Published in final edited form as:

Platelets. 2008 May ; 19(3): 225–233. doi:10.1080/09537100701777311.

Expression of complement components and inhibitors on platelet microparticles

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Abstract

Platelet microparticles (PMP) are released from activated platelets and play an important role in hemostasis, thrombosis and inflammation. Since platelets were recently found to demonstrate an intrinsic capacity for activating both classical and alternative pathways of the complement system, the present study extended these observations to PMP. PMP were generated by treating platelets with 10 μ M A23187 (37°C, 5 min). PMP were identified by flow cytometry, based on size, Annexin V binding, and expression of P-selectin and GPIIb (CD41). PMP expressed gC1qR/p33, a multifunctional cellular protein that was recently described to activate the classical complement cascade. PMP also expressed the classical pathway and contact system regulator, C1 inhibitor (C1-INH), as well as CD55 and CD59. Despite C1-INH expression, PMP supported classical pathway C4 activation in the presence of purified C1 and C4. Moreover, statistically significant deposition of C3b and C5b-9 was detected on PMP exposed to plasma, concurrently with expression of CD55 and CD59. These data provide the first evidence for the ability of PMP to support in situ complement activation. Complement activation contributes to a variety of vascular and inflammatory disease states including atherosclerosis and ischemia/reperfusion injury.

Keywords

Platelets; microparticles; complement

Introduction

Platelets play important roles in hemostasis, thrombosis, inflammation and vascular injury [1,2]. Platelet microparticles (PMP) are small vesicles that are released from activated platelets. They are encountered in a variety of clinical settings, including arterial thrombosis and peripheral vascular disease [3], hypertension, diabetes, stroke [4–6], and atherosclerosis [7]. PMP enhance thrombus formation [8,9] and contribute to platelet activation [10]. PMP have

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been described as a storage pool for disseminating blood-borne bioactive effectors [11] such as tissue factor activity, procoagulant phospholipids and inflammatory mediators. Compared to activated platelets, PMP can express 50- to 100-fold higher procoagulant activity [4]. They retain selected platelet membrane constituents, including glycoproteins Ib, IIb/IIIa, and P-selectin [12,13], which support vascular inflammation by participating in heterotypic communication with leukocytes and vascular endothelial cells [14,15].

Recently, complement activation, an important contributor to innate immune and host inflammatory responses, has been described on activated platelets [16,17], independently of immune complex formation or infection. Platelet gC1qR/p33 (gC1qR) [18] contributes to in situ classical pathway complement activation [19], and P-selectin can activate the alternative pathway [17]. In these studies, platelet mediated complement activation was associated with generation of physiologically relevant levels of inflammatory peptides, C4a, C3a and C5a, which support recruitment of leukocytes and enhance vascular permeability. Moreover, assembly of the terminal C5b-9 membrane attack complex (MAC) was observed. Deposition of sublytic quantities of MAC on platelets has been reported to enhance platelet activation, secretion, and expression of procoagulant activity [20].

Complement activation is increasingly recognized as a major contributor to inflammation [21]. In the vasculature, complement deposition has been observed in atherosclerotic lesions [22], and a growing body of evidence suggests that complement also plays a significant role in ischemia/reperfusion injury [23]. Thus, the ability of platelets and particularly of PMP to support local complement activation is of interest from both pathophysiologic and therapeutic perspectives.

Materials and methods

Antibodies

PMP markers—FITC-conjugated Annexin V (Invitrogen Corporation, Carlsbad, CA, USA) (1:20 dilution) served as a marker of PMP surface phosphatidylserine expression. An Alexa Fluor 488 conjugated mouse anti human CD62P antibody (20 μ g/ml) (Ancell Corporation, Bayport, MN, USA) was used to evaluate PMP P-selectin expression. PMP glyco protein IIb (CD41) expression was identified using a mouse anti human CD41 monoclonal antibody (Beckman Coulter, Inc., Fullerton, CA, USA) that was conjugated with Fluoresceine 518 using a commercial labeling kit (Pierce Biotechnology, Rockford, IL, USA), according to manufacturer instructions. The labeled antibody was used at a concentration of 2 μ g/ml.

