Properdin-Mediated C5a Production Enhances Stable Binding of Platelets to Granulocytes in Human Whole Blood

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Enhanced levels of platelet/granulocyte aggregates (PGAs) are found in patients suffering from many different inflammatory vascular diseases, and their formation in animal models of vascular disease is associated with increased thromboinflammation and worsened outcomes. The complement system, a part of the innate immune system, influences PGA formation, but the mechanisms for its effects are unknown. In this study, we have defined complement-mediated mechanisms that enhance PGA formation in human whole blood stimulated with thrombin receptor-activating peptide (TRAP) using ex vivo flow cytometry assays. We demonstrate that physiological properdin, a positive regulator of complement alternative pathway activity, increases PGA formation when added to TRAP-stimulated blood. All physiological properdin forms increase PGA formation, but properdin tetramers are the most efficient at increasing complement activity and PGA formation. Inhibition of endogenous properdin, either circulating in the blood or produced locally by leukocytes, impairs TRAP-mediated PGA formation to the same level as specific inhibition of either the alternative or classical pathway. Additionally, blocking the interaction of C5a with its cellular receptor prevents properdin-mediated increases in PGA formation. Adding either properdin tetramers or C5a to whole blood increases CD11b expression on granulocytes, and this increase is prevented by blockade of the C5a–C5a receptor axis. Finally, we demonstrate that the effects of properdin on PGA formation are tightly regulated by Factor H. Cumulatively, our data indicate that properdin enhances PGA formation via increased production of C5a, and that inhibition of properdin function has therapeutic potential to limit thromboinflammation in diseases characterized by increased PGA formation. The Journal of Immunology, 2016, 196: 4671–4680.

Platelets are critical for vascular hemostasis and repair in response to blood vessel damage (1). Platelets also help initiate inflammatory responses that induce endothelial regeneration and vessel protection from potential invasion by microorganisms by binding and recruiting leukocytes to sites of vascular damage (1–4). Leukocytes not only promote inflammation, but they have a dynamic role in the regulation of thrombosis. Tissue factor expressed on leukocyte surfaces or leukocyte-derived microparticles increases thrombus formation (5–8), whereas leukocytes limit thrombus formation by directly phagocytosing activated platelets, decreasing local levels of ADP, and preventing fibrinogen binding to platelets (9). Platelets and leukocytes interact to form stable platelet/leukocyte aggregates (PLAs) (10), which involves an ordered series of events where P-selectin on activated platelets binds to P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes. This stimulates the upregulation and activation of complement receptor 3 (CR3; CD11b/CD18; Mac-1) on leukocytes, which then binds multiple ligands on platelets to form stable aggregates (11–14). Excessive PLA formation increases tissue factor expression, secretion of proinflammatory cytokines and chemokines, production of reactive oxygen species, secretion of damaging proteases, and upregulation of adhesion molecules on leukocytes (11–13). Increased levels of circulating PLAs are found in patients suffering from many inflammatory diseases, including cardiovascular, myeloproliferative, and inflammatory bowel diseases (15–20), and inhibiting PLA formation by inhibiting P-selectin or CD11b reduces pathology and improves outcomes in various vascular injury animal models (8, 21–24). Understanding mechanisms by which PLA formation is regulated is imperative to elucidate how PLAs can increase to levels that result in pathologic thromboinflammation and to identify potential therapeutic targets.

The complement system links the innate and adaptive immune systems and helps orchestrate inflammatory reactions. Complement activity can initiate by three distinct pathways (classical, lectin, and alternative) that converge at the cleavage of the central molecule, C3, to C3b and C3a. C3b binds covalently to exposed hydroxyl and amino groups on cell surfaces, enabling the formation...
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of C3/C5 convertases that amplify complement activity and lead to activation of terminal complement components to produce the anaphylatoxin C5a and the membrane attack complex (MAC; C5b-9) (25). Although all three pathways recognize distinct molecular patterns on cell surfaces to activate, the alternative pathway (AP) mainly initiates spontaneously in the fluid phase (25, 26). Properdin, the only known positive complement regulatory molecule, stabilizes the AP convertases, increasing their activity 5- to 10-fold (26). The AP amplifies on any surface on which C3b is bound, including C3b originally deposited by the lectin or classical pathways (CP); thus it is an essential amplification loop for all complement activity. All pathways must be tightly regulated to prevent unwanted tissue damage (25). Factor H (FH) is a key regulator of the AP (27).

Physiological properdin circulates in plasma at 4–25 μg/ml as dimers (P₂), trimers (P₃), and tetramers (P₄) of head-to-tail associations of monomeric subunits; however, repeated freeze/thaw cycles can form higher order nonphysiological aggregates (P₅) that bind nonspecifically to surfaces and consume complement in solution (28–31). Properdin is primarily produced by leukocytes, including neutrophils, which secrete properdin in response to proinflammatory stimuli, leading to higher concentrations in inflammatory locations (26, 32, 33). AP activation on activated platelets leads to terminal complement activation, generating MAC (31), which enhances platelet activation (34–35), and C5a. C5a contributes to formation of platelet/granulocyte aggregates (PGAs) in human whole blood stimulated with thrombin receptor–activating peptide (TRAP) (14, 36); however the complement pathways involved, as well as the role of physiological properdin in this process, are unknown. In this study, we have elucidated a critical role for physiological properdin in amplifying AP and CP activity at the platelet/granulocyte interface to increase PGA formation via C5a production, with resulting C5a-mediated CD11b up-regulation on granulocytes, all of which is tightly controlled by FH.

Materials and Methods

Buffers

Modified HEPES/Tyrode’s (HT) buffer (137 mM NaCl, 2.8 mM KCl, 1 mM MgCl₂, 12 mM NaHCO₃, 0.4 mM Na₂HPO₄, 10 mM HEPES, 0.35% BSA, 5.5 mM glucose [pH 7.4]) and PBS (10 mM NaH₂PO₄, 145 mM NaCl [pH 7.4]) were used as buffers.

Detection Abs

Murine mAbs (all IgG1) included anti-human CD42b-allophycocyanin (BioLegend), anti-human CD45-PE (BioLegend), anti-human/mouse C3/C3b/C4b/FTTC (Cedarlane Laboratories), and anti-human CD11b-PerCP/Cy5.5 (BioLegend). IgG1 isotype controls included allophycocyanin- and PerCP/Cy5.5-labeled (BioLegend), PE-labeled (Chemicon), and FITC-labeled (Cedarlane Laboratories) Abs.

Complement modulators

To inhibit complement-mediated C3 activation, either the originally described C3 inhibitor compstatin (I[C(V)DVQWGHRCT]-NH₂, Tocris Bioscience) (37) or the ~1000-fold more potent analog Cp20 (Ac-[I(C)-1MeW-QDW-Sar]-NH₂) prepared by solid-phase peptide synthesis (38) was used. At concentrations in large excess of C3 as used in our assays (50 μM final), both compstatin analogs were equally effective at inhibiting TRAP-mediated PGA formation and C3 fragment deposition on granulocytes (Supplemental Fig. 1). Samples receiving either compstatin analog were mediated PGA formation and C3 fragment deposition on granulocytes, all of which is tightly controlled by FH.

Complement modulators

Purification of properdin from normal human plasma was carried out as previously described (30). Physiological polymeric forms of properdin (P₂, P₃, P₄) were separated from P₅s by gel filtration chromatography. Briefly, pure properdin (5 mg) was loaded onto a Phenomenex BioSep-SEC-S4000 column (600 × 7.8 mm) with a guard column (75 × 7.8 mm) and eluted at a 0.5 ml/min flow rate in PBS. Purified, physiological forms of properdin were stored at 4°C and used within 2 wk of separation (28, 30).

Measurement of TRAP-mediated PGA formation, C3 fragment deposition, and CD11b expression

Human whole blood was collected via venipuncture from healthy donors. The Institutional Review Board from the University of Toledo College of Medicine and Life Sciences approved the protocols, and written informed consent was obtained from all donors, in accordance with the Declaration of Helsinki. Blood was drawn into BD Vacutainer tubes containing 50 μl of lepirudin (Refudan; Bayer or Celgene; donation from Dr. Sanjay Ram). Blood (20 μl) was immediately gently mixed with 60 μl total of modified HT buffer plus reagents (TRAP [Bachem] with or without properdin, complement modulators, and/or rH19–20), previously added to tubes at room temperature. TRAP was used at a final concentration of 20 μM, unless otherwise indicated. Complement modulators were used at the final concentrations indicated in the figure legends. Compstatin (50 μM final) was used as a positive control for complete complement inhibition. A sample that was not activated with TRAP, but received compstatin immediately after blood collection (designated as nonactivated plus compstatin [NA+Comp]) served as the baseline control for C3 fragment deposition and CD11b expression. The blood plus reagents mixture was incubated at 37°C for 15 min. The reaction was stopped using 800 μl RBC lysis/fixation solution (BioLegend). The samples were then washed with modified HT buffer and stained with detection Abs for 15 min at room temperature. At least 10,000 events (granulocytes and monocytes) were acquired using a Becton Dickinson FACSCalibur flow cytometer, and the data were analyzed using FlowJo 7.6 software (Tree Star). Granulocytes were gated based on CD45-associated fluorescence and side scatter, and the percentages of gated granulocytes positive for CD42b fluorescence, as well as the C3- and CD11b-associated geometric mean fluorescence intensities on gated granulocytes, were determined.

Analysis of C5a levels in reaction supernatants

Whole-blood experiments were set up as described above, in triplicate. Following the 37°C incubation (blood plus reagents), one set of tubes was immediately centrifuged at 300 × g for 10 min at 4°C. The supernatant was then centrifuged at 13,000 × g for 5 min at 4°C and immediately stored at −80°C until use. Supernatants were diluted 1:10 and analyzed using a C5a ELISA kit (Abcam), following the manufacturer’s instructions. The remaining two sets of tubes were processed as described above to determine PGA formation, C3 fragment deposition, and CD11b expression.

Statistical analysis

The data were analyzed with GraphPad Prism 6.0 software. Cumulative raw data were analyzed by repeated measures ANOVA with a Dunnett posttest, using the Geisser–Greenhouse correction, to compare groups to TRAP alone. Individual experiments were analyzed by one-way ANOVA with either a Dunnett or Tukey posttest, depending on the experiment. Statistical tests are indicated in the figure legends.

Results

Physiological properdin forms increase PGA formation

Properdin circulates as P₂, P₃, and P₄ in an approximate 1:2:1 ratio (28). Repeated freeze-thaw cycles cause purified properdin to form P₅s with the abnormal ability to activate complement in solution (28, 29) as well as bind nonspecifically to surfaces (30, 43), including NA platelets (31). In a study conducted by Ruel et al. (44), purified properdin significantly increased PLA formation in citrate-anticoagulated human whole blood. To accurately assess the function of purified properdin in human whole blood, it is essential to separate P₅s from P₂–P₄s and to avoid certain anticoagulants, such as citrate, that dampen complement activation and PLA formation (45, 46). In the present study, we separated P₂–P₄s from P₅s by size
exclusion chromatography to determine the effects of physiological properdin on PGA formation. In our assays, lepirudin, a direct thrombin inhibitor without any complement inhibitory properties (47), was used as the anticoagulant, and human whole blood was stimulated with TRAP, an agonist that directly stimulates thrombin receptors (48).

Fig. 1A shows the gating strategy used to identify granulocyte populations (49). Fig. 1B and 1C are dot plots showing an increase in the percentage of granulocytes with associated CD42b fluorescence (i.e., platelets) upon addition of 25 μg/ml physiological properdin forms (P2–P4) in a 1:2:1 ratio to TRAP-stimulated whole blood. Fig. 1D shows that the addition of P2–P4 leads to an ~2-fold increase in PGA formation compared with TRAP-stimulated whole blood without added properdin. The effect of properdin on TRAP-mediated PGA formation is dose-dependent, as properdin caused a significant increase in PGA formation at concentrations as low as 2 μg/ml and reached its maximal effects by 10 μg/ml (Fig. 1E).

To determine whether the physiological properdin forms had differential effects on PGA formation, each form was individually added to whole blood stimulated with TRAP (Fig. 1F, 1G). A lower dose of TRAP (10 μM) was used in these experiments to increase the sensitivity of detecting properdin-mediated effects on PGA formation. Fig. 1F shows the representative data from one of four independent experiments conducted with different donors' blood. The effects of properdin were dose-dependent, and P4 increased PGA formation to a greater extent than P3 and P2, requiring lower concentrations to achieve maximal effects. P3 caused greater increases in PGA formation than P2 at each concentration tested. There was considerable interindividual variation in the maximal effects of each form on PGA formation (P4, 2.5- to 14-fold; P3, 2.5- to 11-fold; P2, 2- to 5-fold increases in PGA over TRAP alone), but for each individual, P4 always had the greatest effect.

C3 fragments deposited on the granulocytes serve as a marker of the degree of complement activation induced or enhanced by each properdin form. Fig. 1G shows that P4 were the most efficient at increasing, in a dose-dependent manner, C3 fragment deposition (by ~3-fold) on granulocytes in TRAP-stimulated whole blood. P2 and P3 increased C3 fragment deposition on granulocytes to

**FIGURE 1.** Physiological properdin forms increase TRAP-mediated PGA formation. (A–E) Lepirudin anticoagulated whole blood was incubated without (NA) or with TRAP (20 μM) with or without physiological properdin dimers, trimers, and tetramers (P2–P4) in a 1:2:1 ratio (A–D, 25 μg/ml; E, 0–30 μg/ml). (A) Gating strategy to select granulocyte population. (B and C) Percentage PGA formation in TRAP-activated granulocyte population without or with P2–P4. (D) Representative bar graph of (B) and (C). **p < 0.01. (E) Dose curve of effects of P2–P4 on TRAP-mediated PGA formation. (F and G) Effect of different physiological properdin forms on PGA formation and C3 fragment deposition on granulocytes within the same experiment. Lepirudin anticoagulated whole blood was incubated with a submaximal dose of TRAP (10 μM) and increasing concentrations of individual properdin forms (P2, P3, P4; 0–50 μg/ml). Samples were processed as described in Materials and Methods. The p values were <0.05 as compared with TRAP alone (0 μg/ml properdin form), unless indicated in red. Maximal effects of properdin forms on C3 fragment deposition varied depending on the donor (P3, 1.5- to 3-fold; P3 and P4, 2- to 5-fold increases over TRAP alone); one donor shown in (G). (A–G) All experiments shown are representative of at least four independent experiments and are shown as mean and SD of duplicate observations. The data were analyzed by one-way ANOVA with a Dunnett multiple comparison test against TRAP alone. GMFI, geometric mean fluorescence intensity.
approximately the same level, but to a significantly lesser extent than P4s, even when used at concentrations 2-fold higher than the maximal P4 dose (Fig. 1G). Collectively, the data indicate that all physiological properdin forms increase PGA formation, but P4s cause the greatest increases and account for most C3 fragments deposited on the granulocytes in TRAP-stimulated whole blood.

**Inhibition of endogenous properdin reduces TRAP-induced PGA formation**

Because properdin is produced locally by leukocytes upon stimulation, its concentration in inflammatory microenvironments could be significantly higher than its plasma concentration of 4–25 μg/ml (26). Therefore, we sought to determine whether endogenous properdin (produced locally by leukocytes or already circulating in the plasma) modulates the basal level of TRAP-mediated PGA formation. We incubated TRAP-stimulated whole blood with three different anti-properdin mAbs that we recently produced and characterized: 3A3E1, 6E11A4, and 1G6D2 (H. Emch et al., manuscript in preparation). 3A3E1 and 6E11A4 inhibit AP-mediated lysis of rabbit erythrocytes by preventing properdin from binding to C3b. 1G6D2 recognizes properdin, is the same isotype as the inhibitory Abs (IgG1), and does not inhibit AP-mediated lysis of rabbit erythrocytes. Thus, 1G6D2 was used as a negative control for assessing properdin function. Fig. 2A shows that the inhibitory Abs, 3A3E1 and 6E11A4, significantly inhibited PGA formation relative to TRAP alone, whereas 1G6D2 did not. Fig. 2B shows the data for each donor graphed relative to the level of PGA formation induced by TRAP alone in that particular donor (such that TRAP alone = 1 for each donor). The data indicate that the addition of 3A3E1 and 6E11A4 reduced PGA formation by ~50% regardless of the level of PGA formation induced by TRAP alone in each donor’s blood (Fig. 2B). A low level of C3 fragment deposition, most likely due to complement

**FIGURE 2.** Inhibition of endogenous properdin function impairs TRAP-mediated PGA formation. Lepirudin anticoagulated whole blood was incubated with TRAP (20 μM) without or with 3A3E1, 6E11A4, or 1G6D2 (100 μg/ml). A sample that did not receive TRAP (NA) and that was preincubated with compstatin (NA+Comp) was included for (A)/(B) and (C)/(D), respectively. PGA formation (A and B) and C3 fragment deposition on granulocytes (C and D) were determined as described in Materials and Methods. Each graph represents cumulative data from experiments carried out with different human volunteer blood donors. The mean for each group, assessed in duplicate, is graphed in (A) and (C), whereas (B) and (D) represent the mean for each group graphed relative to the mean of TRAP alone (assigned a value of 1) for each independent experiment, and the SD of each group is shown. (A and B) n = 10; (C and D) n = 8. The data were analyzed by repeated measures ANOVA with a Dunnett multiple comparison test against TRAP alone. **p < 0.01, ***p < 0.001. GMFI, geometric mean fluorescence intensity.

**FIGURE 3.** Both AP and CP activity contributes to TRAP-mediated PGA formation. Lepirudin anticoagulated whole blood was incubated with TRAP (20 μM) without or with anti–Factor B no. 1379 [100 μg/ml (A) and (B)] or SALO [2 μM (C) and (D)]. PGA formation was determined as described in Materials and Methods. The mean for each group, assessed in duplicate, is graphed in (A) and (C), whereas (B) and (D) represent the mean for each group graphed relative to the mean of TRAP alone (assigned a value of 1) for each independent experiment, and the SD of each group is shown. (A) n = 10; (B) n = 8. Raw data were analyzed by repeated measures ANOVA with a Dunnett multiple comparison test against TRAP alone. ***p < 0.001.
activation on granulocytes during processing (before the addition of compstatin), was detected on NA+Comp groups (Fig. 2C, 2D; isotype control for C3 fragment detection $\sim 0.3–0.6$ relative to TRAP; not shown). C3 fragment deposition trended toward an increase on TRAP versus NA+Comp groups (Fig. 2C, $p = 0.08$), whereas 3A3E1 and 6E11A4 significantly reduced C3 fragment deposition by $\sim 30\%$ as compared with TRAP alone (Fig. 2C, 2D). C3-covered microparticles released from granulocytes during

FIGURE 4. Inhibition of TRAP-mediated PGA formation with complement inhibitors. Lepirudin anticoagulated whole blood was incubated without [(A and B) NA; (C and D) NA+Comp] or with TRAP (20 $\mu$M) alone or with anticomplement reagents (anti-properdin and anti--Factor B no. 1379, 100 $\mu$g/ml; SALO, 2 $\mu$M; PMX53, 167 $\mu$M; Comp, 50 $\mu$M). Graphs include cumulative data from experiments carried out with four different human volunteer blood samples in which PGA formation (A and B) and C3 fragment deposition (C and D) were determined in the same experiment, as described in Materials and Methods. The mean for each group, assessed in duplicate, is graphed in (A) and (C), whereas (B) and (D) represent the mean for each group graphed relative to the mean of TRAP alone (assigned a value of 1) for each independent experiment, and the SD of each group is shown. The data were analyzed by repeated measures ANOVA with a Dunnett multiple comparison test against TRAP alone. (A and C) $p < 0.05$ compared with TRAP alone unless indicated as nonsignificant. GMFI, geometric mean fluorescence intensity.

FIGURE 5. P4s increase PGA formation via C5a-mediated enhancement of CD11b expression. Lepirudin anticoagulated whole blood was incubated with TRAP alone (10 $\mu$M) or in combination with P4s (5 $\mu$g/ml) and anticomplement reagents (anti-properdin, 100 $\mu$g/ml; PMX53, 16.7 $\mu$M; Comp, 50 $\mu$M). PGA formation (A), C5a in supernatants (B), and CD11b expression (C) were determined as described in Materials and Methods. Results shown in (A) and (C) are representative of one of five independent experiments carried out with different human volunteer blood samples, and in two of these five experiments C5a was also determined [one representative experiment shown in (B)]. Two additional C5a ELISAs were carried out using supernatants harvested from experiments carried out under identical conditions, but that did not include duplicate samples for flow cytometry analysis. In the five independent experiments, effects of P4s on PGA formation varied from 2.5- to 14-fold increases over TRAP alone, depending on the donor (variation not shown). Results are shown as mean and SD of duplicate observations. The data were analyzed by one-way ANOVA with a Dunnett multiple comparison test against TRAP+P4. The $p$ values were $<0.001$ compared with TRAP+P4 unless indicated as nonsignificant. GMFI, geometric mean fluorescence intensity.
PGA formation (50) could reduce the total amount of detectable C3 fragments on granulocytes. This, combined with the inhibition of AP activity, may explain why TRAP plus 3A3E1 and 6E11A4 groups had lower C3 fragment levels than did the NA+Comp control. 1G6D2 did not affect C3 fragment deposition (Fig. 2C, 2D); thus inhibition of endogenous properdin function significantly limits complement activity and PGA formation in TRAP-stimulated whole blood.

AP and CP activation contribute to TRAP-mediated PGA formation

Platelets can activate both the CP and AP on their surface (31, 51, 52) and secrete chondroitin sulfate, which activates the CP in the fluid phase (36, 53). Isolated neutrophils also activate the AP on their surface (32). Because the AP can amplify complement using C3b originally deposited by the CP, we next determined whether the effects of properdin on PGA formation (Fig. 2) were due to AP activation alone or also due to amplification of CP activity.

Anti–Factor B no. 1379, an inhibitory mAb (41), and SALO, a salivary protein produced by the sandfly Lutzomyia longipalpis (54–56) that specifically inhibits the CP (42), were used to determine the effects of AP and CP inhibition on TRAP-mediated PGA formation, respectively. Addition of either anti–Factor B no. 1379 or SALO to whole blood significantly impaired TRAP-mediated PGA formation in each donor’s blood by an average of ∼50% (Fig. 3A, 3B and 3C, 3D, respectively, and Fig. 4A, 4B), as well as C3 fragment deposition (Fig. 4C, 4D) in a mode similar to the anti-properdin monoclonals (Figs. 2, 4). These data, as well as the ability of the CP inhibitor to inhibit PGA formation and C3 fragment deposition to the same level as inhibiting all complement activity with compstatin (Fig. 4), even when AP activity is intact, suggest that complement activity in TRAP-stimulated whole blood may be initiated by the CP and amplified by properdin-mediated AP convertase stabilization.

Properdin-mediated C5a generation enhances TRAP-mediated PGA formation via upregulation of CD11b on granulocytes

We next investigated the potential downstream effector molecules that could account for properdin’s effects on PGA formation. PMX53, a peptide that blocks the interaction of C5a with its receptor (39), was previously shown to impair TRAP-mediated PGA formation (14, 36). We compared the effects of PMX53 to the effects of our other reagents in the same donor’s blood to discern whether C5a and/or MAC generation influences PGA formation. All reagents except 1G6D2 significantly inhibited PGA formation to an average of ∼50% the level seen with TRAP alone (Fig. 4A, 4B) and reduced C3 fragment deposition on granulocytes to a level similar to the NA+Comp control (Fig. 4C, 4D). PMX53 and inhibition of properdin (3A3E1 and 6E11A4) had equivalent inhibitory effects on PGA formation as inhibiting all complement with compstatin (Fig. 4A, 4B), thus indicating that C5a is the key complement effector molecule for PGA formation in TRAP-stimulated blood and properdin is a key enhancer of C5a generation.

To confirm the role of properdin in generating C5a at the platelet/granulocyte interface, we determined whether inhibition of C5a function with PMX53 could prevent P4-mediated increases in PGA formation. Fig. 5A shows that P8 increased PGA formation ∼7-fold over TRAP alone, and PMX53 completely inhibited P4-mediated enhancement of PGA formation. Compstatin, 3A3E1, and 6E11A4 had identical effects as PMX53. C5a levels in reaction supernatants were measured by ELISA (Fig. 5B) and displayed a pattern similar to that of PGA formation (Fig. 5A), with PMX53 being the only exception, as expected, because it inhibits C5a function rather than generation.

Finally, we sought to determine a potential mechanism for the effects of properdin-mediated C5a generation on PGA formation. PMX53 was previously shown to inhibit CD11b (one of two chains that comprise CR3) upregulation on granulocytes in TRAP-stimulated whole blood (36). We measured CD11b expression on granulocytes in the presence of P8 and 3A3E1, 6E11A4, and PMX53, using 1G6D2 as a negative and IgG1 isotype control Ab. 3A3E1, 6E11A4, and PMX53 completely abrogated P4-mediated increases in CD11b expression, whereas 1G6D2 had a partial effect (Fig. 5C), suggesting that properdin-mediated C5a generation enhances PGA formation via upregulation of CD11b on granulocytes. We next assessed the direct effect of C5a on PGA formation and CD11b expression. Although C5a had no effect on PGA formation on its own (Fig. 6A), C5a caused an ∼1.5-fold increase in CD11b expression on granulocytes compared with the NA control (Fig. 6B). C5a significantly increased PGA formation and CD11b expression on granulocytes in the presence of submaximal doses of TRAP (10 μM) by ∼1.5-fold (Fig. 6). The effects of C5a on CD11b expression (either alone or with TRAP), as well as C5a enhancement of PGA formation in the presence of submaximal doses of TRAP, were all abrogated by PMX53 (Fig. 6). Taken together, our results indicate that properdin is key in promoting the generation of C5a at the platelet/granulocyte interface, which enhances PGA formation (in the presence of platelet agonists) by binding to C5a receptor 1 (C5aR1; CD88). This C5a/C5a receptor interaction results in upregulation of CR3 (CD11b/CD18) on granulocytes, contributing to aggregate stabilization.
Properdin-mediated increases in PGA formation are tightly regulated by FH

FH regulates the AP on cell surfaces and is the primary negative AP regulator in the fluid phase. It protects cell surfaces by recognizing and binding to the combination of C3b and polyanions via mainly its C-terminal domains, 19 and 20, while regulating complement with its N terminus (27, 40, 57). A recombinant protein composed solely of FH domains 19 and 20, designated rH19–20, competitively inhibits FH cell surface interactions without affecting fluid-phase regulation and simulates the pathophysiological mechanisms involved in the prothrombotic disease atypical hemolytic uremic syndrome (aHUS) (40, 58–60). The contribution of FH to regulation of PGA formation is unknown.

The addition of rH19–20 to TRAP-stimulated whole blood increased PGA formation and C3 fragment deposition on granulocytes compared with TRAP alone (Fig. 7A, 7B), indicating that FH cell surface protection is key in limiting TRAP-mediated PGA formation despite the presence of membrane-bound complement regulators on granulocytes and platelets. In NA blood, rH19–20 increased C3 fragment deposition on granulocytes and CD11b expression (Fig. 7B, 7C), but did not affect the formation of PGA (Fig. 7A). This is in agreement with the need for initial platelet activation for PGA formation (61), and it suggests that complement effector molecules produced in the absence of FH cell surface protection do not activate platelets. Higher C3 fragment deposition and CD11b expression on granulocytes in NA versus TRAP-stimulated blood, in the presence of rH19–20, may be an indication that bound platelets mask detection of each marker or that TRAP stimulation leads to shedding of C3- and/or CD11b-coated microparticles under these conditions. To determine whether properdin activity could account for rH19–20-mediated increases in PGA formation, the TRAP-stimulated whole blood was incubated with rH19–20 in the presence of the anticomplement reagents (Fig. 7A, 7B). 3A3E1, 6E11A4, anti–Factor B no. 1379, and compstatin significantly inhibited rH19–20-mediated increases in PGA formation and C3 fragment deposition to approximately the same level as NA blood, whereas 1G6D2 had no effect on PGA formation and a partial effect on C3 fragment deposition (Fig. 7A, 7B). Partial effects of 1G6D2 on rH19–20-mediated C3 fragment deposition (Fig. 7B) and PEG-mediated CD11b expression (Fig. 5C) may occur due to steric hindrance of properdin function by Ab–properdin complexes formed as a result of the high Ab concentrations used in our assays. Fig. 7C shows representative data indicating that inhibition of properdin, the AP, or all complement, reduces rH19–20-mediated increases in CD11b expression. These effects were observed in at least three of six experiments, except for 6E11A4, which significantly inhibited CD11b expression twice. The remaining experiments showed identical trends to Fig. 7C but did not reach statistical significance. Collectively, the data indicate that FH regulates properdin-mediated increases in AP activity and subsequent PGA formation by mechanisms including, but not necessarily limited to, increased CD11b expression in TRAP-stimulated whole blood. Fig. 8 shows a proposed model for the overall mechanisms of the effects of properdin on PGA formation.

Discussion

Given that platelets have important roles in hemostasis and in the immune response against microorganisms (2), it is essential to identify novel therapeutic targets to limit thrombosis and inflammation in the...
vasculature without directly affecting platelet function. Granulocytes have both prothrombotic and proinflammatory potential; therefore finding ways to limit the recruitment and activation of granulocytes by platelets could lessen pathology caused by the innate immune response. Long-term blockade of all platelet/granulocyte interactions through the use of P-selectin/PSGL-1 or CR3 antagonists could potentially increase patient susceptibility to infections or even autoimmune diseases (62–64). In this study, we have elucidated a role of properdin in influencing stable PGA formation, thus identifying properdin as a potential target for limiting leukocyte-mediated thromboinflammation without directly affecting platelet function or completely blocking platelet/granulocyte interactions in inflammatory vascular diseases.

The addition of physiological properdin enhanced PGA formation in TRAP-stimulated blood in a dose-dependent manner (Fig. 1). P_4s were the most effective at increasing TRAP-mediated PGA formation and C3 fragment deposition on granulocytes (Figs. 1F, 1G, 5A). P_8 also significantly increased C5a levels recovered in supernatants and CD11b expression on granulocytes (Fig. 5B, 5C). The effectiveness of P_8 is in agreement with their previously described high degree of native properdin activity (ability to stabilize the convertases, promoting AP activation) as compared with P_2s and P_3s (28). Additionally, weak fluid-phase complement activation by P_8 (<7% that of aggregated/nonphysiological properdin) (28) may potentially contribute to C5a generation, thus enhancing PGA formation. Properdin is produced and secreted by many different leukocytes (26), but it is unknown whether different leukocytes secrete properdin forms in the same physiological ratio found in plasma (28). For instance, T cell–derived properdin was shown to be 100-fold more active than serum-derived properdin in an AP hemolytic assay (65). Given our data, specific inhibition of P_8 may have therapeutic potential; thus studies are needed to characterize the distribution of properdin forms in inflammatory microenvironments and to develop novel reagents that specifically inhibit each form. Inhibition of endogenous properdin in TRAP-stimulated whole blood with mAbs 3A3E1 and 6E11A4 limited PGA formation (Fig. 2A, 2B). This effect correlated with an ability to inhibit C3 fragment deposition on granulocytes (Fig. 2C, 2D). Investigation into the mechanisms of complement activation at the platelet/granulocyte interface revealed roles for both the AP and CP (Figs. 3, 4). Properdin is essential for AP activation on platelets and neutrophils (31, 32) (Fig. 8C, 8D, model), and platelets also activate the CP on or near their surface (Fig. 8A) (36, 51, 53). CP activity in the vicinity of the platelet or granulocyte surface could lead to the deposition of C3b on these cells, serving as a site for formation of the AP C3 convertase (C3bBb), which properdin would stabilize, enhancing AP activity (Fig. 8B). Regardless of the activation mechanism, the generation of C5a is critical for PGA formation in TRAP-stimulated whole blood, resulting in enhanced CR3 expression to form stable aggregates (Figs. 4–6, 8E). Inhibition of properdin was as effective at controlling PGA formation as specific inhibition of the C5a–C5aR1 axis (Fig. 4A, 4B), reflecting the ability of the inhibitory properdin Abs to significantly reduce C5a generation at the platelet/granulocyte interface (Fig. 5B). The only complement-specific inhibitor currently in the clinic is an anti-C5 mAb; however direct inhibition of

**FIGURE 8.** Model for the mechanism of properdin’s effects on PGA formation. (A) Activated platelets initially tether to granulocytes via P-selectin/PSGL-1 interactions, where they activate the CP on their surface and/or secrete chondroitin sulfate, a known activator of the CP. (B) Properdin-enhanced AP activity amplifies CP activity initiated by platelets, leading to the deposition of C3b on the granulocyte surface. The AP can then use deposited C3b to amplify its activity directly on the granulocyte surface. The AP also activates spontaneously on (C) neutrophils and activated platelets, and (D) AP activity is enhanced by high levels of properdin oligomers (P_2, P_3, and especially P_4), secreted from neutrophils. (E) Properdin-enhanced AP activity ultimately leads to increased levels of C5a that binds to C5aR1 on neutrophils to enhance CR3 expression. (F) FH regulates AP/properdin-mediated PGA formation.
C5 in clinical trials for myocardial infarction and coronary artery bypass surgery, two conditions associated with increased PGA formation (66, 67), had disappointing results (68, 69). Inhibition of properdin significantly reduces AP activity (C3 fragment degradation [Fig. 2C, 2D] and C5a generation [Fig. 5B]) at the platelet/granulocyte interface. Patients treated with properdin inhibitors could be effectively protected from neisserial infections (the main consequence of properdin deficiency) with the available tetravalent meningococcal vaccine (70). Therefore, properdin inhibitors alone or combined with C5aR1 antagonists may be an option to prevent complement-mediated enhancement of PGA formation in vascular diseases, although the therapeutic window for effects of these antagonists remains to be determined experimentally and would likely vary based on the disease context.

Host cells are normally protected from autologous AP complement activation by various redundant membrane-bound (CD55, CD46, CD59, CD35, CRIg) and fluid-phase (Factors H and I) complement regulators, although the therapeutic window for effects of these antagonists remains to be determined experimentally and would likely vary based on the disease context.

Our study elucidated a critical role for properdin in enhancing PGA formation in whole blood stimulated with TRAP, but platelets can be activated by other agonists (1). Additional studies have demonstrated PGA formation upon activation with LPS and Shiga toxin (50, 71), shear stress (72), and conditions that simulate coronary artery bypass (67, 73). The extent of complement activation on platelets varies depending on the agonist (31, 51, 74, 75); thus the role of properdin in PGA formation may vary based on the agonist used. Because the AP amplifies complement activity initiated by all complement pathways, properdin will likely have a critical role in any effect that depends on complement activation. Collectively, our data implicate properdin as having a key role in enhancing complement activity at the platelet/granulocyte interface, and inhibition of properdin has therapeutic potential to limit thromboinflammation, through limiting PGA formation, even in the absence of FH cell surface protection, such as that which occurs in patients with aHUS.

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