Received: 18 April 2024 | Revised: 21 November 2024 | Accepted: 19 December 2024

https://doi.org/10.1016/j.jtha.2024.12.023

ORIGINAL ARTICLE

Fibrin film on clots is increased by hematocrit but reduced by inflammation: implications for platelets and fibrinolysis

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Funding information
G.A. is supported by a scholarship from King Abdulaziz University, Jeddah, Saudi Arabia. C.D. is supported by the British Heart Foundation (FS/IBSRF/22/25130). F.L.M. is supported by the Wellcome Trust (215861/Z/19/Z). R.A.S.A.'s laboratory is supported by the British Heart Foundation (RG/18/11/34036 and RG/F/23/110112) and the Wellcome Trust (204951/B/16/Z). R.A.S.A. is supported in part by the National Institute for Health and Care Research (NIHR) Leeds Biomedical Research Centre. The views expressed are those of the authors and not necessarily those of the National Health Service, the NIHR, or the Department of Health and Social Care.

Abstract

Background: Blood clot formation, triggered by vascular injury, is crucial for hemostasis and thrombosis. Blood clots are composed mainly of fibrin fibers, platelets, and red blood cells (RBCs). Recent studies show that clot surfaces also develop a fibrin film, which provides protection against wound infection and retains components such as RBCs within the clot. However, the role of fibrin films in thrombi remains poorly understood.

Objectives: To explore the relationship between fibrin films and inflammation, RBC concentration, platelets, and fibrinolysis activity.

Methods: We used laser scanning confocal and scanning electron microscopy, enzyme-linked immunosorbent assay, and turbidity and fibrinolysis assays to investigate the interactions between fibrin film and inflamed endothelium, RBCs, platelets, and fibrinolysis.

Results: We found that plasma clots forming on top of inflamed endothelial cells show less fibrin film coverage and are characterized by higher fiber density and shorter lag time compared with control cells. Blood clots formed under conditions of high hematocrit showed significantly more fibrin film coverage than low hematocrit clots. We found that platelet adhesion was significantly reduced on clots with film compared with clots without film even when platelets were preactivated. Fibrinolysis was faster in clots without film than in clots with film, partly due to reductions in plasmin generation.

Conclusion: Our findings indicate that reductions in fibrin film formation under thromboinflammatory conditions support continued clot growth through effects on increased platelet adhesion and activation. On the other hand, increased fibrin film impairs fibrinolysis. These data show a multifaceted role of the fibrin film in clot growth and stability.

KEYWORDS
blood platelets, fibrinogen, fibrinolysis, inflammation, thrombosis

Manuscript handled by: Patricia Liaw

Final decision: Patricia Liaw, 19 December 2024

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1 | INTRODUCTION

Hemostasis is a critical mechanism that prevents life-threatening bleeding. During hemostasis, fibrin is generated at the site of injury leading to clot formation, followed by clot dissolution via fibrinolysis in later stages of wound healing [1]. Fibrin is generated by thrombin cleavage of fibrinogen during coagulation, producing a fiber network [2]. Importantly, abnormal clot structure and increased clot stability are linked to thrombosis [3]. Thrombosis is a severe complication that is the most common cause of death in patients with cardiovascular disease [2]. Thrombi are heterogeneous in composition, consisting of fibrin fibers, platelets, red blood cells (RBCs), and leukocytes [4]. The composition of thrombi changes from platelet-rich to fibrin-rich during their development over time. This adjustment occurs as the thrombus transitions from a rapidly growing to a stable thrombus. It has been previously reported that platelet migration toward the thrombus surface is reduced and that the accumulation of fibrin on the surface, in a so-called "fibrin cap," could limit thrombus growth [5]. These data indicate that following thrombus formation, its surface may not be thrombogenic. Furthermore, contracted clots impair external fibrinolysis, but not internal fibrinolysis [6]. Therefore, the question arises as to whether the dynamics of blood components and vasculature influence clot surface properties and whether this may determine the size and stability of thrombi. Does the surface of the clot limit thrombus growth by controlling blood cell adhesion and interactions, and what is the effect of the surface of clots on fibrinolysis?

We previously discovered a new structure of fibrin on blood clots called fibrin film [7]. We found that fibrin forms a sheet-like structure at phase interfaces on the surface of the clot that protects against bacterial invasion and helps to retain blood cells [7]. Several studies have shown that fibrin film formation also occurs within the vasculature. Fibrin film–type structures have been identified in *ex vivo* thrombi obtained from patients with myocardial infarction [8], ischemic stroke [9,10], and abdominal aortic aneurysm [4]. A study by Di Meglio et al. [10] showed that the shell- or film-like structures on the surface of thrombi retrieved from patients with stroke impair thrombolysis. Langmuir–Blodgett fibrinogen films have previously been shown at the interface between liquids and phospholipids [11], providing a potential mechanism for intravascular film formation in the absence of air–blood interfaces. However, little is known about the interactions between fibrin films and endothelial cells, RBCs, platelets, or fibrinolysis.

Therefore, the aims of this study were to 1) analyze the effect of inflamed endothelial cells on fibrin film formation, 2) identify the effect of RBCs on fibrin film coverage, 3) investigate the role of fibrin film in the context of platelet adhesion, and 4) characterize the effect of fibrin film on fibrinolysis. We found that inflammation of endothelial cells reduces fibrin film coverage of the clot through a tissue factor (TF)–dependent mechanism, while the coverage of fibrin film on clots is modulated by RBC content. We also found that fibrin film coverage decreases the adhesion of resting and activated platelets to clots, while increasing the resistance of the clot to fibrinolysis through reductions in plasmin generation (PG). Our data thus reveal a multifaceted role for fibrin film by limiting thrombus growth yet enhancing thrombus stability.

2 | MATERIALS AND METHODS

2.1 | Materials

Human fibrinogen (plasminogen-depleted, von Willebrand factor (VWF)–depleted, and fibronectin-depleted) and Glu-plasminogen were from Enzyme Research Laboratories. Normal pooled platelet-poor plasma was purchased from First Link UK Ltd. Human thrombin was from Merck Diagnostics; tissue-plasminogen activator (tPA) was obtained from Pathway Diagnostics, while Alexa Fluor 594-fibrinogen and Alexa Fluor 488-phalloidin were from Invitrogen. Platelet poor plasma reagent (a mixture of TF and phospholipids) was obtained from Stago. All other chemicals were purchased from Sigma-Aldrich unless stated otherwise.

2.2 | Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from PromoCell. Three vials from different pools were used, each pooled from 3 to 4 donors. Cells were used between passages 3 and 5. To knockdown TF, HUVECs were treated with 20 pmol ON-TARGET small interfering RNA (siRNA; Dharmacon) in RNAiMAX Lipofectamine (Thermo Fisher Scientific) overnight, targeting TF or a scrambled control 24 hours after seeding. Cells were then left to recover for 72 hours after transfection. Knockdown efficiency was confirmed through quantitative polymerase chain reaction TaqMan (Thermo Fisher Scientific) analysis and a TF-specific enzyme-linked immunosorbent assay (ELISA; R&D Systems). To induce inflammation, HUVECs were treated with tumor necrosis factor (TNF)-α, lipopolysaccharide (LPS) from *Escherichia coli* O111:B4, or interleukin (IL)-1β (final concentrations, 20 ng/mL, 1 μg/mL, and 5.5 ng/mL, respectively) for 24 hours prior to experiments. These inflammatory mediator concentrations were based on previously published data [12].

2.3 | ELISA

TF and thrombomodulin (TM) were measured in the cell lysates, and VWF, TF pathway inhibitor (TFPI) and monocyte chemoattractant protein-1 (MCP-1) were measured in the cell supernatants using ELISA kits according to the manufacturer's instructions. All ELISA kits were purchased from R&D, except VWF, which was from Abcam. See Supplementary Methods for sample preparations.

2.4 | Clot structure on top of HUVECs

HUVECs were seeded in gelatin-coated ibidi μ-Dish slides (35 mm; Thistle Scientific) at 10 000 cells/well with TNF-α, LPS, or IL-1β as detailed above. After removing supernatants, HUVECs were stained with Calcein-AM (1 μM; Thermo Fisher Scientific) for 30 minutes at 37 °C with 5% CO_2. Normal pooled platelet-poor plasma was diluted 1:2 in cell supernatant and supplemented with Alexa Fluor 594-fibrinogen (50 μg/mL). Clotting was triggered by addition of thrombin (0.1 U/mL) and $CaCl_2$ (5 mM), or $CaCl_2$ alone, on top of the cells, and the cells were incubated for 2 hours. HUVEC and clot density images were taken using an inverted laser scanning confocal microscope (LSCM; LSM880, Carl Zeiss) with a 40× oil lens. Z-stacks (43 slides, 21.5 μm) were taken in 3 areas per clot. For fibrin density measurements, stacks were merged into 2-dimensional images and analyzed using ImageJ/Fiji software (Public Domain, originally developed by Wayne Rasband, National Institutes of Health) and an in-house macro to calculate the number of fibers per 100 μm. Clots were then prepared for scanning electron microscopy (SEM) (Hitachi High-Technologies) by fixation in 2% glutaraldehyde overnight, washing in sodium cacodylate buffer (10.7 g/L sodium cacodylate; pH 7.4) for 1 hour and dehydrated in increasing acetone percentages (30%-100%, 15 minutes each) [7]. Finally, clots were subjected to critical point drying with CO_2 and coated with 10 nm iridium. Images were taken in 5 areas per clot at 600× magnification and fibrin film coverage was analyzed using ImageJ/Fiji. Three to 5 replicates were performed.

2.5 | Turbidity assay

A turbidity assay for fibrin polymerization was adapted from a standard protocol [13]. See Supplementary Methods for further information.

2.6 | Hematocrit experiments

Blood samples were obtained from the antecubital vein of healthy volunteers and collected in 3.2% sodium citrate vacutainers (BD Biosciences). Samples were centrifuged at 100*g* for 6 minutes to separate platelet-rich plasma and RBCs. RBCs (10%-70%) were then resuspended in the autologous plasma with the volume topped up to replace missing RBCs using extra autologous plasma or saline to provide a range of hematocrit (10%-70%). Clotting was initiated by a platelet poor plasma reagent (final concentration of 5 pM TF and 4 pM phospholipids) and $CaCl_2$ (10 mM), and the cells were left to clot for 2 hours in a humidity chamber. Clots were washed in saline solution and prepared for SEM, as described above. Images were taken in 5 areas per clot, with 4 images at 600× in each area. Average fibrin film coverage was analyzed by ImageJ/Fiji. Experiments were performed in 6 replicates.

2.7 | Platelet adhesion

Washed platelets were prepared (see Supplementary Methods) and added on top of coverslips coated with a fibrin monomer, fibrin clot with film, and fibrin clot without film. Recombinant wild-type (WT) and γ'/γ' fibrinogens were expressed in Chinese hamster ovary cells and purified in house as previously described [14,15]. Plasma-purified, recombinant WT or γ'/γ' fibrinogen (final concentration, 200 μg/mL) was coated on coverslips for 1 hour, and then, thrombin (0.1 U/mL) and $CaCl_2$ (5 mM) were added for 30 minutes to produce a fibrin monomer. For clot preparation, fibrinogen (final concentration, 200 μg/mL) or platelet-poor plasma from the platelet donor (1:6 dilution) were incubated with Alexa Fluor 594-fibrinogen (10 μg/mL) and clots were triggered with thrombin (0.1 U/mL) and $CaCl_2$ (5 mM). The mixture (30 μL) was immediately transferred to the coverslip and left for 2 hours. Clots without film were generated by immediately covering clots with a thin layer of plastic wrap, preventing the air–clot interface, which was carefully removed after clot formation (Supplementary Figure S1A, B). Then, fibrin monomers and clots were washed with high salt solution (40 mM Tris base, 1 M NaCl, 0.05% Tween 20, pH 7.4) to neutralize thrombin and blocked with bovine serum albumin (BSA, 5 mg/mL) for 1 hour. Subsequently, resting platelets (5×10^6), platelets pretreated with calcium (2 mM) with thrombin (0.1 U/mL) or adenosine diphosphate (ADP, 20 μM) for 30 seconds, or platelets pretreated with eptifibatide (25μM), to block fibrinogen–platelet interaction via $\alpha_{IIb}\beta_3$ integrin, were added to the surface of clots for 45 minutes at 37 °C. Cells and clots were then fixed with 4% paraformaldehyde for 10 minutes, permeabilized with 0.1% Triton X-100 for 5 minutes, and stained with Alexa Fluor 488-phalloidin (1:3000) for 1 hour in the dark. Coverslips were mounted on microscope slides and imaged using an upright LSM880 (Carl Zeiss) with a 40× oil immersion lens at 5 areas per slide. Platelet number, total surface area per field, and surface area per platelet were analyzed using ImageJ/Fiji. Experiments were performed at least in triplicates.

2.8 | External fibrinolysis

The fibrinolysis experiment was adapted from a previously described protocol [16]. See Supplementary Methods for further information.

2.9 | Plasmin generation assay

PG was carried out as previously described [17] on the surface of preformed plasma clots in a 96-well plate with or without fibrin films using TF (platelet poor plasma-Reagent LOW, ~1 pM TF; Diagnostica Stago) and calcium (10 mM). PG was triggered by the addition of exogenous tPA (1.25 μg/mL) and a substrate/calcium solution

(0.5 mM Boc-Glu-Lys-Lys-AMC substrate, 16.6 mM $CaCl_2$, 60 mg/mL BSA in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES, 0.02% NaN_3, pH 7.3). The plates were then shaken for 10 seconds. PG was determined using fluorogenic substrate cleavage over time using a fluorometer (Fluoroskan Ascent, Thrombinoscope) to monitor reactions at 20-second intervals. Data analysis was performed similarly to the thrombin generation assay: lag time, time to peak, peak plasmin, and endogenous plasmin potential.

2.10 | Fibrin film components

Plasminogen and tPA components within the clots were analyzed by LSCM. See Supplementary Methods for further information.

2.11 | Data analysis

Data were tested for normal distribution (Shapiro–Wilk test) before application of parametric or nonparametric tests as appropriate (detailed in figure legends). GraphPad Prism 10 (GraphPad Software incorporated) was used for all statistical analyses, with $P < .05$ considered as significant.

3 | RESULTS

3.1 | TNF-α, LPS, and IL-1β trigger a proinflammatory and prothrombotic profile in endothelial cells

HUVECs were treated with LPS, IL-1β or TNF-α for 24 hours to trigger inflammation. We measured the inflammatory and prothrombotic response through the expression of TF and TM in cell lysates and VWF, MCP-1, and TFPI in cell supernatants by ELISA. MCP-1 levels were increased while TM levels were decreased in cells treated with all inflammatory mediators (Supplementary Figure S2A, B). TF expression, however, was only elevated in TNF-α–treated cells (Supplementary Figure S2C). These data indicate that cytokines tested increased the proinflammatory and procoagulant state of the endothelial cells, while TNF-α was able to trigger a stronger procoagulant phenotype on HUVECs by also increasing TF levels and by having the strongest effect on decreasing TM. There were no significant changes in the VWF and TFPI secretions (Supplementary Figure S2D, E).

3.2 | Clots on top of inflamed endothelial cells form faster, with higher fiber density and less fibrin film coverage

Plasma clots triggered with thrombin and $CaCl_2$, or $CaCl_2$ alone, were generated on the top of endothelial cells. LSCM was used to analyze the density of the fibrin fiber network, followed by SEM imaging of fibrin film coverage (Figure 1A, B). Plasma clots formed with thrombin and $CaCl_2$ on top of TNF-α–treated cells showed increased fiber density (Figure 1C) and reduced fibrin film coverage (Figure 1D) compared with those produced on top of control cells. Similar outcomes were observed when clotting was triggered with $CaCl_2$ alone (Supplementary Figure S3A–C). There were no significant differences in fibrin fiber density and film coverage of clots on top of LPS-treated and IL-1β–treated cells (Figure 1A, C, D and Supplementary Figure S3A–C).

Next, fibrin polymerization in plasma on top of endothelial cells stimulated with inflammatory cytokines was monitored by turbidity assay. Clotting lag-time was shorter for clots forming on top of TNF-α– and IL-1β–treated cells compared with control cells, with TNF-α treatment resulting in the shortest lag-time (Figure 2A–D). LPS-treated cells also reduced lag time, but only for clots formed with $CaCl_2$ alone. Time to 50% clotting was shorter for clots forming on top of TNF-α–treated cells compared with control cells (Figure 2E, F), while time to 50% clotting was also shorter for clots on top of LPS-treated and IL-1β–treated cells in the presence of $CaCl_2$ alone. There were no significant changes in maximum optical density (Figure 2G, H). Without HUVECs, TNF-α alone did not affect clot density, fibrin film, and lag time compared with control clots (Supplementary Figure S4).

3.3 | TF is the main mediator of TNF-α–mediated increased clotting potential

To investigate the role of TF in regulating film development further, HUVECs were treated with siRNAs to target TF and reduce its expression. Following this, the cells were treated with TNF-α for 24 hours to trigger inflammation. We measured the expression of TF in cell lysates by ELISA. We found that TF expression was reduced back to basal levels in inflamed cells treated with TF-targeting siRNA when compared with inflamed control cells or inflamed cells treated with a nonspecific siRNA (Supplementary Figure S5A). Plasma clots triggered with thrombin and $CaCl_2$, or $CaCl_2$ alone, were generated on top of these endothelial cells. LSCM was again used to analyze the density of the fibrin fiber network, followed by SEM imaging of fibrin film coverage (Figure 3A and Supplementary Figure S5B). Plasma clots formed with thrombin and $CaCl_2$ on top of cells treated with TF-targeting siRNA and TNF-α showed decreased fiber density (Figure 3B) and increased fibrin film coverage (Figure 3C) compared with those produced on top of inflamed control cells or inflamed cells treated with a nonspecific siRNA. Similar outcomes were observed when clotting was triggered with $CaCl_2$ alone (Supplementary Figure S5B–D). The scrambled siRNA or TF-silencing siRNA had no impact on fiber density or film coverage in noninflamed cells (Supplementary Figure S5E–G).

Altogether, these data suggest that plasma clots forming on top of TNF-α–treated cells show less fibrin film coverage and are characterized by higher fiber density and shorter lag time compared with control cells due to increased TF expression. This increased endothelial procoagulant phenotype after TNF-α stimulation is further

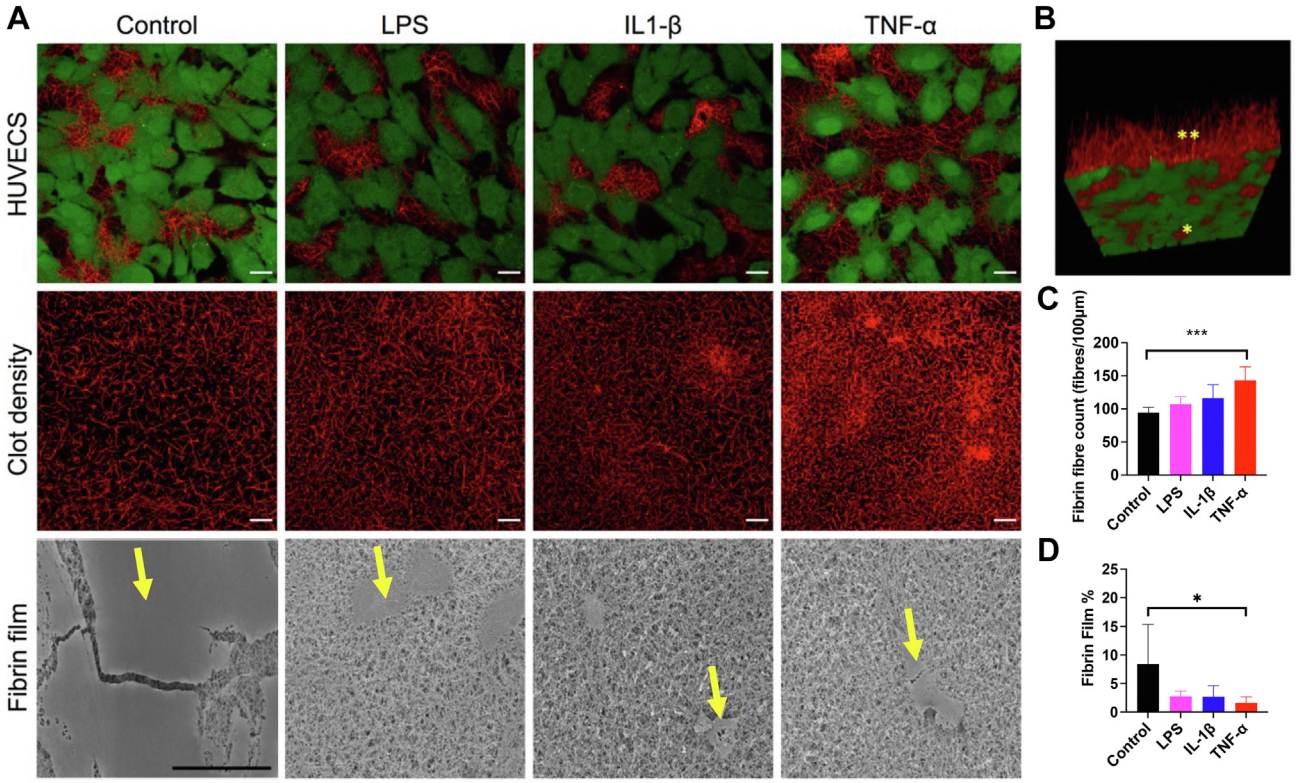

FIGURE 1 Plasma clot structure on top of inflamed human umbilical vein endothelial cells (HUVECs). (A) Representative images of HUVECs (top row) and fibrin fibers (middle row) imaged by laser scanning confocal microscopy and of fibrin film (bottom row) imaged by scanning electron microscopy (yellow arrows indicate fibrin film), following treatment with cell culture media (control), lipopolysaccharide (LPS), interleukin (IL)-1β, or tumor necrosis factor (TNF)-α. Clotting was triggered with 0.1 U/mL thrombin and 5 mM $CaCl_2$. (B) 3-dimensional confocal image showing fibrin clot (**, red [Alexa-594 labeling]) on the top of HUVECs (*, green [Calcein-AM labeling]). (C) Fibrin fiber density was significantly increased on top of HUVECs treated with TNF-α. (D) Fibrin film coverage on top of the clot was significantly reduced when HUVECs were treated with TNF-α. Confocal microscopy and electron microscopy images were obtained at 40× magnification (scale bar: 20 μm) and 1000× magnification (scale bar: 50 μm), respectively. $n = 5$, analyzed by 1-way analysis of variance: $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$.

supported by decreased TM expression. Thus, TNF-α modulates clot structure via its effects on the proinflammatory and procoagulant phenotype of the endothelial cell.

3.4 | RBCs support fibrin film coverage

Blood samples were obtained from healthy volunteers, and hematocrit was adjusted to 10, 30, 50, and 70% by mixing the RBC fraction with the plasma from the same donor and then topped up with extra plasma or saline to replace the volume of missing RBCs. This allowed us to compare the effects of hematocrit on film formation in conditions where either the concentration or the number/quantity of platelets and proteins was maintained. Fibrin film coverage of clots made with the different hematocrits was investigated by SEM. All blood clots within the hematocrit range developed fibrin film; however, blood clots formed with 10% hematocrit showed less fibrin film coverage than those with a higher (50%-70%) hematocrit when RBC volume was replaced with either plasma or saline (Figure 4). Moreover, fibrin film coverage appeared more variable at low hematocrit, while being more consistent between samples at higher hematocrit.

3.5 | Fibrin film reduces platelet interactions with the clot

Next, we assessed the adhesion and spreading of platelets on fibrin films. Clots with or without film were produced using purified fibrinogen on coverslips before washed platelets were added to their surface. Platelet adhesion was reduced by the presence of film on top of fibrin clots (Figure 5A, B). There was a trend toward a reduction in average aggregate area on clots in the presence of film (Figure 5C), but this finding was not significant. Similar findings were observed with plasma clots with the number of platelets binding to the clot significantly reduced by the presence of the fibrin film (Supplementary Figure S6A, B). However, with plasma clots, there was also a significant decrease in the average platelet aggregate size (Supplementary

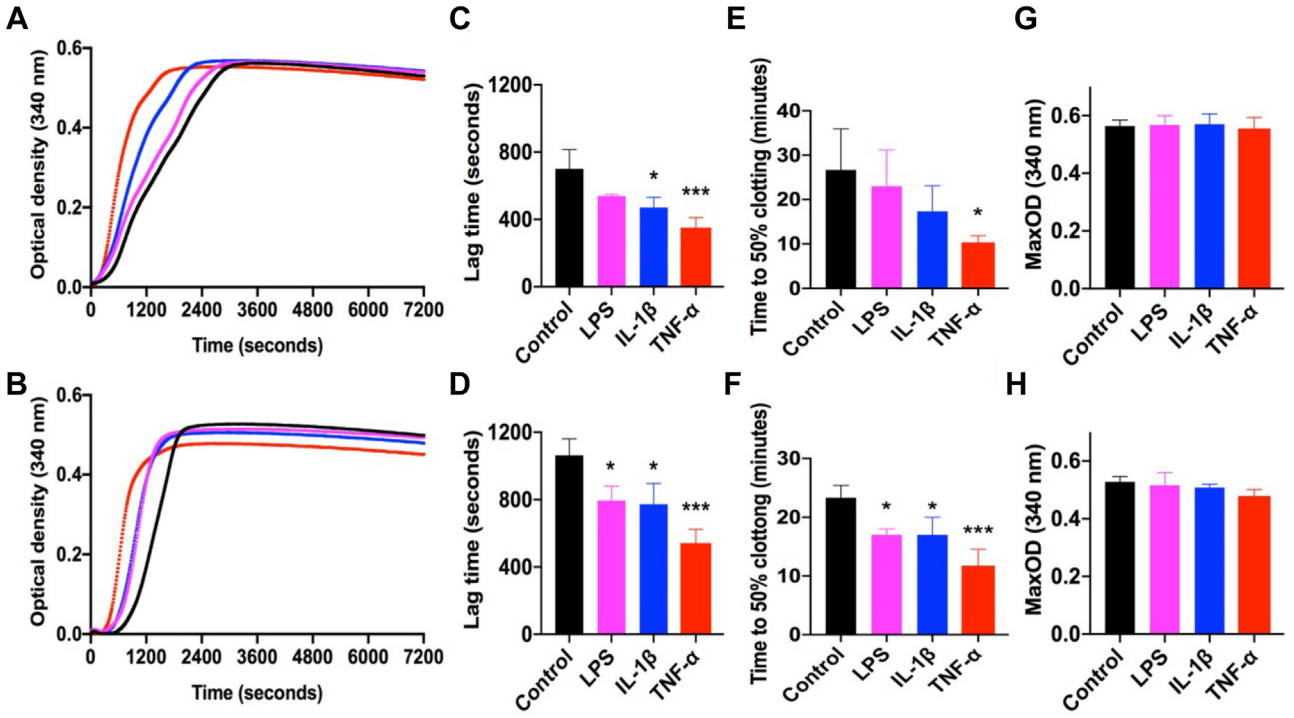

FIGURE 2 Plasma clot formation on top of inflamed human umbilical vein endothelial cells. Turbidity curves of normal pool plasma activated with 0.1 U/mL thrombin and 5 mM (A) $CaCl_2$ or (B) 5 mM $CaCl_2$ alone. Lag time and time to 50% clotting were decreased when the clots were formed on top of inflamed cells, compared with controls, in the presence of (C, E) thrombin and $CaCl_2$ or (D, F) $CaCl_2$ alone. Maximum optical density (Max OD) was unchanged for clots formed in the presence of (G) thrombin and $CaCl_2$ or (H) $CaCl_2$ alone. n = 3, analyzed by 1-way analysis of variance: ***$P < .001$; *$P < .05$. IL, interleukin; LPS, lipopolysaccharide; TNF, tumor necrosis factor.

Figure S6C). Furthermore, the fibrin film also reduced the number of preactivated platelets, with thrombin or ADP, binding to clots (Supplementary Figure S6A, B), but the effect on the average aggregate size was not seen with preactivated platelets (Supplementary Figure S6C). These data indicate that fibrin films reduce the binding of resting and activated platelets and reduce aggregate size in the absence of platelet activation.

Following on from this, we investigated mechanisms by which fibrin films reduce platelet adhesion using an inhibitor of fibrin–platelet interactions (eptifibatide, inhibitor of $\alpha_{IIb}\beta_3$). Eptifibatide reduced platelet adhesion on the surface of clots formed without film, but no difference was observed on clots with film (Figure 5A–C). These findings suggest that the integrin binding site of fibrin is hidden within the film. The effects of film on platelet adhesion and spreading were analyzed further using recombinant γ'/γ' fibrin, which lacks the Ala-Gly-Asp-Val (AGDV) binding site on the C-terminus of the γA chain that is required for $\alpha_{IIb}\beta_3$ interaction. In agreement with the previous experiment using eptifibatide, platelet adhesion was decreased on γ'/γ' fibrin clots compared with WT fibrin (γA; containing AGDV) in the absence of film. However, platelet adhesion was not different on WT and γ'/γ' fibrin clots in the presence of film, or γ'/γ' fibrin clots without film (Figure 6A–C). These data further confirm that reduced platelet adhesion to fibrin films is caused by the lack of the γ-chain Arg-Gly-Asp (RGD) sequence exposure in the film. The platelet aggregate size was slightly reduced on WT fibrin film or γ'/γ' fibrin clots (Figure 6D), but this did not reach significance. Platelet adhesion and aggregation on fibrin monomers were also investigated as control for the above experiments to verify that platelet interactions are reduced by eptifibatide or γ'/γ' fibrin. As expected, platelet adhesion was reduced on coverslips coated with fibrin monomers with eptifibatide compared with those without inhibitor (Supplementary Figure S7A, C, D). In addition, platelet adhesion was decreased on γ'/γ' compared with WT fibrin monomer (Supplementary Figure S7B, E, F). These experiments confirm that eptifibatide and γ'/γ' fibrin effectively reduce platelets' ability to adhere to fibrin.

Together these data indicate that fibrin films reduce platelet adhesion via the loss of interaction with $\alpha_{IIb}\beta_3$ and reduce aggregate size in the absence of platelet activation.

3.6 | Fibrin film and fibrinolysis

We next studied the effect of fibrin film on fibrinolysis. Purified fibrin (+plasminogen) and plasma clots with or without film were formed in microcentrifuge tubes. After clot formation, tPA was added to the clots and lysis percentage was analyzed over time. Percentage lysis was higher in fibrin and plasma clots without film than in clots with film, indicating that the presence of fibrin film on clots impedes fibrinolysis (Figure 7A and Supplementary Figure S8A). Next, we investigated whether plasminogen and tPA are present in the fibrin

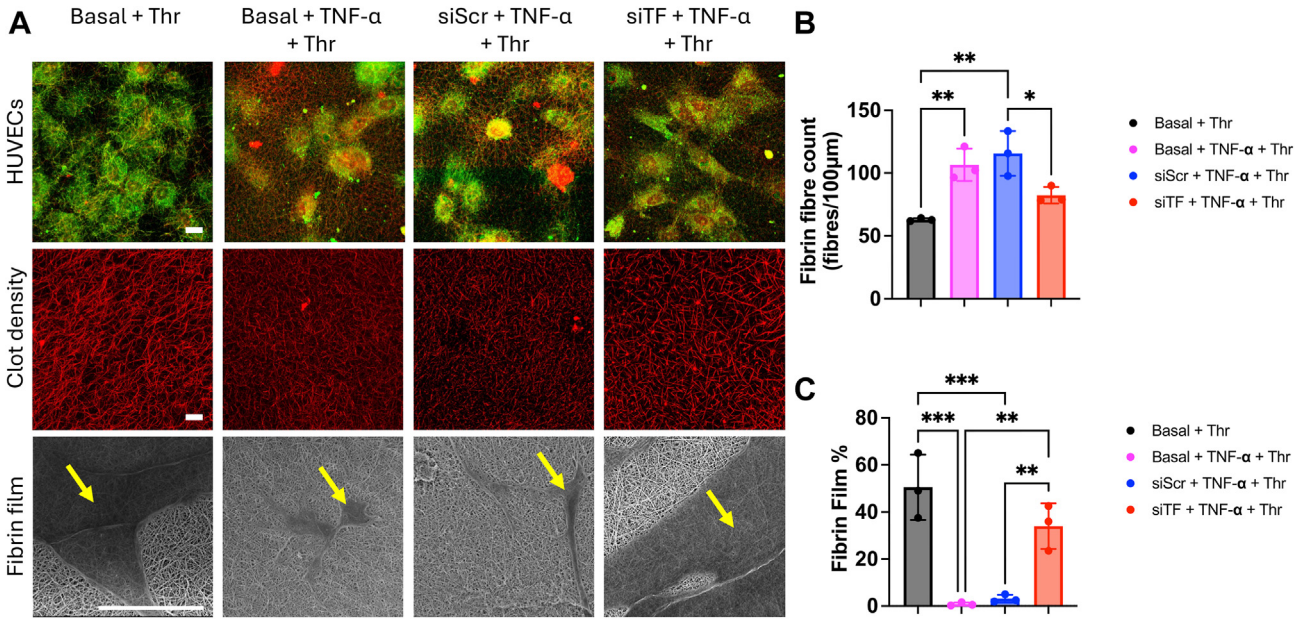

FIGURE 3 Silencing tissue factor (TF) reduces the impact of inflamed cells on film formation. (A) Representative images of human umbilical vein endothelial cells (HUVECs; top row) and fibrin fibers (middle row) imaged by laser scanning confocal microscopy and of fibrin film (bottom row) imaged by scanning electron microscopy (yellow arrows indicate fibrin film) following treatment of cells with culture media (basal), scramble small interfering RNA (siScr), and TF small interfering RNA (siTF), followed by treatment with culture media or tumor necrosis factor (TNF)-α. Clotting was triggered with 0.1 U/mL thrombin (Thr) and 5 mM $CaCl_2$. (B) Fibrin fiber density was significantly reduced following silencing of TF and TNF-α treatment compared with that on cells without siTF treatment. (C) Fibrin film coverage on top of the clot was significantly increased following silencing of TF and TNF-α treatment compared with that on cells without siTF treatment. Confocal microscopy and electron microscopy images were obtained at 40× magnification (scale bar: 20μm) and 1000× magnification (scale bar: 50 μm), respectively. $n = 3$, analyzed by 1-way analysis of variance: $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$.

film via LSCM. Clots were formed with fluorescently labeled fibrinogen, plasminogen, or tPA. We found that only plasminogen colocalized with fibrin film, while tPA was evenly distributed across the clot (Supplementary Figure S8B). These data indicate a sequestration of plasminogen in the fibrin film, which may reduce fibrinolysis in the remainder of the fibrin network due to a lack of plasminogen associated with the fibers. To explore this further, we carried out PG assays on the surface of preformed clots with or without fibrin films. PG was reduced in clots with fibrin films compared with clots without films, with increased lag time, reduced peak plasmin and endogenous PG, and a longer time-to-peak PG (Figure 7B–F). These data indicate that sequestration of plasminogen into fibrin films reduces PG and slows fibrinolysis.

4 | DISCUSSION

Our study shows that a proinflammatory endothelial environment reduces the formation of fibrin films on the clot surface via TF-dependent mechanisms, while RBCs enhance fibrin film coverage. The fibrin film prevents platelet adhesion and delays fibrinolysis through reductions in PG, indicating that fibrin films may play an important multifaceted role in hemostasis and thrombosis.

We used 3 inflammatory mediators, LPS, IL-1β, or TNF-α, to induce endothelial inflammation *in vitro*. These mediators increase the expression of endothelial adhesion molecules, cytokines, and modulators of coagulation [12,18–20]. We confirmed that all treated cells showed higher levels of prothrombotic MCP-1 and lower levels of antithrombotic TM than control cells. There was no change in VWF and TFPI secretions between control cells and cells treated with inflammatory mediators. We demonstrated that TNF-α–treated cells associated with highest levels of TF, resulting in denser clots, compared with unstimulated cells. We examined for the first time the effect of inflammation on fibrin film coverage. Plasma clots forming on the top of TNF-α–treated cells presented with increased fibrin fiber density and reduced film formation on their surface compared with control cells. Together with our previous findings, showing that in the absence of cells faster clot formation results in denser clots [21] and reduced fibrin film formation [7], we hypothesized that inflammatory conditions in cells increased clot formation rates, resulting in denser clots and reduced film formation. Faster clot formation was demonstrated by shorter clotting times in all clots formed on top of inflamed cells compared with control cells. TNF-α was found to accelerate clot formation the most among all the inflammatory mediators and resulted in the largest increase in TF expression. To explore this further, RNA silencing was used to reduce TF expression back to basal

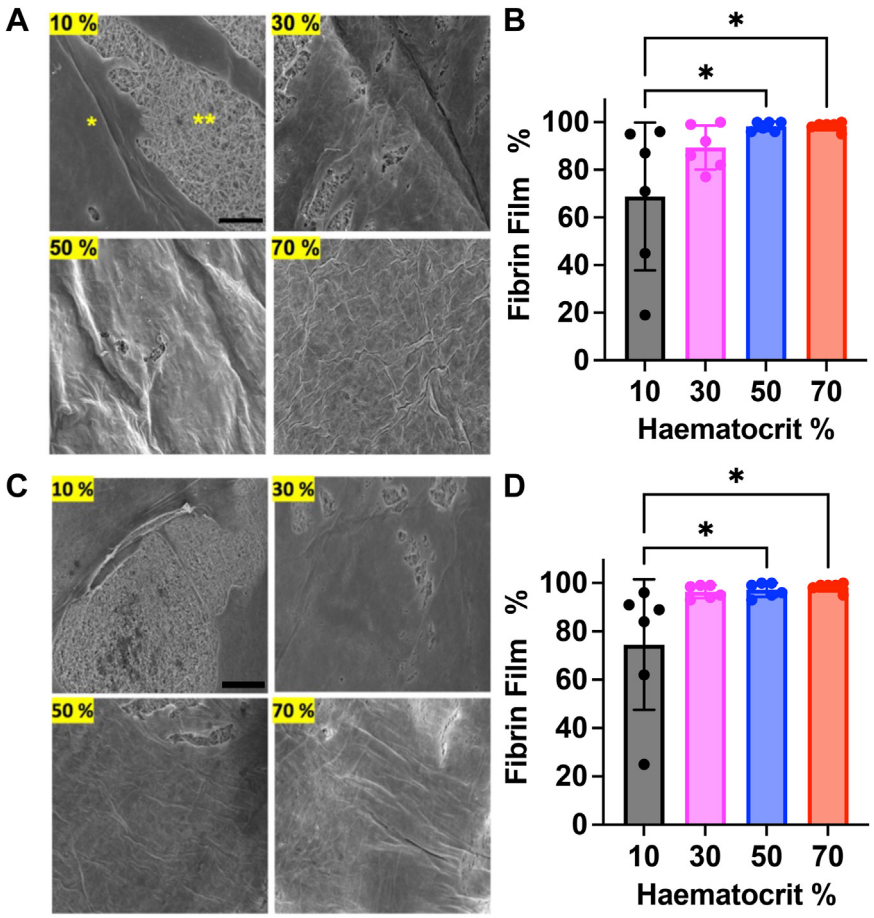

FIGURE 4 Effect of hematocrit on fibrin film coverage of blood clot. Hematocrit was adjusted to 10% to 70% by dilution with autologous plasma or saline. (A) Representative scanning electron microscope images of the surface of blood clots with 10% to 70% hematocrit diluted with plasma. (B) In clots diluted with plasma, the percentage of fibrin film coverage was higher with increased hematocrit. (C) Representative scanning electron microscope images of the surface of blood clots with 10% to 70% hematocrit diluted with saline. (D) In clots diluted with saline, the percentage of fibrin film coverage was also higher with increased hematocrit. Areas with fibrin film (*) and without (**). Magnification 600× (scale bar: 50 μm). $n = 6$, analyzed by Kruskal–Wallis' test: $^*P < .05$.

levels and we demonstrated that this almost completely prevented TNF-α–induced increases in fibrin clot density and reductions in fibrin film coverage. This was in agreement with a previous study by Campbell et al. [22], showing that anti-TF significantly reduced fibrin density and stability in the presence of TNF-α–stimulated cells. However, density and film formation did not completely return to basal levels, which may be an indicator of other factors such as TM playing a role in fibrin film formation. Together, these data show a direct relationship between inflammation-driven TF expression and altered fibrin clot structure and film coverage, where inflamed endothelial cells trigger faster formation of the fibrin network via their prothrombotic phenotype, which in turn decreases the fibrin film formation potential.

Since RBCs occupy a large proportion of a thrombus, we studied the effect of RBC density on fibrin film coverage. We used between 10% and 70% hematocrit to include the full pathophysiological range. Normal hematocrit in humans can vary (normal hematocrit: adult males 41%-50%, females 36%-44%, and infants 30%-42%). However, pathologically, hematocrit can range from 10% to 70%, with both the extremes of 10% and 70% obviously imposing a high mortality risk. Extremely high hematocrit between 65% and 70% can occur in patients with excessive polycythemia, while low hematocrit of around 10% can occur in patients with hematological cancers who refuse blood transfusion based on religious grounds. We have previously shown that fibrin films are involved in the retention of RBCs within the blood clot [7]. In this study, we found that clots made with high hematocrit support more fibrin film coverage compared with low hematocrit clots. Reductions in film formation due to lower hematocrit were comparable when the volume difference between hematocrits, due to reduced number of RBCs, was replaced with autologous plasma or saline. This indicates that the effect of hematocrit on film formation is independent of changes in platelet number and protein concentration and is likely due to alterations in RBC number. The cell membranes of RBCs may provide a surface that stimulates fibrin film formation. RBCs can expose phosphatidylserine on the surface of their membranes, and this can support thrombin generation and increase adhesive properties [23–26]. Fibrinogen has been shown to adsorb on phospholipid surfaces forming film-like structures [11]. Because fibrinogen forms film-like structures on phospholipid surfaces, it is possible that fibrin could do the same on RBC phosphatidylserine surfaces and other cells that expose phosphatidylserine, providing a potential mechanism for film formation in the vasculature. It is possible that other mechanisms beyond the phospholipid surfaces of RBCs impact film formation. RBCs may secrete factors that could trigger film

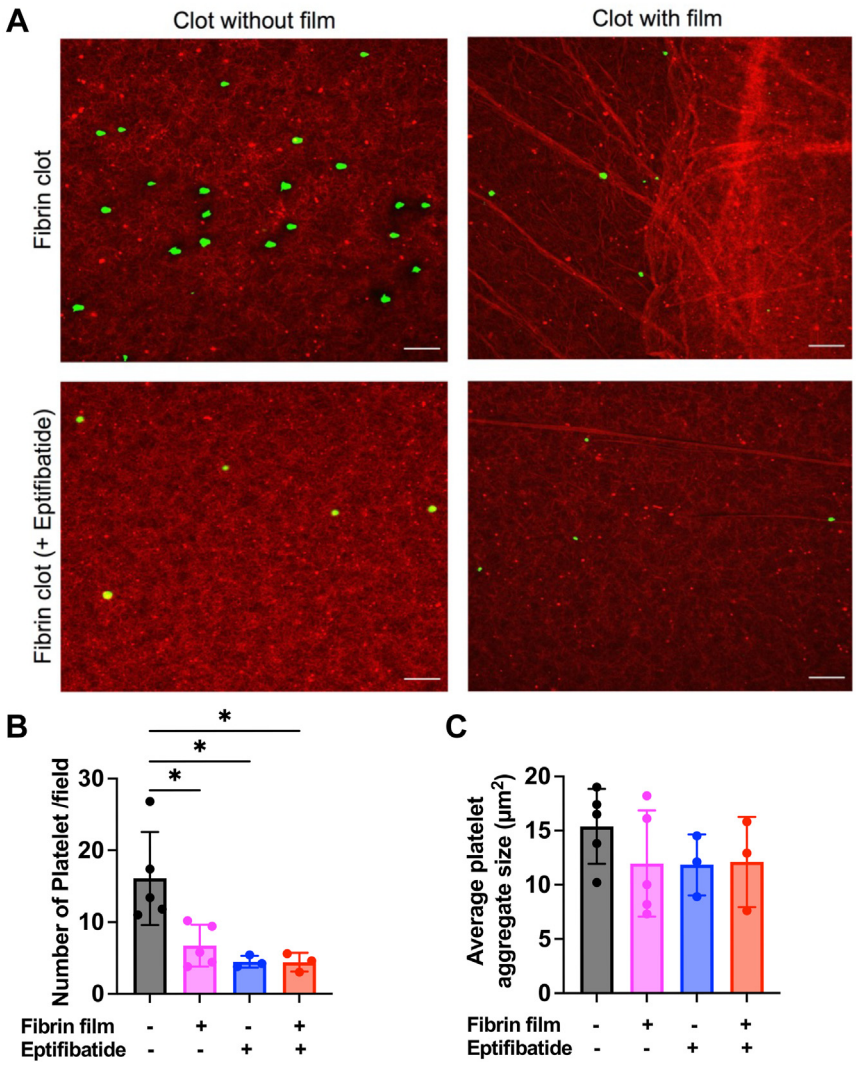

FIGURE 5 Platelet adhesion on purified fibrin clots without and with fibrin film, with or without eptifibatide. (A) Representative laser confocal microscopy images showing platelet adhesion on purified fibrin clot ± film, with or without eptifibatide. Fibrinogen was labeled with Alexa-594 (red), and platelets were labeled with phalloidin (green). The number of platelets per field (B) was significantly decreased in the presence of film, or in the presence of eptifibatide. The surface area per platelet (C) showed no significant change. Magnification 40× (scale bar: 20 μm). n = 3-5, analyzed by 1-way analysis of variance: $^*P < .05$.

formation, while RBCs also form pseudopodium-like projections that interact and fuse with fibrin fibers and fibrin strands (producing similar structures as the fibrin film) [27]. These mechanisms could provide alternative mechanisms that support the interactions between fibrin film and RBCs and need to be explored further in the future.

Platelets play an important role in blood clot growth, and there is increasing evidence of fibrin films forming on the surface of thrombi *in vivo* [4,8,10]. Thus, the effect(s) of fibrin films on platelet adhesion and spreading was investigated to understand the role of fibrin films in regulating thrombus growth. We found that fibrin films limited platelet adhesion at the surface of the clot, even when platelets were preactivated with thrombin or ADP. Integrin $\alpha_{IIb}\beta_3$ is the most abundant fibrinogen receptor on platelets, mediating their adhesion, spreading, and aggregation [28]. We established that the reduced binding ability of platelets on the surface of fibrin films is due to the lack of exposure of the RGD sequence located in the C-terminus of fibrin γ-chain, resulting in the absence of a binding partner for $\alpha_{IIb}\beta_3$. Platelets modify $\alpha_{IIb}\beta_3$ integrin outside-in signaling in response to fibrin(ogen) substrates [29,30]. Fibrin(ogen) orientation when adhered to a surface may play a role in platelet–fibrin(ogen) interaction via $\alpha_{IIb}\beta_3$. A lower density of fibrin(ogen) exposes more binding sites for $\alpha_{IIb}\beta_3$ due to a horizontal orientation on the surface, resulting in platelet activation, whereas high density fibrin(ogen) coating appears to vertically align fibrin(ogen) on the surface hindering the access to the $\alpha_{IIb}\beta_3$ binding site, indicating that fibrin(ogen) orientation is important for the exposure of the binding sites [29]. This agrees with our previous study showing that in the fibrin film, fibrin monomers adopt a perpendicular, or vertical, orientation to the clot surface [7]. This provides further evidence that the fibrin γ-chain RGD motif is obstructed by the molecular orientation of fibrin in the film, thereby blocking the ability of platelets to adhere. These findings suggest that fibrin film may contribute to minimizing thrombus growth by limiting platelet interactions. In a previous report, the percentage of fibrin film coverage was relatively low with an average of 15% on thrombi obtained from patients with myocardial infarction [8]. Lack of fibrin film may support platelet interactions, as shown here, which could

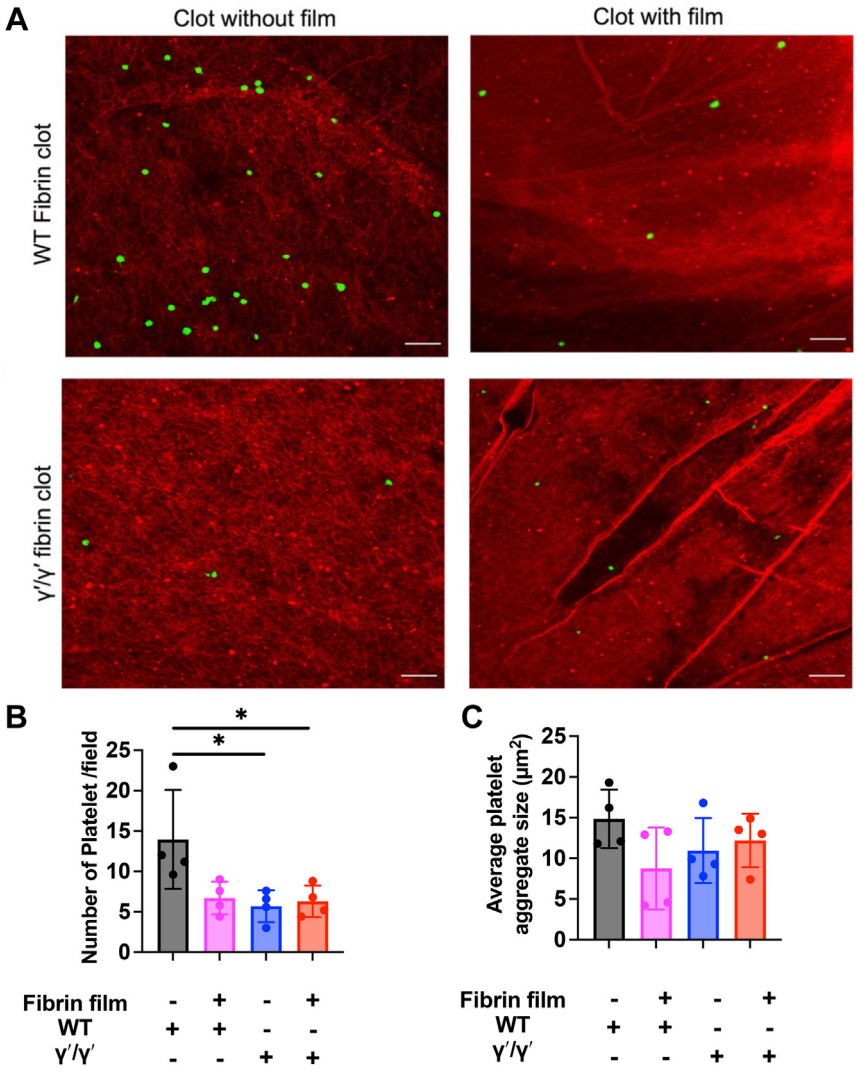

FIGURE 6 Platelet adhesion on fibrin clots formed with recombinant wild type (WT) and γ'/γ' fibrin. (A) Representative laser confocal microscopy images showing platelet adhesion on WT or γ'/γ' fibrin clots, with or without the film. Fibrinogen was labeled with Alexa-594 (red), and platelets were labeled with phalloidin (green). The number of platelet per field (B) was significantly decreased in the presence of film or in the presence of γ'/γ' fibrin. The surface area per platelet (C) showed no significant change. Magnification 40× (scale bar: 20 μm). $n = 4$, analyzed with 1-way analysis of variance: $^*P < .05$.

potentially contribute to continued thrombus growth and thrombosis in areas where the clot is not covered by fibrin film.

Our data also showed that fibrin films on the clot's surface contributed to delaying fibrinolysis. This may in part be due to delays in tPA diffusion into the clot. The relative thickness of the fibrin film could further impact the efficiency of clot breakdown and potentially thrombolysis. We have previously shown that fibrin film thickness is influenced by multiple factors, including thrombin, $CaCl_2$, and fibrinogen concentrations [7], and this needs to be explored further. Our data agree with previous work by Di Meglio et al. [10], showing that fibrin films on the outer surface of thrombi from patients with ischemic stroke delay clot breakdown. They went on to show that antifibrinolytic proteins protease nexin-1 and plasminogen activator inhibitor-1 accumulated in the film contributing to the delay in clot breakdown. Here, we found that plasminogen, but not tPA, also accumulates in fibrin films. This may be expected to accelerate fibrinolysis due to the abundance of plasminogen at the lysis front [31]. However, we found that the plasminogen captured within films may be shielded from tPA and/or plasminogen activator inhibitor-1 incorporated into the film may be inhibiting tPA, reducing PG. In addition, plasminogen distribution appeared to be reduced throughout the rest of the clot, further delaying PG and the lysis process. These data demonstrate a novel way in which clot structure impacts fibrinolysis and has potential importance for the efficiency of thrombolytic agents.

It appears that fibrin films may have opposing antithrombotic and antifibrinolytic mechanisms, making it difficult to understand its physiological and/or pathophysiological role. However, similar Janus-faced mechanisms occur elsewhere in coagulation; for example, TM and fibrinogen γ' that have both been associated with procoagulant and anticoagulant effects. We hypothesize that fibrin films form on the surface of intravascular clots following the drop in initial thrombin generation, going on to play a controlling role in reducing platelet binding at the clot surface, thereby slowing thrombus growth (Figure 8). In prothrombotic or inflammatory conditions, increased and prolonged thrombin generation hinders film formation, leading to amplified thrombin activity and platelet binding, promoting thrombus growth. We hypothesize that the link between film coverage and

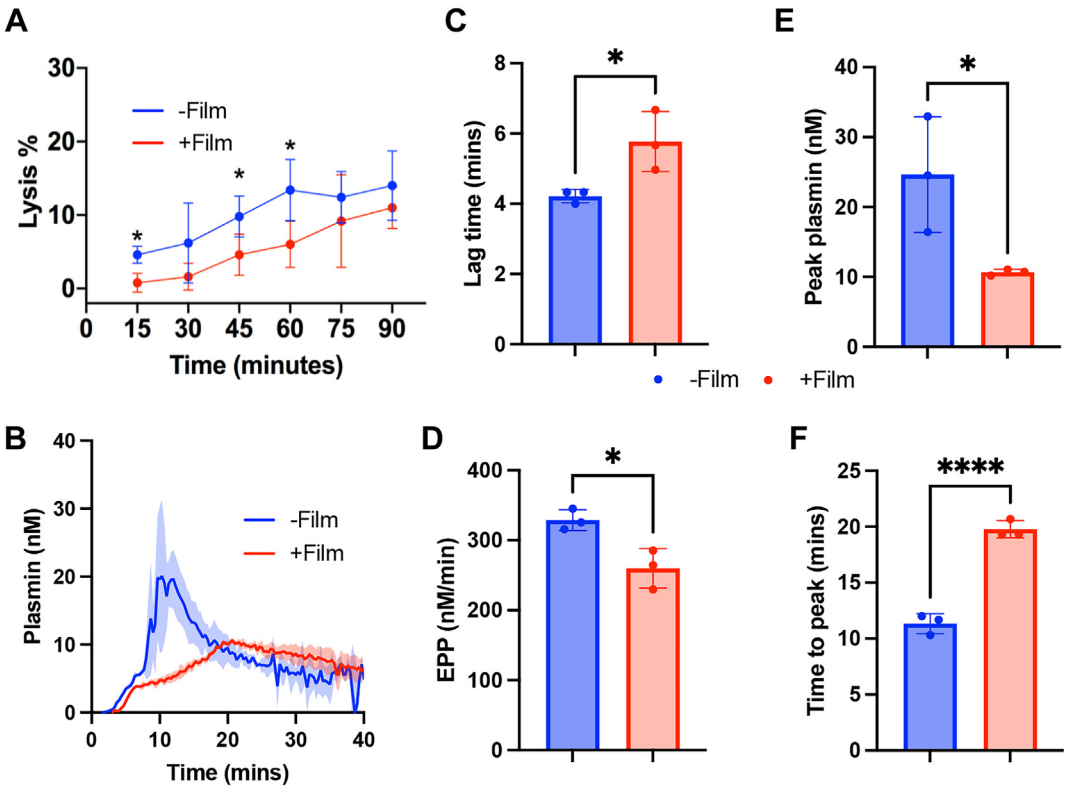

FIGURE 7 The effect of fibrin film on fibrinolysis. (A) The effect of fibrin film on fibrinolysis over time initiated with tissue-plasminogen activator (225 ng/mL) added to the top of preformed clots with or without fibrin films. Breakdown was quantified by weight of the remaining clot. Fibrinolysis was slowed down by the presence of fibrin films. $n = 5$, 2-way analysis of variance. $^{*}P < .05$. (B–F) Plasmin generation assays were carried out on the surface of preformed clots with and without fibrin films. Plasmin generation was reduced in the presence of fibrin films. (B) Plasmin generation traces on the surface of clots with or without films. (C) Plasmin generation lag time. (D) Endogenous plasmin potential (EPP). (E) Peak plasmin generation. (F) Time-to-peak plasmin generation. $^{*}P < .05$; $^{****}P < .0001$, $n = 3$, analyzed by unpaired t-test.

fibrinolysis is a separate mechanism that is only significant in pathological conditions driven by stasis as a thrombus grows. We have data showing positive correlation of fibrin film coverage on thrombi removed from ST-elevation myocardial infarction patients with increasing ischemia time (time from ambulance call to thrombus removal, under review). Therefore, we hypothesize that as a thrombus grows and occludes, increased stasis and disturbed flow promote late-stage fibrin film formation. This late-stage film formation occurs at a pivotal time, contributing to the thrombus's occlusive potential by impairing fibrinolysis and the effectiveness of potential thrombolytic treatments. Thus, fibrin films can play distinct roles during clot formation (limiting clot growth at the boundary), versus clot stability (reducing fibrinolysis and increasing clot stability).

In summary, our data show that fibrin films play a multifaceted role in thrombus growth and stability. Firstly, fibrin film limits platelet adhesion, thus potentially reducing thrombus growth in the early phases of thrombus formation. Reduced film formation under thromboinflammatory conditions may therefore support continued clot growth. Secondly, RBCs support fibrin film formation on the periphery of the blood clot, although the mechanism driving this remains to be determined. Thirdly, once the clot is formed, the fibrin film contributes to impaired external fibrinolysis, increasing clot stability.

ACKNOWLEDGEMENTS

We would like to thank Bas de Laat and Dana Huskens from the Synapse Research Institute for their contributions to the PG assay. We thank all the volunteers providing blood for the study. We are grateful to the Naseem laboratory (Leeds Institute of Cardiovascular and Metabolic Medicine) for washed platelet preparations and thank Martin Fuller (Faculty of Biological Sciences, University of Leeds) for SEM preparation. SEM was performed using the microscopes of the Leeds Electron Microscopy and Spectroscopy Centre (LEMAS).

ETHICS STATEMENT

The study protocol was approved by the Ethics Committee of the University of Leeds (reference number: HSLTLM12045). Written informed consent was received from each subject prior to inclusion in the study in accordance with the Declaration of Helsinki.

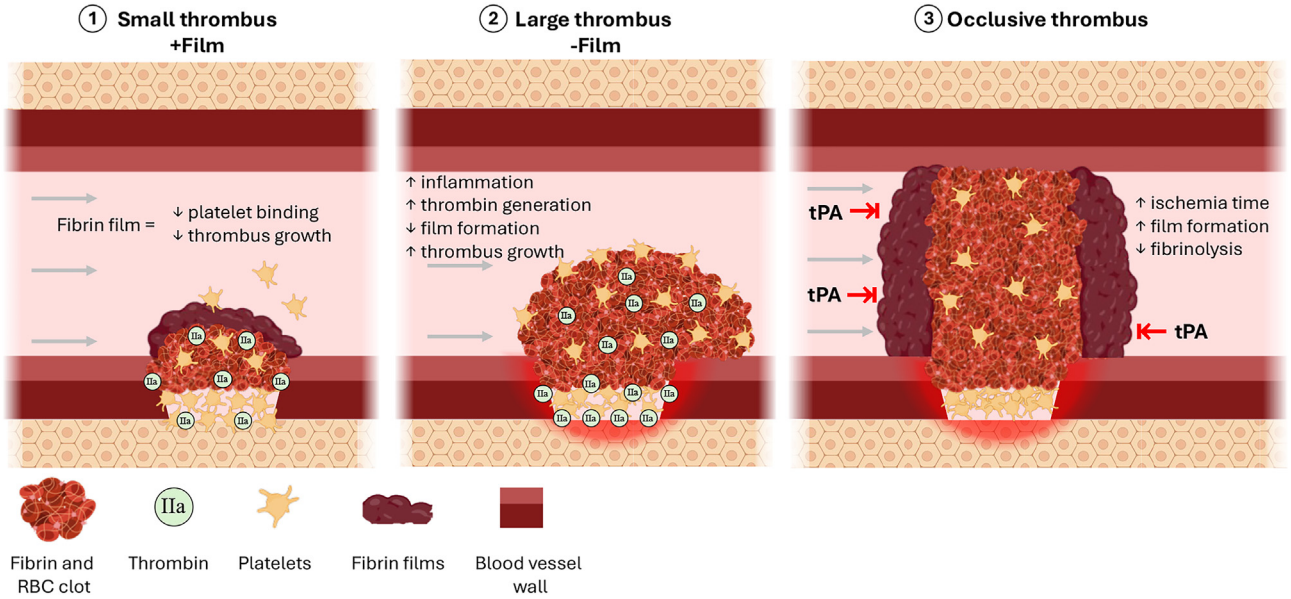

FIGURE 8 The role of fibrin films in thrombus formation and breakdown. (1) Fibrin films form on the clot surface following the drop in thrombin (IIa) generation, reducing platelet binding at the clot surface, therby reducing further thrombus growth. (2) Inflammation-driven increases in thrombin generation hinder film formation, resulting in increased thrombin activity and platelet binding. (3) As a thrombus grows and occludes, increased stasis and disturbed flow promote late-stage fibrin film formation, impairing fibrinolysis and the effectiveness of thrombolytic treatments through reduced plasmin generation. Created in BioRender. Alkarithi G (2025), https://biorender.com/q64j535. RBC, red blood cell; tPA, tissue-plasminogen activator.

AUTHOR CONTRIBUTIONS

G.A. wrote the first draft of the manuscript. G.A., H.R.M., L.S., C.D., and F.L.M. performed experiments. H.R.M. expressed recombinant fibrinogens and assisted in cell culture experiments. I.D.S. produced and provided key reagents. G.A., H.R.M., L.S., I.D.S., C.D., F.L.M., and R.A.S.A. contributed to discussion, interpretation of results, and the writing and revision of the manuscript. C.D., F.L.M., and R.A.S.A. conceived and supervised the project.

DECLARATION OF COMPETING INTERESTS

There are no competing interests to disclose.

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SUPPLEMENTARY MATERIAL

The online version contains supplementary material available at https://doi.org/10.1016/j.jtha.2024.12.023