Complement components—C4d, C3b and C5b-9 deposition was investigated with murine monoclonal antibodies against human C4d (10 μ g/ml), iC3b (10 μ g/ml) and SC5b-9 (10 μ g/ml) (Quidel Corporation, Santa Clara, CA, USA). These represent stable and/or neoepitopes exposed following complement activation. Nonimmune mouse IgG (10 μ g/ml) (MOPC21, Sigma-Aldrich, St. Louis, MO, USA) served as a control antibody. Primary antibody binding was detected using 1 : 100 Alexa Fluor 488 labeled goat anti mouse antibody (Molecular Probes, Eugene, OR, USA). In some experiments, C4 deposition was examined using a polyclonal rabbit anti human C4 antibody, detected with an Alexa Fluor 488 labeled goat anti rabbit antibody (Molecular Probes) (1:100).

Complement regulators—Fluoresceine 518-labeled polyclonal rabbit anti human C1-INH antibody (20 μ g/ml), conjugated using a commercial labeling kit from Pierce Biotechnology, was used to measure PMP surface C1-INH expression. Fluoresceine 518 conjugated rabbit anti mouse IgG (Sigma) was used as a nonspecific antibody control. Murine monoclonal anti human CD55 and CD59 antibodies were obtained from Ancell Corporation (Bayport, MN, USA) and used at a concentration of 10 μ g/ml. Primary antibody binding was detected using 1:100 Alexa

Fluor 488 labeled goat anti mouse antibody (Molecular Probes). All antibodies were diluted in HEPES (hydroxyethyl piperazine ethanesulfonic acid) buffered modified Tyrode's solution (HBMT) [24].

Complement activators—gC1qR expression was measured with a fluorescein 518 labeled monoclonal antibody, 74.5.2 [16] (15 μ g/ml), conjugated using a commercial kit (Pierce Biotechnology). A rabbit anti human IgG antibody (Fc specific, Sigma-Aldrich, Saint Louis, MO, USA) (10 μ g/ml) was used to measure PMP associated IgG.

PMP preparation

Blood was obtained from healthy volunteers in accordance with protocols approved by the institutional review board of Weill Medical College of Cornell University, and in accordance with the Helsinki Declaration of 1975, as revised in 1983. Blood was collected into 3.2% sodium citrate (1:9 ratio anticoagulant: blood). Washed platelets were prepared in 0.01 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffered, modified Tyrode's solution, containing 1 mM MgCl₂ and 2.0mg/ml bovine serum albumin (Sigma) (HBMT) as described previously [16]. PMP were generated by stimulating washed platelets with 10 μ M ionophore A23187 [25] (Sigma-Aldrich) in the presence of 1 mM CaCl₂ for 5 minutes at 37°C. PMP were identified by flow cytometry based on their size and light scattering properties. A PMP gate was established for each experiment, and the surface membrane properties of PMP were characterized by examining particles within this gate.

Preparation of platelet poor plasma (PPP)

PPP was prepared by centrifugation of anticoagulated whole blood for 20 min at 1000g [16].

Flow cytometric analysis of PMP

Samples were analysed in a Becton Dickinson FACS Calibur flow cytometer (Franklin Lakes, Becton Dickinson, NJ, USA). PMP were distinguished from platelets based on forward and side light scattering properties, as shown in Figure 1. A platelet gate was established using unstimulated platelets. The microparticle gate was defined as events smaller than intact single platelets.

PMP were characterized by Annexin V binding. A23187 stimulated platelet suspensions were washed twice in 0.01 M phosphate buffered 0.15M NaCl, pH 7.5 (PBS) by centrifugation (8000g, 5 min) and resuspension. The final resuspension occurred in Annexin-binding buffer (140 mM NaCl, 10 mM HEPES, 2.5 mM CaCl₂, pH 7.4). The platelet/PMP suspension was subsequently incubated with FITC-conjugated Annexin V for 15 minutes at room temperature. Samples were diluted five times in Annexin-binding buffer and analysed.

PMP were also evaluated for expression of P-selectin (CD62P), glycoprotein IIb (CD41), gC1qR, surface associated IgG, C1-INH, CD55, and CD59. Activated platelet suspensions were incubated with antibodies described above for 30 minutes at room temperature, and diluted five-fold in HBMT for flow cytometric analysis. Negative fluorescence gates were established using the staining intensity of PMP exposed to species matched, nonimmune primary antibodies.

Complement activation on PMP

Activated platelet suspensions were incubated (45 min at 37°C) with platelet poor plasma (PPP), diluted 1/10 with HBMT. Samples were subsequently washed twice by centrifugation (8000 g, 5 min) and resuspension in HBMT containing 13 mM EDTA. C4d, C3b and C5b-9 deposition was measured using murine monoclonal anti human C4d, iC3b and SC5b-9

antibodies (30 min at room temperature). Primary antibody binding was detected using an Alexa Fluor 488 labeled goat anti mouse antibody (1:100) (30 min, room temperature). Platelet suspensions were fixed with 1% paraformaldehyde (Electron Microscopy Sciences, Washington, PA, USA) in 0.15 M NaCl and analysed by flow cytometry. Background complement activation was assessed in the absence of plasma. MOPC21 was used as a nonimmune control antibody.

Classical pathway complement activation on PMP was assessed after exposure of activated platelet suspensions to purified C1 (5 µg/ml) and C4 (5 µg/ml) (45 min, 37°C). Complement proteins were obtained from Quidel Corporation (CA, USA). A polyclonal rabbit antibody against human C4 (2 µg/ml, raised by one of the investigators, BG) was used to measure activated C4 deposition. Primary antibody binding was detected using an Alexa Fluor 488 labeled goat anti rabbit antibody (1 : 100) (Invitrogen, Carlsbad, CA, USA). Nonimmune rabbit IgG served as a primary antibody control. To assess the dependence of C4 deposition on C4 activation by C1, additional studies were conducted in the absence of C1, or the presence of 13 mM EDTA. To quantify positively staining PMP, negative fluorescence gates were set using the staining intensity of control PMP exposed to species matched, nonimmune primary antibodies.

Statistics

The paired Student's *t*-test was used to compare data sets. All analyses were performed using software "Primer of Biostatistics" (Version 4.02).

Results

Studies were performed to evaluate the capacity of PMP to activate the classical complement pathway. PMP were generated by A23187 activation of platelets and identified based on their light scattering properties. PMP demonstrated the expected reactivity with antibodies to GPIIb (CD41) and P-selectin (CD62P), and bound Annexin V (Figure 1). As shown in Figure 2, PMP also expressed potential complement activators, gC1qR and IgG, as well as complement regulators, C1-INH, CD55, and CD59. The data are summarized in Table I. Compared to PMP reactivity with species matched control antibodies, significant increases in fluorescence intensity were noted when PMP were exposed to antibodies against gC1qR (~five-fold increase), IgG (10–60-fold increase), C1-INH (~two-fold increase), CD55 (four-12-fold increase) and CD59 (10–15 fold increase). gC1qR expression was measured by 74.5.2 binding, a monoclonal antibody that recognizes the N-terminal domain of gC1qR [26].

Despite the expression of complement regulators, classical complement pathway activation, measured as a function of C4 deposition, occurred on PMP in the presence of purified C1 and C4. C4 deposition required C1 and failed to occur in the presence of EDTA (data not shown), demonstrating a requirement for classical pathway C1 activation. A two-four-fold increase in fluorescence intensity and a statistically significant increase in the proportion of PMP staining positively for C4 were seen following exposure of PMP to C1 and C4. The data are summarized in Figure 3 and Table II.

Similar deposition of C4 on PMP was noted when A23187 activated platelet suspensions were exposed to diluted plasma. Moreover, complement activation on PMP proceeded to completion in this setting, with deposition of C3b and C5b-9. Our previous studies [16] demonstrated that similar levels of complement activation were achieved on platelets exposed to plasma anticoagulated with 0.32% sodium citrate and serum, when both were diluted (1/10) in HBMT to restore divalent cations. HBMT contains 1 mM MgCl₂ [27]. In the present study, an approximately four-fold increase in PMP reactivity (mean fluorescence intensity) with antibodies against C4d, iC3b, and SC5b-9 was observed following exposure to plasma (Figure

3). Maximum complement activation occurred within 30–60 min at 37°C. Statistically significant increases in the proportion of PMP staining positively with each antibody were noted (Table II). Considering that the surface area of PMP is approximately two-orders of magnitude smaller than that of activated platelets (4), the observed fluorescence intensity for PMP associated complement components exceeds that observed on A23187 activated platelets by at least one order of magnitude (Table III).

Although the extent of complement activation on PMP varied between experiments, complement activation was consistently detected. Interestingly, an increase in the expression of C1 INH was also noted on PMP relative to activated platelets (Table III). Comparative data were not collected for constitutively expressed complement regulators, CD55 and CD59.

Discussion

Platelet activation plays an important role in thrombosis and inflammation [28]. We previously demonstrated that activated platelets support classical pathway complement activation [16]. The present study provides the first evidence that complement activation also occurs on PMP. Despite expression of complement regulators, C1-INH, CD55 and CD59, completion of the complement cascade was noted with deposition of C3b and the membrane attack complex (MAC, C5b-9). Given the small surface area of PMP relative to intact platelets, PMP may present concentrated activated complement components to targets in the vasculature.

Our studies are limited to an investigation of PMP generated by activation of platelets with calcium ionophore, A23187. It will be interesting to explore whether PMP generated by platelet stimulation with combinations of other agonists express differing capacities for classical complement pathway activation. Expression of procoagulant activity and Factor V on activated platelets, for example, is especially pronounced in subsets of platelets referred to as “coated” platelets, activated by thrombin and collagen or thrombin and A23187 [29,30].

An additional limitation of the present study relates to the identification of PMP by flow cytometry. PMP were not separated from intact platelets. However, in some studies the A23187 activated platelet suspensions were washed by centrifugation and resuspension. This will have eliminated the smallest microparticles from analysis.

C4 deposition on PMP, and its dependence on active C1, demonstrates the intrinsic capacity of PMP to activate the classical complement pathway. This is consistent with the expression of gC1qR. gC1qR was previously shown to directly activate the classical complement system *in vitro* [26]. Moreover, gC1qR antibodies partially blocked complement activation on stimulated platelets [16]. Whether PMP associated IgG, which is unlikely to reflect immune complex formation in healthy individuals, triggers classical pathway complement activation, remains to be determined.

PMP are best known for their procoagulant properties. Increasing evidence for cross-talk between coagulation and complement systems is emerging [21,31]. Thrombin, for example, has been shown to activate C5 directly [32]. Thus, thrombin generation and the assembly of complement components on PMP could significantly increase the *in situ* generation of inflammatory mediators. In addition, the expression of P-selectin on PMP may engage the alternative pathway, as has been described on platelets [17], to further enhance local complement activation.

Under physiologic conditions, complement activation may contribute to the clearance of PMP from the circulation, via deposition of C1q and generation of PMP associated C3b [33]. In the process of complement activation, however, potent inflammatory mediators, C3a and C5a are generated [34]. These peptides have cytokine like properties and enhance leukocyte

recruitment and support the host inflammatory response. In nM quantities, C3a and C5a bind endothelial cell receptors and stimulate upregulation of interleukins 8 and 1 β in addition to RANTES, and strongly activate the MAP-kinase signaling pathway [35]. Thus, the rolling or association of PMP on or with endothelial cells [36] may carry activated complement components along the vasculature and accelerate endothelial responses to vascular injury.

Comparisons of complement activation on PMP relative to platelets suggest enhanced complement deposition on PMP when fluorescence was normalized for differences in platelet and PMP size. Interestingly, C4 activation exceeded C3b and C5b-9 activation on PMP, suggesting potential regulation of C3 and C5 convertases, as well as assembly of C5b-9 by intrinsic platelet complement regulators. The present study demonstrates that PMP express complement regulatory proteins, C1-INH, CD55 and CD59. In addition, clusterin, a regulator of the assembly of the terminal complement complex, is packaged in platelet alpha granules [37], and is expressed on the surface of platelets [38] and microparticles [39].

Like clusterin, C1-INH is present in platelet alpha granules and is released following platelet activation [40]. C1-INH can inhibit classical pathway complement activation by binding to C1r and C1s. It also binds to P- and E- selectins and interferes with leukocyte adhesion [41]. Since its anti proteolytic activity appears to be preserved after binding to P-selectin, C1-INH expression on PMP may limit the generation of C3a and C5a [42,43]. The expression of CD55 and CD59 may further modulate complement activation on PMP. CD55 disassembles C3 and C5 convertases (31), whereas CD59 prevents the formation of the terminal complement complex (C5b-9), which can contribute to platelet and endothelial cell activation at sublytic concentrations [44,45].

A growing body of evidence supports the hypothesis that immune mechanisms, including complement activation, are involved in inflammatory conditions associated with vascular injury [46,47]. Deposition of complement components, C1q, C3, and C4, and generation of the terminal complement complex C5b-9 has been described in human atherosclerotic lesions [48]. The extent of C5b-9 deposition appears to correlate with the severity of the lesion [49], with the greatest deposition of iC3b reported in vulnerable and ruptured plaques [49,50]. Indeed, elevations in circulating C5a levels have been associated with increased cardiovascular risk in patients with advanced atherosclerosis [51]. Results from the present study suggest that PMP can transport complement components to sites of vascular injury, and support local inflammation by supporting classical pathway complement activation.

Acknowledgments

This work was supported in part by grants HL67211 (EIBP) and AI060866 (BG) from the National Institutes of Health, and an American Heart Association Heritage Affiliate postdoctoral award # 0625900T (WY).

References

1. Franchini M, Veneri D, Lippi G. Inflammation and hemostasis: A bidirectional interaction. *Clin Lab* 2007;53:63–67. [PubMed: 17323827]
2. Esmon CT. Inflammation and thrombosis. *J Thromb Haemost* 2003;1:1343–1348. [PubMed: 12871267]
3. Zeiger F, Stephan S, Hoheisel G, Pfeiffer D, Ruehlmann C, Koksche M. P-Selectin expression, platelet aggregates, and platelet-derived microparticle formation are increased in peripheral arterial disease. *Blood Coagul Fibrinolysis* 2000;11:723–728. [PubMed: 11132650]
4. Sinauridze EI, Kireev DA, Popenko NY, Pichugin AV, Pantelev MA, Krymskaya OV, Ataulakhov FI. Platelet microparticle membranes have 50- to 100-fold higher specific procoagulant activity than activated platelets. *Thromb Haemost* 2007;97:425–434. [PubMed: 17334510]

5. Piccin A, Murphy WG, Smith OP. Circulating microparticles: Pathophysiology and clinical implications. *Blood Rev* 2007;21:157–171. [PubMed: 17118501]
6. Choudhury A, Chung I, Blann AD, Lip GY. Elevated platelet microparticle levels in nonvalvular atrial fibrillation: Relationship to p-selectin and antithrombotic therapy. *Chest* 2007;131:809–815. [PubMed: 17356097]
7. Tan KT, Lip GY. The potential role of platelet microparticles in atherosclerosis. *Thromb Haemost* 2005;94:488–492. [PubMed: 16268460]
8. Diamant M, Tushuizen ME, Sturk A, Nieuwland R. Cellular microparticles: New players in the field of vascular disease? *Eur J Clin Invest* 2004;34:392–401. [PubMed: 15200490]
9. Tans G, Rosing J, Thomassen MC, Heeb MJ, Zwaal RF, Griffin JH. Comparison of anticoagulant and procoagulant activities of stimulated platelets and platelet-derived micro-particles. *Blood* 1991;77:2641–2648. [PubMed: 2043766]
10. VanWijk MJ, VanBavel E, Sturk A, Nieuwland R. Microparticles in cardiovascular diseases. *Cardiovasc Res* 2003;59:277–287. [PubMed: 12909311]
11. Morel O, Toti F, Hugel B, Freyssinet JM. Cellular microparticles: A disseminated storage pool of bioactive vascular effectors. *Curr Opin Hematol* 2004;11:156–164. [PubMed: 15257014]
12. Gawaz M, Ott I, Reiningner AJ, Heinzmann U, Neumann FJ. Agglutination of isolated platelet membranes. *Arterioscler Thromb Vasc Biol* 1996;16:621–627. [PubMed: 8963718]
13. George JN, Pickett EB, Saucerman S, McEver RP, Kunicki TJ, Kieffer N, Newman PJ. Platelet surface glycoproteins. Studies on resting and activated platelets and platelet membrane microparticles in normal subjects, and observations in patients during adult respiratory distress syndrome and cardiac surgery. *J Clin Invest* 1986;78:340–348. [PubMed: 2942561]
14. Martinez MC, Tesse A, Zobairi F, Andriantsitohaina R. Shed membrane microparticles from circulating and vascular cells in regulating vascular function. *Am J Physiol Heart Circ Physiol* 2005;288:H1004–H1009. [PubMed: 15706036]
15. Barry OP, Pratico D, Savani RC, FitzGerald GA. Modulation of monocyte-endothelial cell interactions by platelet microparticles. *J Clin Invest* 1998;102:136–144. [PubMed: 9649567]
16. Peerschke EI, Yin W, Grigg SE, Ghebrehwet B. Blood platelets activate the classical pathway of human complement. *J Thromb Haemost* 2006;4:2035–2042. [PubMed: 16961611]
17. Del Conde I, Cruz MA, Zhang H, Lopez JA, Afshar-Kharghan V. Platelet activation leads to activation and propagation of the complement system. *J Exp Med* 2005;201:871–879. [PubMed: 15781579]
18. Peerschke EI, Murphy TK, Ghebrehwet B. Activation-dependent surface expression of gC1qR/p33 on human blood platelets. *Thromb Haemost* 2003;89:331–339. [PubMed: 12574814]
19. Ghebrehwet B, Peerschke EI. cC1q-R (calreticulin) and gC1q-R/p33: ubiquitously expressed multi-ligand binding cellular proteins involved in inflammation and infection. *Mol Immunol* 2004;41:173–183. [PubMed: 15159063]
20. Sims PJ, Rollins SA, Wiedmer T. Regulatory control of complement on blood platelets. Modulation of platelet procoagulant responses by a membrane inhibitor of the C5b-9 complex. *J Biol Chem* 1989;264:19228–19235. [PubMed: 2808422]
21. Markiewski MM, Lambris JD. The role of complement in inflammatory diseases from behind the scenes into the spotlight. *Am J Pathol* 2007;171:715–727. [PubMed: 17640961]
22. Bhakdi S. Complement and atherogenesis: The unknown connection. *Ann Med* 1998;30:503–507. [PubMed: 9920350]
23. Weiser MR, Williams JP, Moore FD Jr, Kobzik L, Ma M, Hechtman HB, Carroll MC. Reperfusion injury of ischemic skeletal muscle is mediated by natural antibody and complement. *J Exp Med* 1996;183:2343–2348. [PubMed: 8642343]
24. Peerschke EI, Smyth SS, Teng EI, Dalzell M, Ghebrehwet B. Human umbilical vein endothelial cells possess binding sites for the globular domain of C1q. *J Immunol* 1996;157:4154–4158. [PubMed: 8892652]
25. Bode AP, Hickerson DH. Characterization and quantitation by flow cytometry of membranous microparticles formed during activation of platelet suspensions with ionophore or thrombin. *Platelets* 2000;11:259–271. [PubMed: 11030460]
26. Ghebrehwet B, Lu PD, Zhang W, Lim BL, Eggleton P, Leigh LE, Reid KB, Peerschke EI. Identification of functional domains on gC1q-R, a cell surface protein that binds to the globular

- “heads” of C1q, using monoclonal antibodies and synthetic peptides. *Hybridoma* 1996;15:333–342. [PubMed: 8913782]
27. Peerschke EI, Grant RA, Zucker MB. Decreased association of ⁴⁵ calcium with platelets unable to aggregate due to thrombasthenia or prolonged calcium deprivation. *Br J Haematol* 1980;46:247–256. [PubMed: 6775678]
 28. Steinhubl SR. Platelets as mediators of inflammation. *Hematol Oncol Clin North Am* 2007;21:115–121. [PubMed: 17258122]
 29. