Introduction

The coagulation system and innate immunity are coordinately activated and highly integrated during venous and arterial thrombus formation and progression (von Bruhl et al., 2012; Engelmann and Massberg, 2013; Fuchs et al., 2012). Vascular endothelial activation or damage causes release of ultralarge von Willebrand factor (VWF) and P-selectin from Weibel-Palade bodies, and local activation of complement with liberation of anaphylatoxic and chemotactic factors C3a and C5a. These pathways cooperate to trigger platelet, neutrophil, and monocyte recruitment and activation (von Bruhl et al., 2012). The locally accumulated cells release proteases, reactive oxygen species, and nucleosomes, which provide a scaffold for aggregating platelets and red blood cells and further promote coagulation and fibrin formation (Fuchs et al., 2012). Several complement factors, including C3, C4, C3a, C5a and factor H are incorporated into the thrombus, where they modulate thrombus stability and the inflammatory process (Distelmaier et al., 2009; Howes et al., 2012). The fibrinolytic system and plasmin-mediated proteolysis are also intimately coupled to the axis of thrombus development and inflammation by controlling fibrin degradation, activation of matrix metalloproteinases, infiltration of monocytes/macrophages and other immune mediators, vessel wall remodeling, and ultimately thrombus resolution (Wakefield et al., 2008).

The mechanisms by which leukocytes are recruited early in thrombus formation and later during thrombus extension or resolution, are poorly understood. However, C5a, the most potent chemotactic complement activation fragment, is released following proteolytic cleavage of C5 and is considered a critical determinant of neutrophil recruitment and activation in thrombosis (Distelmaier et al., 2009; Salmon et al., 2002; Pierangeli et al., 2005). Moreover, terminal complement pathway complexes formed as C5 is activated, have multiple procoagulant properties (Langer et al., 2013; Hamilton et al., 1990). Thus, there is interest in understanding how C5a and the other major complement-derived
chemotactic factor, C3a, are generated, so that novel therapeu-
tic strategies may be designed to prevent thrombosis.

Complement activation typically proceeds via three pathways —
classical, lectin and alternative — which converge to form C3 convertases
that proteolyse C3 into C3b with release of C3a (Ricklin et al., 2010). As
complement activation exceeds a threshold, and the density of C3b
increases, the specificity of the convertase shifts from C3 to C5. The
resultant C5 convertases — C3bBbC3b for the alternative pathway and
C4b2aC3b for the classical/lectin pathway — efficiently cleave C5 at argi-
nine 751 (R751), liberating C5a and generating C5b, the initiating factor
for assembly of the lytic C5b–9 membrane attack complex (MAC).
Although the C3/C5 convertases are well-recognized for their capacity
to cleave C3 and C5, other serine proteases reportedly also exhibit
convertase activity (Huber-Lang et al., 2006; Amara et al., 2010; Wiggins
et al., 1981). Notably, thrombin was implicated in providing a “new
pathway” to activate complement by cleaving C5 in a C3-independent
manner; thereby bypassing the bona fide C5 convertases (Huber-Lang et
al., 2006). However, C5 is a relatively poor substrate for thrombin
cleavage at R751 (Krisinger et al., 2012), raising questions as to its im-
tance in contributing to C5a generation during thrombus formation
in vivo. We therefore explored the mechanisms by which C3a and C5a
are generated using biochemical approaches and in vivo models of
venous and arterial thrombosis.

2. Materials and Methods

2.1. Materials

Human complement C3a and C5a were measured using Quidel MicroVue C3a Plus or C5a ELISA kits (Cedarlane Laboratories, Burlington,
Ontario). Murine thrombin–antithrombin (TAT) levels were measured
using Enzygnost TAT micro ELISA (Siemens, Munich, Germany).
Human complement proteins C3 and C5 were obtained from Complement
Technology, Inc. (Tyler, TX) and human hemostatic enzymes
(plasmin, factor Xa and thrombin) were from Haematologic Technolo-
gies, Inc. (Essex Junction, VT).

2.2. ELISAs to Measure C3a and C5a

Murine complement C5a levels were determined using the mouse
complement component C5a duoset and accompanying standard from R&D systems (catalog #DY2150; Minneapolis, MN). An ELISA for
murine complement C3a was established using a murine C3a standard
and antibodies from BD Biosciences (Mississauga, Canada). The capture
rat monoclonal anti-mouse C3a antibody (catalog # 55820, clone: 187-
1162) was coated overnight onto 96-well plates in 100 μL of PBS at
a concentration of 2 μg/mL. Wells were washed ×3 with wash buffer
(R&D catalog #WA126) followed by blocking for 2 h with 300 μL of
reagent diluent (R&D systems catalog #DY995). Plasma samples in
duplicate were diluted 1/50 and 1/125 in sample diluent to a final
volume of 100 μL and incubated for 2 h. After 3 washes, 100 μL of the
biotinylated detection monoclonal rat anti-mouse C3a antibody (clone
187-419, catalog #55821) 0.5 μg/mL in reagent diluent was incubated for
1 h. Wells were washed ×4 and incubated for 20 min at room
temperature with 100 μL of streptavidin-biotinylated horseradish per-
oxidase (1:3000), followed by 2 washes, and development with the
substrate solution containing o-phenylenediamine using a plate reader
set to 450 nm. A standard curve was generated with purified murine
C3a (catalog # 558618). The sensitivity range of the assay was 0.1 nM
to 2.5 nM. Intra-assay and inter-assay precision was 8–10%.

2.3. Animal Models

All experiments with animals were approved by the University of
North Carolina at Chapel Hill Institutional Animal Care and Use Commit-
tees. The mice (C57Bl/6) were male and between 6 and 8 weeks of age.

The number of animals for each model was determined based on previ-
ous work that showed a broad range of TAT levels in the respective
models (Machlus et al., 2011a, 2011b). On each day of the experiments,
animals were randomly assigned to receive the stated treatment or to
be used for baseline measurements. Quantification of biomarkers was
performed in a blinded fashion wherein an experimenter, different
from the one who performed the procedures on the animals, carried
out the ELISAs on coded samples that were only de-coded after results
had been generated.

2.4. Murine model of Venous Thrombosis

The inferior vena cava (IVC) stasis model was performed as previ-
ously described (Aleman et al., 2013). Briefly, mice were anesthetized
with 1–5% isoflurane in oxygen and human prothrombin (to 300%,
final, mouse plus human prothrombin) or vehicle was infused via tail
vein injection. Prothrombin was infused to give a broader range of
thrombin generation and clot weight. Following sterile laparotomy,
the intestines were exteriorized, the IVC was dissected bluntly, and
side branches were ligated with 8–0 prolene suture and lumbar branches
closed by cautery. The IVC was separated from the aorta by blunt
dissection and completely ligated with 8–0 prolene suture. After
replacing the intestines, the muscle layer was closed with 5–0 vicryl
suture and skin closed with 8–0 prolene suture and skin glue. Mice re-
covered with analgesia (buprenorphine, 0·05 mg/kg subcutaneous).
After 12 h, blood was drawn from the IVC above the ligation site into
3.2% sodium citrate and processed to platelet-poor plasma by centrifu-
gation at 5000 × g for 10 min. Thrombi were collected and weighed.
Plasmas were stored at −80 °C for analysis of TAT, C3a and C5a levels
by a person that was blinded to the treatment group. Two samples
showing hemolysis were excluded.

2.5. In Vitro Generation of C3a or C5a by Hemostatic Enzymes

The relative efficiency, time course, and rate of complement cleavage
by plasmin, factor Xa or thrombin were determined using a series of
assays. Briefly, complement C3 (20 μM) or C5 (2 μM) was incubated
with 100 nM plasmin or 250 nM factor Xa or thrombin. Reactions
were quenched at various time points and C3a or C5a levels were quan-
tified by ELISA. To determine the relative efficiency of cleavage, 2 μM
of C5 was incubated with 100 nM of plasmin, factor Xa or thrombin at
37 °C. After 10 min the reaction was quenched with the appropriate
chloromethylketone (Val-Phe-Lys-chloromethylketone (VFKck) for
plasmin and Phe-Pro-Arg-chloromethylketone (PPACK) for factor Xa
and thrombin). In similar experiments, aliquots of the reaction mixture
were sub-sampled into chloromethylketones at various time points to
determine the time courses of C3 and C5 cleavages by plasmin, factor
Xa and thrombin. The kinetics of C5a generation were determined by
incubating 0–3 μM of C5 with 100 nM of plasmin at 37 °C. Reactions
were quenched after 1 min and C5a levels were quantified by ELISA.
Similar experiments substituting factor Xa or thrombin for plasmin
were conducted, but the amount of C5a generated in these assays over
30 min (for factor Xa) or 1 h (for thrombin) was below the limit of
detection for the assay.

C5a generation occurring during clot formation and degradation
was assessed in vitro by incubating physiological concentrations of
fibrinogen (9 μM), plasminogen (2 μM), antiplasmin (1 μM) and
C5 (400 nM) at 37 °C. High (1 μM) or low (10 nM) concentra-
tions of thrombin were added to induce clot formation and 10 nM of
tPA was used to induce plasminogen activation and clot lysis. The
IPA concentration chosen resulted in complete clot lysis in 30 min.
At the end of the 30-minute incubation period, enzymes were
quenched with PPAck/VFKck, the sample centrifuged and supernatant or
sample stored at −80 °C for quantification of C5a levels. Background
C5a signal when PPAck/VFKck was added at t = 0 were subtracted.
2.6. In Vitro Generation of C5b,6 by Hemostatic Enzymes

Complement factors C5 (400 nM) and C6 (500 nM) were incubated with 100 nM of plasmin or 250 nM of factor Xa or thrombin. Parallel reactions were quenched over time with an appropriate chloromethylketone. The amount of functional C5b,6 generated was subsequently quantified using a chicken erythrocyte hemolytic assay (Wat et al., 2014). Since plasmin readily cleaves C5, we also incubated 500 nM of C5b,6 with 100 nM of plasmin to determine if C5b,6 activity diminishes over time. In these experiments, plasmin was quenched with VFKck.

2.7. Ferric Chloride Model of Arterial Thrombosis

Mice were anesthetized with 1.5–2% isoflurane in oxygen. Ferric chloride injury to the carotid artery was performed as described (Aleman et al., 2013). Briefly, the right common carotid artery was exposed, dried, and treated with ferric chloride (7.5% or 10% on 0.5 × 1.0-mm filter paper) for 2 min. The artery was washed with warm saline and blood flow was continuously monitored by Doppler ultrasonic flow probe (Indus Instruments). The time to vessel occlusion was defined as the time between FeCl3 administration and lack of flow for 60 s. Blood was sampled into citrate from the IVC 5 min after stable vessel occlusion (defined as continuous occlusion for 1 min) or after 40 min if no occlusion occurred.

2.8. Thrombolysis Model

Thrombolysis was assessed in mice subjected to FeCl3 carotid artery thrombosis. After 5 consecutive minutes of vessel occlusion, mice were infused with Tenecteplase (5 ng/kg, generous gift of Genentech, CA) through a saphenous vein intravenous catheter constructed of pulled PE-10 tubing (Braintree Scientific, Braintree, MA) with a 3.0-mil (0.076-mm diameter) cleaning wire (Hamilton Company, Reno NV) placed into the lumen as a stylet, as described (Machlus et al., 2011a), while continuously monitoring carotid blood flow. Blood was sampled from the IVC 5 min after the return of blood flow or 30 min after Tenecteplase infusion if the clot did not lyse.

2.9. Statistics

The relationships between prothrombotic and complement activation markers were assessed using Spearman rank correlation. C3a and C5a levels were compared using t-tests or the Wilcoxon rank sum test (Tenecteplase-treated versus untreated mice). P < 0.05 was considered statistically significant.

3. Results

We studied the role of thrombin in complement activation using an in vivo murine model of ligation (stasis)-induced IVC thrombosis (von Bruhl et al., 2012; Aleman et al., 2013). Plasma levels of activation markers of coagulation and complement were measured by ELISA. Baseline TAT levels were 3.8 ± 4.0 ng/mL (n = 5), as previously reported (Aleman et al., 2013). 24 h after IVC ligation, plasma TAT levels rose to 21.2 ± 11 ng/mL (mean ± SD, n = 6). Prothrombin was infused in some mice to give a broader range of thrombin generation and clot weight (Aleman et al., 2013) (Fig. 1 – open circles – mice infused with prothrombin; solid circles – mice infused with vehicle). When prothrombin was infused just prior to IVC ligation (Aleman et al., 2013), TAT levels measured at 24 h were significantly higher (47.9 ± 19 ng/mL, n = 8, p < 0.009). As expected, clot weights directly correlated with TAT levels (r = 0.66, p < 0.01). Plasma levels of C5a and C3a in venous thrombosis were elevated as compared to baseline (unchallenged) levels (C5a = 0.43 ± 0.15 nM; C3a < 0.1 nM; n = 6). Interestingly, circulating levels of complement activation markers C3a and C5a correlated poorly with TAT levels (Fig. 1a, b), suggesting that thrombin does not directly activate complement in this experimental model of venous thrombosis. Notably, C5a also correlated poorly with C3a (Fig. 1c), suggesting that C5a was generated to a large extent via C3/C5 convertase-independent pathways. Furthermore, there was no relationship between clot weight and C3a levels (Fig. 1d). There was, however, a strong direct correlation between clot weight and C5a levels (Fig. 1e). Taken together, these findings show that processes triggered during venous thrombosis are associated with C5a generation, but suggest that thrombin is not the major activator of C5 under these conditions.

To determine the potential mechanisms of C5a generation in vivo, we used in vitro assays in purified systems to compare C5a generation following cleavage of C5 by thrombin, factor Xa or plasmin. Plasmin was considered a likely candidate of complement activation in the setting of a fibrin clot because, 1) plasmin is known to cleave C5 to yield chemotactically-active C5a in vitro (Amaro et al., 2010), and 2) fibrin is an essential cofactor for tissue-type plasminogen activator (tPA)-mediated plasmin generation (Horrillo et al., 1997).

Incubation of C3 or C5 with thrombin, factor Xa or plasmin revealed that plasmin is much more effective than thrombin or factor Xa in cleaving C3 and C5 to generate C3a and C5a, respectively (Fig. 2a, b). Plasmin more readily cleaved C5 than C3, with ~30% of C5 (~700 nM) being converted to C5a, and only ~2% of C3 (~450 nM) cleaved to form intact C3a. The low turnover of C3 by plasmin, thrombin and FXa precluded further in vitro interrogation. The incomplete cleavage of C5 to C5a under the conditions employed may reflect competitive inhibition by an abundance of cleavage products (molecular weight >30–70 kDa) that were detected following SDS-PAGE (not shown and (Barthel et al., 2012)). We compared the efficiency of C5 cleavage by various concentrations (0–100 nM) of each of the three enzymes (Fig. 2c). During a 10-minute incubation period, plasmin generated substantially more C5a than factor Xa or thrombin.

In kinetic assays, the rate of C5a generation by plasmin increased linearly as the concentration of C5 increased (Fig. 2d). The catalytic efficiency, inferred from the slope of the plot, was 2.3 ± 0.6 × 10^4 M^−1 s^−1 (Distelmaier et al., 2009). This is similar to the published rate of C5 cleavage by the bona fide alternative pathway C5 convertase and the soluble monomeric classical/lectin pathway C3/C5 convertase (Rawal and Pangburn, 1998, 2001). This rate of C5 cleavage by plasmin is therefore consistent with the premise that plasmin has a physiologically relevant role in generation of C5a.

Since plasmin is rarely, if ever, free in circulation, we next tested whether plasmin is capable of generating C5a in the presence of physiological concentrations of antiplasmin and fibrinogen, that when converted to fibrin, binds plasminogen and plasmin with high affinity. We first confirmed that even at very high concentrations, thrombin generates almost undetectable amounts of C5a (Fig. 2e, condition i). In the absence of plasminogen, tPA was incapable of cleaving C5 to yield C5a (Fig. 2e, condition ii). When C5 was incubated with tPA and plasminogen in the presence of fibrinogen (Fig. 2e, condition v), readily detectable amounts of C5a were generated, and this occurred even in the presence of physiological concentrations of antiplasmin (condition iv). Absence of fibrinogen, a cofactor for tPA-mediated conversion of plasminogen to plasmin, resulted in the generation of measurable, but less, C5a than with fibrinogen (condition v versus vi). Overall, the data confirm that in the presence of physiological concentrations of hemostatic proteins, C5a can be generated in a plasmin-dependent manner.

Conversionase-mediated release of C5a from C5 occurs in parallel with generation of C5b6 that is required for MAC formation. Using a terminal pathway hemolytic assay (Wat et al., 2014), we showed that in the presence of excess C6, cleavage of C5 by factor Xa or plasmin yielded a C5b6 complex that could assemble with C7, C8 and C9 to form a fully functional lytic MAC. The efficiency of C5b6 generation by plasmin, factor Xa and thrombin mirrored that observed for C5a (Fig. 3a). Over 60 min, 100 nM of plasmin generated 3·0 ± 0.6 nM C5b6, whereas even 250 nM of factor Xa (which is ~2-fold higher than the plasma
concentration of factor X) generated only 1·1 ± 0·7 nM of C5b,6. Consistent with its inefficient cleavage of C5, thrombin (250 nM) did not generate any detectable C5b,6. Thus, although plasmin can cleave C5 at several sites (Barthel et al., 2012), exposure of C5b,6 to plasmin for 1 h did not appreciably decrease the functionality of the MAC (Fig. 3b). In view of these in vitro findings, we tested the association between thrombin generation and complement activation in a second, independent thrombosis/thrombolysis model, and used this model to determine whether C5 is activated by thrombolytic pathways in vivo. Using wild-type mice with stable carotid artery thrombi induced by ferric chloride, we first showed that 5 min after occlusion, TAT levels correlated poorly with systemic levels of C3a and C5a (r = −0·36, p = 0·39 for C3a; r = −0·05, p = 0·91 for C5a; n = 8), consistent with our observations in the venous thrombosis model. To determine the impact of plasminogen activation in this setting, we intravenously administered the tPA analog Tenecteplase (n = 8) into mice that had been challenged with arterial occlusion and measured C3a and C5a 5 min after restoration of blood flow, or 30 min after infusion if blood flow was not restored (Fig. 4a, b). Of these, 3 mice did not re-perfuse and 5 mice did re-perfuse. Regardless of outcome, all mice that received Tenecteplase were included in the analysis. As compared to ferric chloride-challenged mice that were not infused with Tenecteplase (n = 9), Tenecteplase caused a significant (~2-fold) increase in C3a and C5a levels. Notably, this increase is in line with published studies in a small group of patients treated with recombinant tPA following acute myocardial infarction (Bennett et al., 1987). Our finding that C5 could not be cleaved by recombinant tPA (Fig. 2d, iii) makes it highly unlikely that the elevated C5a levels were attributable to Tenecteplase-mediated

Fig. 1. Coagulation and complement activation in venous thrombosis. Following stasis-induced thrombosis of the inferior vena cava (see Materials and Methods section), levels of thrombin-antithrombin complexes (TAT), C3a, and C5a were measured by ELISA. C3a (a) and C5a (b) levels correlated poorly with TAT levels. C3a and C5a levels also poorly correlated (c), implying the existence of C3-independent pathways to generate C5a. Clot weight poorly correlated with C3a (d), but strongly correlated with C5a (e). Each dot represents a separate mouse, n = 14. Solid circles are untreated mice (infused with vehicle); open circles are mice that were infused with prothrombin to increase thrombin generation (see Materials and Methods section). Correlation coefficients (r) with 95% confidence levels and p-values are indicated on each panel.

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generation of C5. We further excluded this possibility by showing in vitro that exposure of C5 to high concentrations of tPA (up to 200 nM) for 10 and 30 min, did not yield measurable amounts of C5a (data not shown). Taken together, these data are consistent with a direct effect of plasmin on complement activation during thrombosis.

4. Discussion

Thrombus formation leading to pathological vaso-occlusive events (e.g., acute coronary syndrome, stroke, deep vein thrombosis and pulmonary embolus) is a major cause of death worldwide (Mozaffarian et al., 2015; Raskob et al., 2014). Initiation, propagation, and resolution of a thrombus rely on the recruitment of platelets and inflammatory cells, and this is mediated partly by local release of the complement activation factor C5a. C5a is a potent anaphylatoxic peptide, inducing a range of pro-inflammatory and pro-thrombotic effects via its cognate G-protein-coupled receptors, C5aR and C5L2. C5a activates platelets, leukocytes and endothelial cells, upregulates expression of adhesion molecules, induces secretion of pro-inflammatory and procoagulant cytokines, promotes tissue factor expression by neutrophils and release of tissue factor-containing microparticles, induces the formation of neutrophil extracellular traps (NETs), and amplifies complement

Fig. 2. Hemostatic enzymes generate C5a. a, b, c. In a purified system, plasmin (100 nM) (▴), factor Xa (250 nM) (●) or thrombin (250 nM) (■) was incubated with C3 or C5 for varying periods of time and C3a (a) or C5a (b) was measured by ELISA. Plasmin efficiently generated C3a (a) (inset is higher magnification to show relative initial rates of C3a generation) and C5a (b) within 1 min, whereas factor Xa or thrombin took much longer to generate appreciable amounts of C3a or C5a. c. C5 (2 μM) was incubated for 10 min with varying concentrations of each of the three enzymes, after which C5a levels were measured by ELISA. Plasmin was significantly more efficient at cleaving C5 than factor Xa or thrombin. d. The rate of C5a generation by plasmin increased linearly as the concentration of C5 increased. The slope of the line implies that plasmin cleaves C5, generating C5a with a catalytic efficiency of 2.3 ± 0.6 × 10^4 M⁻¹ s⁻¹ (Distelmaier et al., 2009). e. Thrombin (10 nM, 1 μM; i, ii, respectively) or tPA (10 nM) (iii) did not generate significant levels of C5a from C5. Addition of plasminogen (2 μM) to the system enabled plasmin generation and was associated with C5a generation in the absence (iv) or presence (v) of 1 μM of antiplasmin. Fibrinogen (9 μM) (vi) is not essential for C5a generation when tPA, plasminogen and C5 are present, but it enhances C5a generation in the context of tPA induced plasminogen activation (v). The data presented in a, b are a single representative experiment and the data for all other experiments (c–e) represent means ± SD for 3 replicates. n.d. = not detectable.
Fig. 3. Hemostatic enzymes generate C5b-6. a. Cleavage of C5 by plasmin (▴), factor Xa (●), or thrombin (■) in the presence of excess C6, C7, C8 and C9, resulted in the generation of functional C5b-6, measured by a terminal pathway erythrocyte hemolytic assay. Thrombin did not generate any measurable C5b-6 in this experimental system. b. The functional integrity of C5b-6, measured by the terminal pathway hemolytic assay, did not decay appreciably over a 1-hour period when incubated with plasmin. The data presented represent means ± SD for 3 replicates.

Fig. 4. Effects of plasminogen activator-mediated thrombolysis on C3a and C5a levels. a, b. Stable carotid artery thrombosis was induced in wild-type mice using the ferric chloride model (see Materials and Methods section), after which the plasminogen activator Tenecteplase was administered intravenously as noted. C3a (a) and C5a (b) levels were significantly increased by Tenecteplase infusion. Each dot represents a separate mouse (n = 9 controls, n = 8 infused with Tenecteplase).

Fig. 5. Proposed contributions of plasmin-mediated C5 activation in thrombosis. During a. thrombus formation, progression, and b. resolution, fibrin deposition promotes plasmin generation, which activates complement. The traditional complement activation pathways likely also participate (not shown). The effects of plasmin-mediated complement activation on thrombus growth and resolution depend on the timing and localization of C5a generation, and assembly of the membrane attack complex (MAC). a. Thrombus formation: Following endothelial cell activation/damage, C5 (and C3) is activated, generating C5a and C5b (1), the latter of which binds to C6-C9 to form the membrane-damaging, procoagulant MAC. C5a is a potent chemoattractant for platelets and neutrophils (2) and activates cells to express monocyte chemoattractant protein-1 (MCP-1) and interleukin (IL)-8 (3). These pathways cooperate to recruit and activate platelets, monocytes and neutrophils (4), with release of reactive oxygen species, proteases and nucleosomes, all of which enhance thrombus formation (5). b. Thrombus resolution: As plasmin degrades fibrin into fibrin degradation products (FDPs) (6), it also generates C5a and C5b (1). C5a induces the release of factors (e.g. MCP-1, IL-8) (3) that recruit macrophages and neutrophils (7), which promote clot resolution by augmenting plasmin generation (8), fibrin degradation, and phagocytic clearance of clot-associated debris (9).
activation through positive feedback loops (Oikonomopoulou et al., 2012). Given the current findings, we propose a model in which plasmin, via liberation of C5a, contributes to leukocyte trafficking during thrombus formation, propagation and/or resolution (Fig. 5). The precise local contribution of C5a (and C3a) to thrombus formation and resolution, is difficult to ascertain, particularly since clearance of these peptides is short and likely dynamically changes in this setting. Nonetheless, with generation of C5a, terminal complement pathway complexes form which also regulate coagulation. C5b-7 induces tissue factor expression by monocytes (Langer et al., 2013), while the MAC induces WVF and P-selectin secretion, platelet microparticle release, and endothelial cell and platelet membrane changes that favor prothrombinase assembly and thrombin generation (Hamilton et al., 1990; Wiedmer et al., 1986; Sims et al., 1988). Since C5 activation is associated with many disease states, including acute lung injury, arthritis, sepsis (Huber-Lang et al., 2006; Kessel et al., 2014; Yan and Gao, 2012), and thrombosis (Distelmaier et al., 2009; Cheung et al., 1994), the present studies suggest that interventions at the level of plasmin may have broad clinical utility.

From studies with mouse models (Huber-Lang et al., 2006; Hoth et al., 2014; Khan et al., 2013; Auger et al., 2012; Zecher et al., 2014; Borkowska et al., 2014), several groups have concluded that thrombin is the major coagulation enzyme that generates C5a under pathologic conditions. This role for thrombin was supported by observations that thrombin generates C5 in vitro (Huber-Lang et al., 2006), and inhibition of thrombin dampens severity of disease and reduces C5a levels in murine models of disease (Huber-Lang et al., 2006; Hoth et al., 2014; Khan et al., 2013; Auger et al., 2012; Zecher et al., 2014; Borkowska et al., 2014). How do we reconcile these findings with the fact that the residues flanking the R751 C5 convertase cleavage site necessary to generate C5a lack similarity to thrombin cleavage sites in all other classic thrombin substrates (e.g., protein C, PAR1, fibrinogen, factor V, factor VIII) (Krisinger et al., 2012), and that thrombin is an inefficient cutter of C5 at that site (Krisinger et al., 2012)?

That thrombin participates in C5a generation during coagulation is not challenged by the present findings. However, this reaction likely does not occur via direct C5 cleavage. Indeed, in our experiments, with thrombin concentrations that more closely approximate the dynamics of thrombin generation in plasma and blood (Brummel et al., 2013; Dielis et al., 2008), C5a could not be measured. Moreover, the C5T of thrombin generation in plasma and blood (Brummel et al., 2002; Hamilton et al., 1990; Wiedmer et al., 1986; Sims et al., 1988) since C5 activation is highly efficacious in preventing complement-mediated thrombosis in atypical hemolytic uremic syndrome and paroxysmal nocturnal hemoglobinuria (Wong and Kavanagh, 2015). Similar successes were not, however, observed for acute myocardial infarction (APEX AMI Investigators et al., 2007), which might be due to the fact that therapy was initiated early at presentation. The variable responses in these reports underline the need for further study, using models that represent different vascular disorders.

Contributors
JHF, ASW and EMC designed experiments, analyzed data and wrote the manuscript and managed the project. JHF, MMA, BLW, AMO, VL and MH performed experiments. KAF analyzed data and edited the manuscript.

Declaration of Interests
We declare no competing interests.

Ethical Research Conduct
All aspects of the work covered in this manuscript were conducted with the ethical approval of all relevant bodies and these are acknowledged within the manuscript.
Role of the Funding Sources

None of the funders had any input into the design of the experiments, the collection, analysis or interpretation of the data, the writing of the manuscript. or the decision to submit it for publication.

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References

Alemán, M.M., Walton, B.L., Byrne, J.R., et al., 2013. Elevated prothrombin promotes venous, but not arterial, thrombosis in mice. Arterioscler. Thromb. Vasc. Biol. 33 (8), 1829–1836.

Amara, U., Flierl, M.A., Rittirsch, D., et al., 2010. Molecular intercommunication between the complement and coagulation systems. J. Immunol. 185 (9), 5628–5636.

APECXI Immunologists, Armstrong, W.P., Granger, C.B., et al., 2007. Pexelizumab for acute ST-elevation myocardial infarction in patients undergoing primary percutaneous coronary intervention: a randomized controlled trial. JAMA 297 (1), 43–51.

Augier, J.L., Haaksman, S., Binstadt, B.A., 2012. Autoantibody-mediated arthritis in the absence of C3 and activating Fcgamma receptors: C5 is activated by the coagulation cascade. Arthritis Res. Ther. 14 (6), R269.

Barthel, D., Schindler, S., Zipfel, P.F., 2012. Plasminogen is a complement inhibitor. J. Biol. Chem. 287 (22), 18331–18342.

Bennett, W.R., Yawn, D.H., Migliore, P.J., et al., 1987. Activation of the complement system by recombinant tissue plasminogen activator. J. Am. Coll. Cardiol. 10 (3), 627–632.

Borkowska, S., Suszyńska, M., Miętjewiecka, K., et al., 2014. Novel evidence that crossstalk between the complement, coagulation, and fibrinolysis proteolytic cascades is involved in mobilization of hematopoietic stem/progenitor cells (HSPCs). Leukemia.

Brummel, K.E., Paradis, S.G., Butenas, S., Mann, K.G., 2002. Thrombin functions during tissue factor-induced blood coagulation. Blood 100 (1), 148–152.

Brummel-Ziedins, K.E., Vossen, C.V., Butenas, S., Mann, K.G., Rosendaal, F.R., 2005. Thrombin generation profiles in deep venous thrombosis. J. Thromb. Haemost. 3 (11), 2497–2505.

Cheung, A.K., Faesi-Jenkin, B., Leyoldt, J.K., 1994. Effect of thrombosis on complement activation and neutrophil degranulation during in vitro hemolysis. J. Am. Soc. Nephrol. 5 (1), 110–115.

Dielis, A.W., Castoldi, E., Spronk, H.M., et al., 2008. Coagulation factors and the protein C pathway in sepsis. J. Thromb. Haemost. 3 (11), 3510–3517.

Engelmann, B., Massberg, S., 2013. Thrombosis as an intravascular effector of innate immunity. Nat. Rev. Immunol. 13 (1), 34–45.

Fuchs, T.A., Brilli, A., Wagner, D.D., 2012. Neutrophil extracellular trap (NET) impact on deep vein thrombosis. Arterioscler. Thromb. Vasc. Biol. 32 (8), 1777–1783.

Hamilton, K.K., Hattori, R., Esmon, C.T., Sims, P.J., 1990. Complement protein C5b-9 induces vesiculation of the endothelial plasma membrane and expose catalytic surface for assembly of the prothrombinase enzyme complex. J. Biol. Chem. 265 (7), 3809–3814.

Horrevoets, A.J., Pannekoek, H., Neshim, M.E., 1997. A steady-state template model that describes the kinetics of fibrin-stimulated [Glu1]- and [Lys78]plasminogen activation by native tissue-type plasminogen activator and variants that lack either the finger or kringle-2 domain. J. Biol. Chem. 272 (4), 2183–2193.

Hoth, J.J., Wells, J.D., Jones, S.E., Yozu, B.K., McCall, C.E., 2014. Complement mediates a primed inflammatory response after traumatic lung injury. J. Trauma Acute Care Surg. 76 (3), 601–608 (discussion 8-9).

Khan, M.A., Maasch, C., Vater, A., et al., 2013. Targeting complement component 5a promotes vascular integrity and limits airway remodeling. Proc. Natl. Acad. Sci. U. S. A. 110 (15), 6061–6066.

Kislinger, T., Goebeler, V., Lu, Z., et al., 2012. Thrombin generates previously unidentified C5 products that support the terminal complement activation pathway. Blood 120 (8), 1717–1725.

Kyrle, P.A., Mannhalter, C., Begen, S., et al., 1998. Clinical studies and thrombin generation in patients homozygous or heterozygous for the G20210A mutation in the prothrombin gene. Arterioscler. Thromb. Vasc. Biol. 18 (8), 1287–1291.

Langer, F., Spath, B., Fischer, C., et al., 2013. Rapid activation of monocyte tissue factor by antifibrinogen globulin is dependent on complement and protein disulfide isomerase. Blood 121 (12), 2324–2333.

Machlus, K.R., Cardenas, J.C., Church, F.C., Wolberg, A.S., 2011a. Causal relationship between hyperfibrinogenemia, thrombosis, and resistance to thrombolysis in mice. Blood 117 (18), 4953–4963.

Machlus, K.R., Lin, F.C., Wolberg, A.S., 2011b. Procoagulant activity induced by vascular injury determines contribution of elevated factor VIII to thrombosis and thrombus stability in mice. Blood 118 (14), 3960–3968.

Moazzafarian, D., Benjamin, E.J., Go, A.S., et al., 2015. Heart disease and stroke statistics-2015 update: a report from the American Heart Association. Circulation 131 (4), 292–322.

Oikonomopoulou, K., Ricklin, D., Ward, P.A., Lambris, J.D., 2012. Interactions between coagulation and complement—their role in inflammation. Semin. Immunopathol. 34 (1), 151–165.

Pierangelis, S.S., Girardi, G., Vega-Ostertag, M., Liu, X., Espinola, R.C., Salmon, J., 2005. Requirement of activation of complement C3 and C5 for antiphospholipid antibody-mediated thrombosis. Arthritis Rheum. 52 (7), 2120–2124.

Raskob, G.E., Angihausikuri, P., Blanco, A.N., et al., 2014. Thrombosis: a major contributor to global disease burden. Arterioscler. Thromb. Vasc. Biol. 34 (11), 2363–2371.

Rawal, N., Pangburn, M.K., 1998. C5 conversion of the alternative pathway of complement. Kinetic analysis of the free and surface-bound forms of the enzyme. J. Biol. Chem. 273 (27), 16828–16835.

Rawal, N., Pangburn, M., 2001. Formation of high-affinity C5 convertases of the alternative pathway of complement. J. Immunol. 166 (4), 2635–2642.

Ricklin, D., Hajishengallis, G., Yang, K., Lambris, J.D., 2010. Complement: a key system for immune surveillance and homeostasis. Nat. Immunol. 11 (9), 785–797.

Salmon, J.E., Girardi, G., Holers, V.M., 2002. Complement activation as a mediator of antiphospholipid antibody induced pregnancy loss and thrombosis. Ann. Rheum. Dis. 61 (Suppl. 2), i46–i50.

Sims, P.J., Fiaison, E.M., Wiedmer, T., Shattil, S.J., 1986. Complement proteins C5b-9 stimulate coagulation factor Va and express procothrombinase activity. J. Biol. Chem. 261 (34), 18205–18212.

van den Eijnden-Schrauwen, Y., Kooistra, T., de Vries, R.E., Emeis, J.J., 1995. Studies on the role of tissue factor-induced blood coagulation. Thromb. Res. 76 (5–6), 601–608 (discussion 8-9).

Wat, J., Foley, J.H., Krisinger, M.J., et al., 2014. Polyphosphate suppresses complement via thrombin-dependent activation of complement C5a receptor. Arterioscler. Thromb. Vasc. Biol. 35 (2), 380–388.

Yan, C., Gao, H., 2012. New insights for C5a and C5a receptors in sepsis. Front. Immunol. 3, 182.

Zecher, D., Cumpelik, A., Schifferli, J.A., 2014. The C5a receptor: a seven-transmembrane receptor for C5a in the absence of C3: a new complement activation pathway. Nat. Med. 12 (6), 682–687.