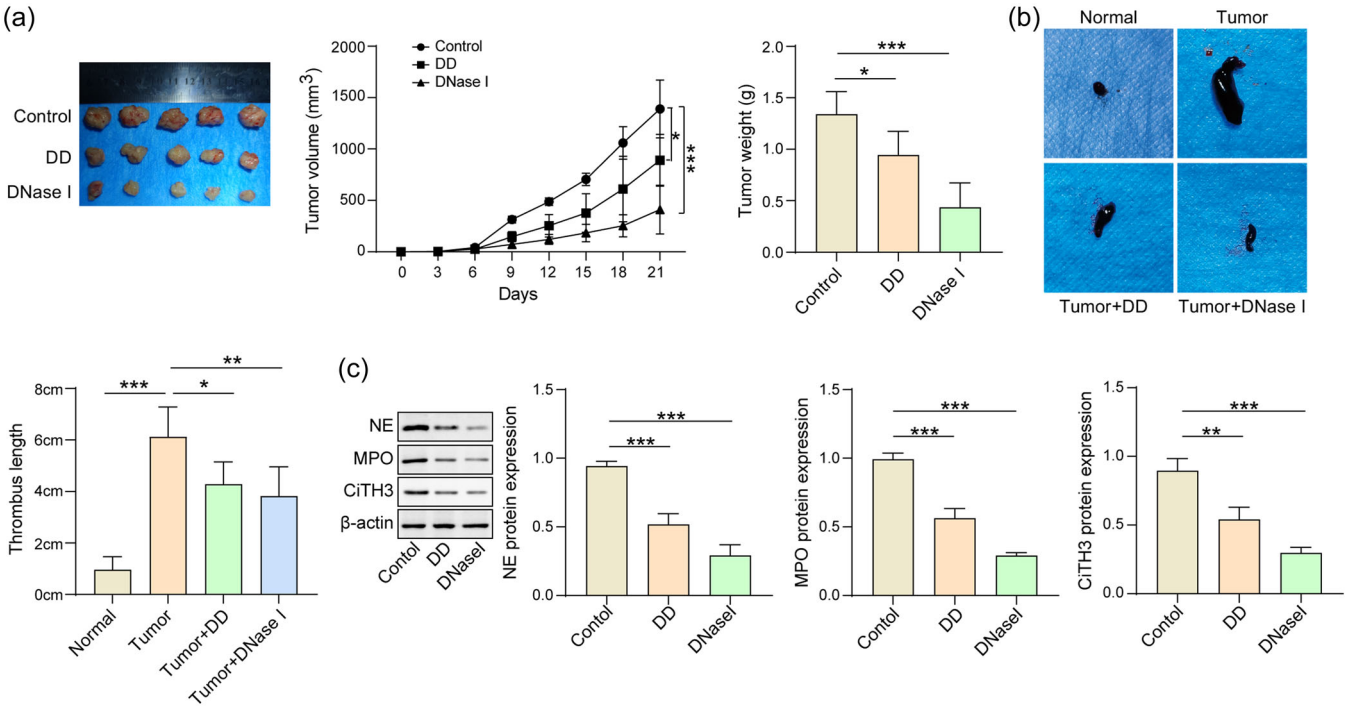

FIGURE 1 DD attenuated lung cancer-associated thrombosis in mice. (a) Images of tumors derived from xenograft mice, and xenograft mice with DD or DNase I treatment. Tumor weight and volume were quantified in each group. (b) Images of thrombus derived from xenograft mice, and xenograft mice with DD or DNase I treatment. ImageJ was used for quantification of the length of thrombus. $n = 5$. (c) Western blot detection of NE, citH3, and MPO protein in tumors derived from xenograft mice, and xenograft mice with DD or DNase I treatment. $n = 3$, citH3, citrullinated histone H; DD, Didang decoction; MPO, myeloperoxidase; NE, neutrophil elastase. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus Control, Normal, or Tumor group.

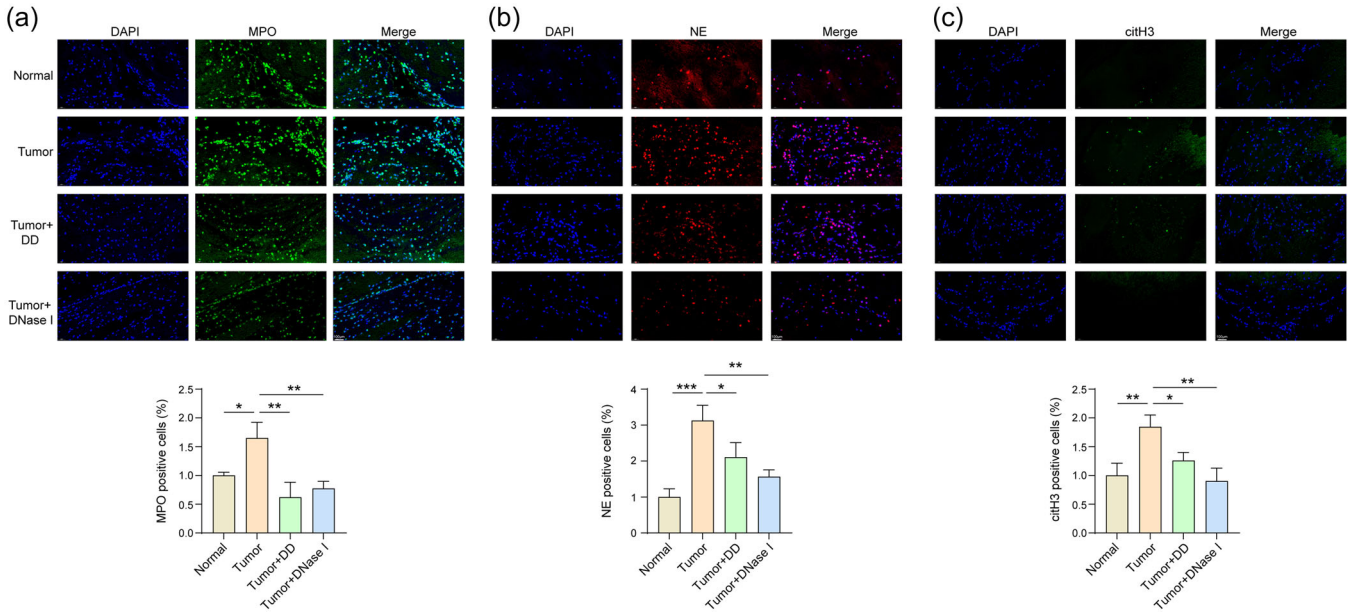

FIGURE 2 DD decreased NETs in the thrombotic tissues from xenograft mice. (a–c) Immunofluorescence analysis for MPO, citH3, and NE in thrombus tissues from each group of mice. The quantification analysis was performed using ImageJ. Nuclei were stained with DAPI. Scale bar: 100 μm. citH3, citrullinated histone H; DD, Didang decoction; MPO, myeloperoxidase; NE, neutrophil elastase; NET, neutrophil extracellular trap. $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus Normal or Tumor group.

DD reduced the formation of NETs in neutrophils derived from xenograft mice

To investigate the impact of DD on the formation of lung cancer-induced NETs in vitro, neutrophils were isolated from xenograft mice treated with either DD or DNase I. Following the induction of NET formation through PMA stimulation, immunofluorescence staining was conducted on the neutrophils. The results indicated that the levels of MPO, citH3, and NE proteins in neutrophils from xenograft mice were significantly higher than those from normal mice. In contrast, treatment with DD or DNase I markedly reduced these NET markers (Figure 3a–c). Collectively, these findings suggest that DD can inhibit the formation of NETs induced by lung cancer.

DD suppressed neutrophil counts through G-CSF

As indicated in Figure 4a,b, there were no significant differences in the number of red blood cells or hemoglobin levels in blood samples between normal and xenograft mice. Neither DD treatment nor DNase I treatment had a significant effect on the number of red blood cells or hemoglobin levels. However, the number of monocytes and neutrophils was markedly elevated in xenograft mice compared to normal mice, and this elevation was mitigated by treatment with either DD or DNase I (Figure 4c,d). Furthermore, both DD and DNase I significantly reduced the concentration of G-CSF in the serum of xenograft mice, while having no significant effect on GM-CSF levels (Figure 4e,f). Regarding NET markers, a dramatic increase in MPO and NE concentrations was observed in the serum of xenograft mice, which were significantly reduced by DD or DNase I (Figure 4g,h). Collectively, these findings suggest that DD can suppress neutrophil counts, which may be associated with G-CSF rather than GM-CSF.

DD inhibited the formation of NETs via the TF-thrombin-platelet pathway

TF is a key initiator of coagulation and has been shown to be closely associated with the formation of NETs.[20] The ELISA data indicated a significant increase in TF levels in the serum of xenograft mice, which could be partially reversed by the treatment with either DD or DNase (Figure 5a). The TAT complex, platelets, and cell-free DNA are recognized as biomarkers of immune thrombosis and have been reported to contribute to NET formation. Significantly elevated levels of NETs were observed in the xenograft model group, as indicated by increased levels of the TAT complex, platelets, and cell-free DNA. In contrast, treatment with DD or DNase downregulated these NET markers (Figure 5b–d).

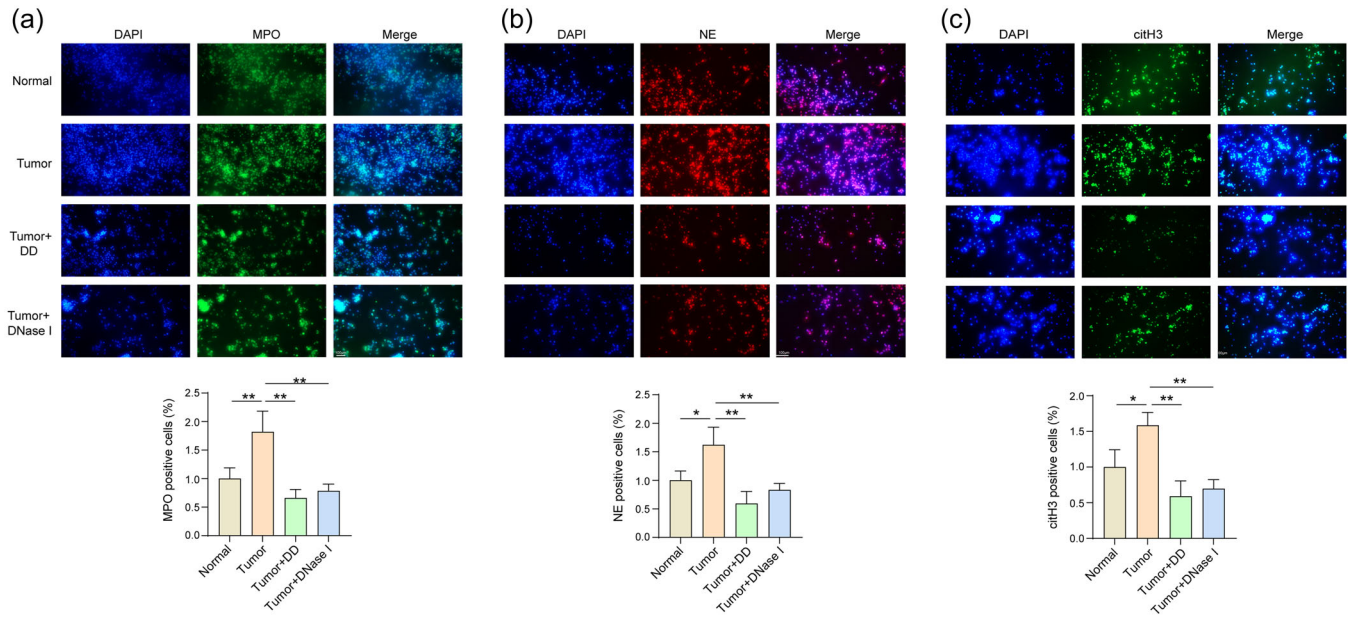

FIGURE 3 DD reduced the formation of NETs in neutrophils derived from xenograft mice. (a–c) Neutrophils were isolated from each group of mice and stimulated with PMA (50 nM) for 3 h. Immunofluorescence analysis for MPO, citH3, and NE in neutrophils was performed. The quantification analysis was carried out using ImageJ. Nuclei were stained with DAPI. Scale bar: 100 μm. citH3, citrullinated histone H; DAPI, 4′,6-diamidino-2-phenylindole; DD, Didang decoction; MPO, myeloperoxidase; NET, neutrophil extracellular trap; PMA, phorbol-12-myristate-13-acetate. $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$ versus Normal or Tumor group.

Overall, DD appears to inhibit the formation of NETs by regulating the TF-thrombin-platelet pathway.

DD reduced the formation of NETs in vitro

Subsequently, we investigated the impact of DD on the formation of NETs in vitro. The results indicated that the MPO level in the supernatants of neutrophils increased in the LLC CM treatment group compared to the RPMI-1640 group. However, this change was negated by the treatment with DD or DNase I (Figure 6a). Immunofluorescence staining results demonstrated that the levels of MPO, citH3, and NE proteins in neutrophils were upregulated, and the extracellular fibrous network structure was clearly observed (Figure 6b–d). Nevertheless, the enhanced markers of NETs and the fibrous network structure induced by CM were partially reversed by the treatment with DD or DNase I (Figure 6b–d). The data suggest that DD may reduce the formation of NETs induced by lung cancer cells.

DD inhibited the formation of NETs by downregulating PAD4

PAD4 is expressed in neutrophils, and upon activation, it can promote the formation of NETs.[21] To investigate whether DD influences NETs through the regulation of PAD4, neutrophils were pretreated with the PAD4 inhibitor CI-amidine. It was observed that the mRNA and protein levels of PAD4 were significantly elevated in CM-treated neutrophils; however, these levels were markedly reduced when the cells were treated with either DD or CI-amidine (Figure 7a,b). Simultaneously, the protein expressions of MPO, citH3, and NE were significantly increased in neutrophils following CM induction. However, treatment with CI-amidine inhibited the upregulation of these proteins in CM-induced cells (Figure 7c). These data suggest that DD may inhibit the formation of NETs by suppressing the expression of PAD4.

DISCUSSION

Over the past few decades, numerous studies have demonstrated the antithrombotic effects of DD in various thrombogenic diseases; however, its impact on cancer-associated thrombosis remains largely unexplored. In this study, the results from animal models indicated that DD treatment reduced tumor growth, inhibited thrombus formation, and decreased the levels of NET markers in serum, tumor tissues, neutrophils, and thrombi of xenograft mice. Moreover, DD inhibited thrombosis via PAD4-mediated NET formation through TF-mediated

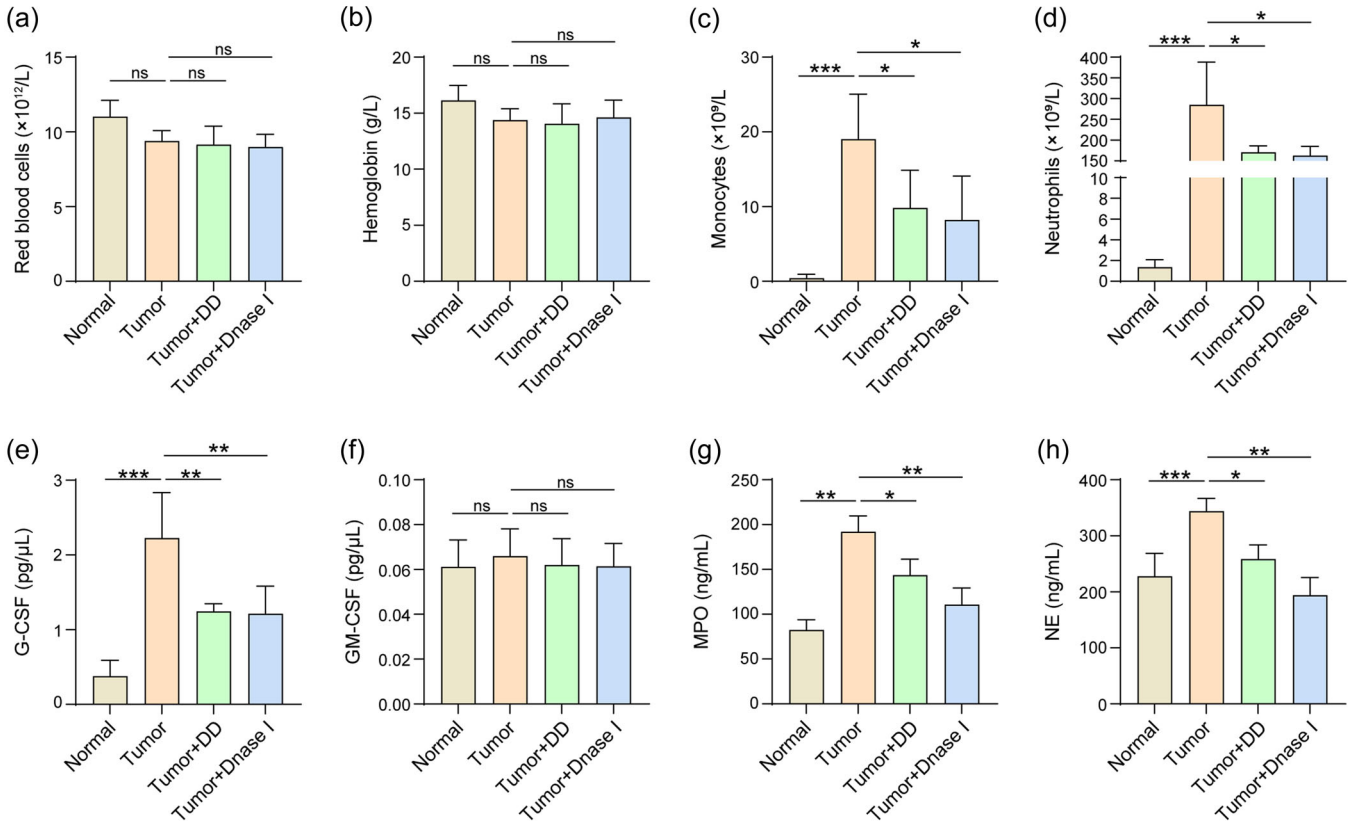

FIGURE 4 DD suppressed neutrophil counts through G-CSF. (a, b) Comparison of red blood cell and hemoglobin levels in each group. (c, d) Peripheral neutrophil and monocyte counts were measured by an automated hematology analyzer. (e, f) Concentrations of G-CSF and GM-CSF in serum were assessed by ELISA. (g, h) Serum MPO and NE levels were detected by ELISA. ELISA, enzyme-linked immunosorbent assay; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; MPO, myeloperoxidase; NE, neutrophil elastase; ns, no significance. $n = 5$, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus Normal or Tumor group.

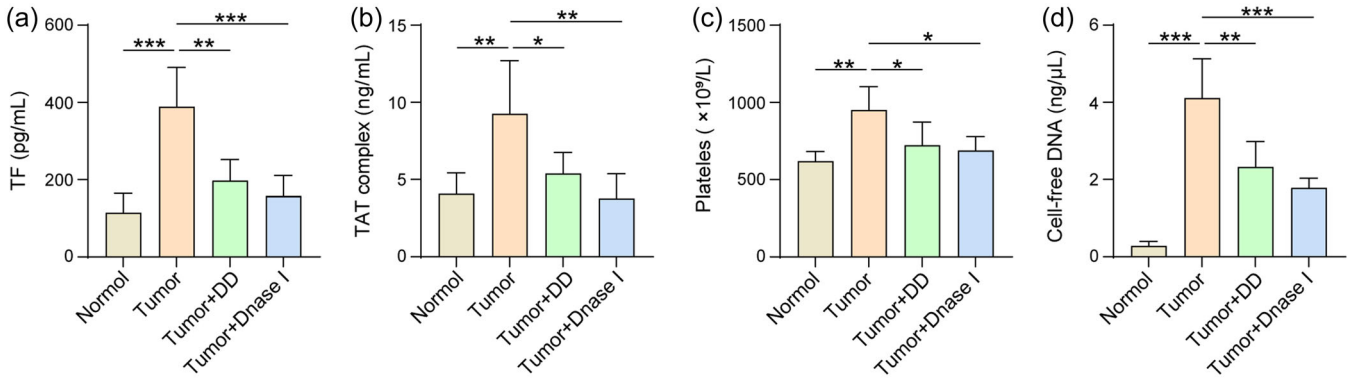

FIGURE 5 DD reduced the formation of NETs via the TF-thrombin-platelet pathway. (a, b) ELISA detection of TF and TAT complex concentrations in each group. (c) Comparison of platelet counts in each group. (d) Systemic levels of cell-free DNA were evaluated by the Quant-iT PicoGreen dsDNA kit in the serum. DD, Didang decoction; NET, neutrophil extracellular trap; TF, tissue factor. $n = 5$. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus Normal or Tumor group.

thrombin-platelet activation. In vitro, DD downregulated markers of NETs and reduced the extracellular fibrous network structure compared to treatment with CM derived from LLC cell supernatants. Therefore, inhibiting the formation of NETs may represent a potential therapeutic strategy for treating lung cancer-associated thrombosis.

Previous reports have demonstrated the involvement of NETs in various pathological progresses, including thrombosis, atherosclerosis, and wound healing.[22–24]

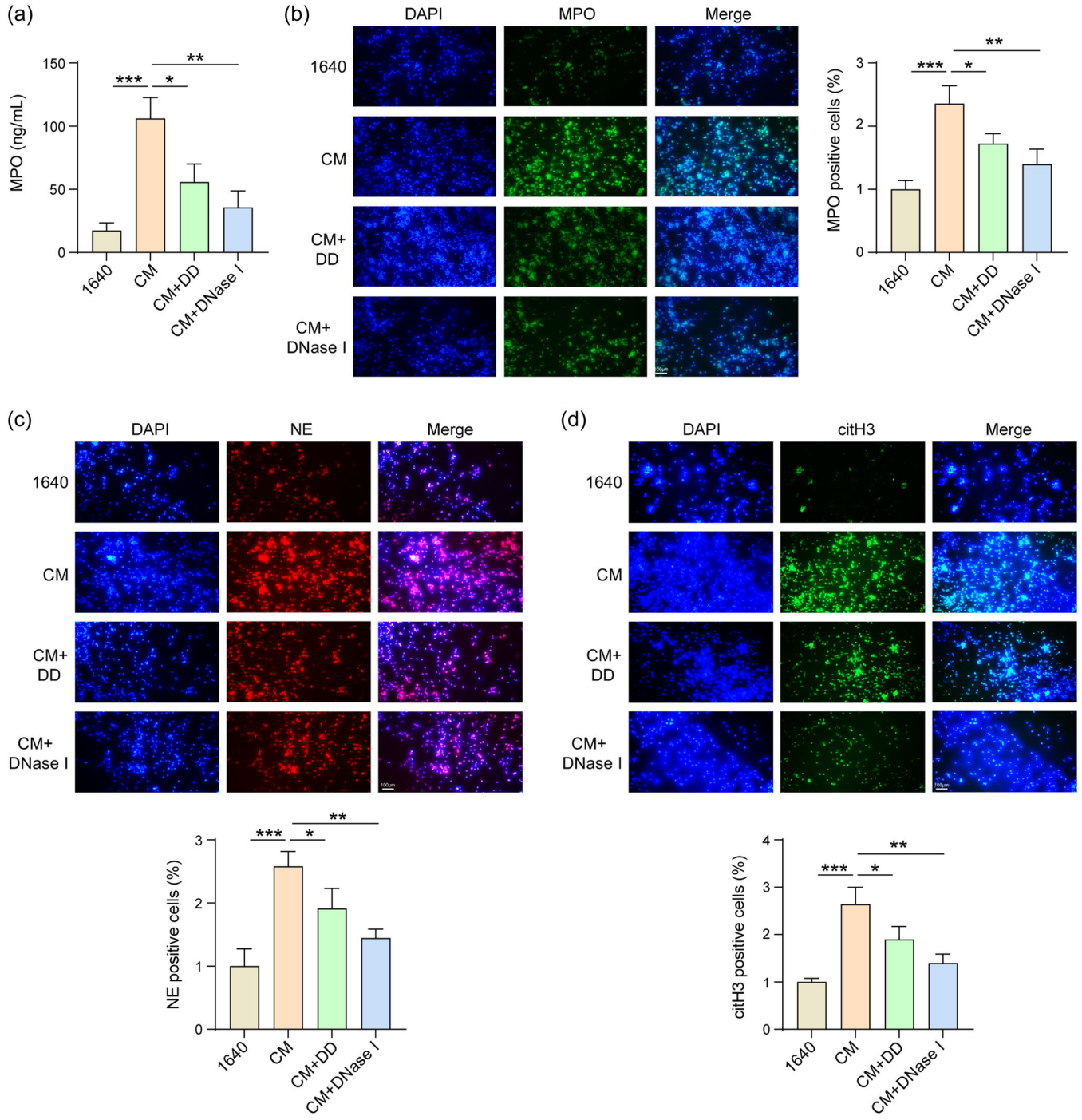

FIGURE 6 DD reduced the formation of NETs in vitro. The primarily isolated neutrophils were incubated with CM from LLC cells and/or treated with DD or DNase I. (a) MPO level in the neutrophil supernatants was measured by ELISA. (b–d) Immunofluorescence analysis and quantitative data for MPO, citH3, and NE levels in neutrophils. Nuclei were stained with DAPI. Scale bar: 100 μm. citH3, citrullinated histone H; CM, conditioned medium; DAPI, 4′,6-diamidino-2-phenylindole; DD, Didang decoction; ELISA, enzyme-linked immunosorbent assay; LLC, Lewis lung carcinoma; MPO, myeloperoxidase; NE, neutrophil elastase; NET, neutrophil extracellular trap. $n = 3$. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus RPMI-1640 or CM group.

Recently, studies have confirmed the critical role of NETs in cancer immunoediting, cancer metastasis, and cancer-associated thrombosis.[25,26] Circulating NETs can cause organ damage in cancer patients and have been recognized as significant contributors to the exacerbation of various thrombogenic diseases.[27] In addition, the formation of NETs has been observed in blood samples and tumor tissues from both animal models and cancer

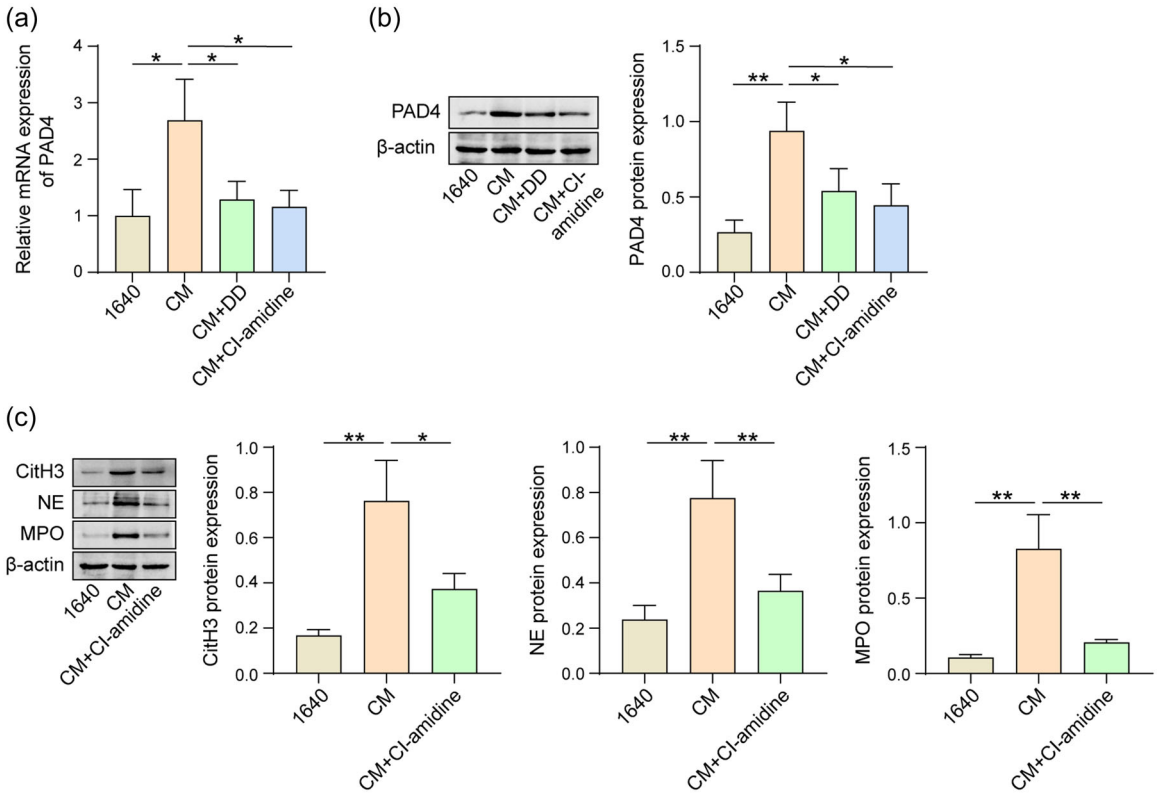

FIGURE 7 DD reduced the formation of NETs by downregulating of PAD4. Neutrophils were treated with CM and/or treated with DD or PAD4 inhibitor CI-amidine. (a) qPCR and (b) western blot were carried out to measure PAD4 mRNA and protein level. (c) Western blot analysis of MPO, citH3, and NE levels in neutrophils. citH3, citrullinated histone H; CM, conditioned medium; DD, Didang decoction; MPO, myeloperoxidase; NE, neutrophil elastase; NET, neutrophil extracellular trap; qPCR, quantitative real-time PCR; $n = 3$. $^{*}p < 0.05$, $^{**}p < 0.01$ versus RPMI-1640 or CM group.

patients.[28] This study investigated the relationship between NETs and thrombosis in lung cancer. It was found that the levels of NET markers MPO, citH3 and NE in the blood, tumors, or tumor-associated thrombus tissues of xenograft mice were elevated. This suggests a positive correlation between NET formation and lung cancer-associated thrombosis, which is consistent with findings from previous studies.[23,29] Thus, inhibiting the formation of NETs may offer protection against cancer-associated thrombosis in lung cancer. Research has demonstrated that NETs play multiple roles in the context of cancer-related inflammation. For example, NETs can directly or indirectly promote tumor metastasis to distant sites and protect cancer cells, thereby hindering the effects of cytotoxic lymphocytes and contributing to anti-inflammatory responses.[13] Another study confirmed that gallbladder cancer cells produced IL-8, which stimulated the formation of NETs, thereby accelerating thromboembolism.[30] This study found that DD significantly inhibited NF-κB phosphorylation in tumor tissues and decreased serum IL-8 levels in xenograft mice, demonstrating an anti-inflammatory effect in lung cancer.

The components of DD are crucial in the prevention of macroangiopathy.[18] Recent studies have confirmed that rhubarb contains active compounds, including rhein, emodin, catechin, and epicatechin, which play a pivotal role in modulating the abnormal accumulation of extracellular matrix.[31] Leech contains an extract of hirudin, which has the ability to inhibit platelet aggregation and thereby prevent thrombosis. The extract of gadfly reduces the activity of blood coagulation factors and inhibits the formation of thrombi. Peach seed is thought to promote the degradation of collagen and downregulate the expression of α-SMA and fibronectin.[15,16] This study initially investigated the effects of DD on thrombosis and NET formation in lung cancer. The results demonstrated that DD treatment significantly inhibited tumor growth, prevented thrombosis, and reduced the levels of NET markers MPO and NE levels in the serum and tumor tissues of xenograft mice. As previously described, NETs have been shown to promote angiogenesis and enhance tumor growth in various cancers.[32,33] Hence, the inhibition of DD or DNase I on tumor growth may be associated with the inhibition of NET formation. The direct antithrombotic effect of DD in

certain inflammatory diseases, such as atherosclerosis and pulmonary interstitial fibrosis, has been substantiated through rigorous research.[17,18] A recent study demonstrated that the pathomechanism of thrombosis in glioma is likely due to platelet activation induced by extracellular vesicles released from cancer cells.[34] In addition, cancer cells can induce neutrophils to produce NETs, which contribute to the formation of thrombosis. Therefore, DD may have a direct antithrombotic effect on thrombosis and may also exert an inhibitory effect on tumor growth, indirectly leading to a reduction in thrombosis.

Neutrophils are the most abundant polymorphonuclear leukocytes in human blood, and they release NETs.[35] Our study found that DD gavage reduced the levels of NET markers MPO, citH3, and NE in primary neutrophils and thrombus tissues from xenograft mice. Additionally, CM prepared from LLC cell supernatants significantly induced the upregulation of NET markers and the formation of a prominent extracellular fibrous network, which was inhibited by treatment with DD. It has been reported that NET components, such as MPO, histones, and proteinases, can inhibit tumor growth and metastasis.[36] Combined with previous evidence, this study ultimately concludes that the inhibition of cancer-associated thrombosis in lung cancer by DD may be due to its effect on NET formation. Nevertheless, the underlying mechanisms by which DD stimulates neutrophils to generate NETs in lung cancer still require further exploration. NETs are web-like structures composed of DNA released by activated neutrophils.[37] This study found that the number of neutrophils was significantly higher in the whole blood of xenograft mice compared to that of normal mice. However, the DD treatment partially reversed this change. Additionally, treatment with DD also reduced the monocyte counts and G-CSF concentration in xenograft mice, but did not affect the red blood cell counts, hemoglobin levels, or GM-CSF concentrations. Taken together, these data suggest that DD may suppress neutrophil counts, potentially in association with G-CSF.

TF is a transmembrane glycoprotein that serves as the primary trigger for coagulation activation. It is often upregulated in cancer and has been extensively studied in relation to cancer-associated thrombosis.[38] Platelets play a crucial role in immune thrombosis, with activated platelets capable of inducing neutrophils to release NETs.[7] Coagulation activation is a common concern in cancer patients, leading to thromboembolic events and intravascular coagulation activation. Moreover, the concentration of TAT complexes is regarded as an early indicator of deep vein thrombosis.[39] In this study, the concentrations of TF and TAT complexes, as well as platelet counts, were significantly elevated in the serum of xenograft mice; however, the administration of DD reversed these changes. Cell-free DNA is a major structural component of NETs, and we confirmed that DD reduced the level of cell-free DNA in xenograft mice. These discoveries highlight the significant role of the TF-thrombin-platelets axis in the regulation of NET formation and subsequent immune thrombosis. Furthermore, previous evidence has demonstrated that the frequency of TF-positive monocytes/macrophages in thrombosis is higher in cancer-associated VTE compared to VTE without cancer.[40] Our study also found that the number of monocytes was markedly elevated in xenograft mice compared to normal mice. Therefore, the TF-positive monocytes/macrophages may represent a potential mechanism for NET formation, warranting further investigation. PAD4 is a critical enzyme that plays a significant role in posttranslational modifications by facilitating the citrullination of proteins in various cellular compartments. This process contributes to the modulation of gene expression and the formation of NETs.[41] This study demonstrated that both the PAD4 inhibitor CI-amidine and DD effectively suppressed NET formation in CM-treated neutrophils. These findings suggest that the reduced expression of PAD4 is responsible for the inhibitory effect of DD on the formation of NETs.

DD has been confirmed to be significant in the treatment of atherosclerosis, hyperlipidemia, and type 2 diabetes.[15,17] In addition, early intervention with DD delays the development of macrovascular lesions in diabetic rats without causing bleeding events.[16] Although there is limited evidence regarding the effect of DD on cancer, our study found that DD treatment reduced tumor growth and inhibited thrombus formation in xenograft mice. Furthermore, DD treatment exhibited effects similar to those of the positive control, DNase I treatment. Therefore, DD is considered effective and safe in xenograft mice. However, the risks and efficacy of DD in clinical settings need to be confirmed through further clinical trials. Though adenocarcinoma is the type of lung cancer with the highest risk of thrombus formation, previous reports have demonstrated that LLC-induced mouse lung cancer models can promote cancer-associated thrombosis.[42,43] In future studies, we will explore the role of DD in cancer-associated thrombus formation in lung adenocarcinoma cells.

In summary (Figure 8), we observed a significant inhibitory effect on the formation of NETs and lung cancer-associated thrombosis following the intervention with the traditional Chinese medicine DD. Therefore, DD may impede the progression of lung cancer-associated thrombosis by inhibiting PAD4-dependent

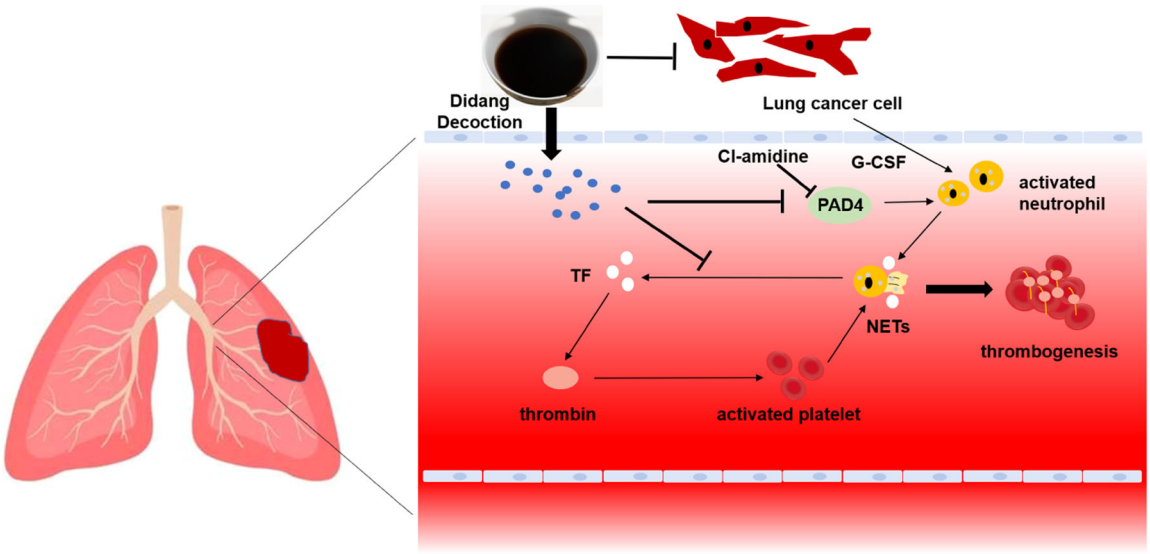

FIGURE 8 Schematic diagram of DD inhibiting cancer-associated thrombosis. DD treatment reduces cancer-associated thrombosis in lung cancer. Lung cancer increases thrombosis by inducing PAD4-mediated NET formation via the TF-thrombin-platelet pathway. DD, Didang decoction; NET, neutrophil extracellular trap; TF, tissue factor.

NET formation through the TF-thrombin-platelet pathway. Furthermore, additional research is necessary to elucidate the various roles and mechanisms of DD in the prevention and treatment of cancer-associated thrombosis in lung cancer.

AUTHOR CONTRIBUTIONS

Guarantor of integrity of the entire study: Ting Zhou and Xiaoyan Zeng. Study concepts: Ting Zhou. Study design: Ting Zhou. Definition of intellectual content: Ting Zhou. Literature research: Xiaoyan Zeng. Clinical studies: Ting Zhang. Experimental studies: Xiaoyan Zeng. Data acquisition: Liyuan Pei and Ting Zhou. Data analysis: Jiuxi Li and Yaping Yang. Statistical analysis: Ya Chen and Xuejing Wang. Manuscript preparation: Xiaoyan Zeng. Manuscript editing: Xiaoyan Zeng and Ting Zhou. Manuscript review: Xiaoyan Zeng and Ting Zhou.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data sets used or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

The animal experiments were permitted by the Experimental Animal Ethics Committee of Hunan University of Traditional Chinese Medicine and in accordance with standard protocols (LLBH2024030132). This article does not contain any studies with human participants performed by any of the authors.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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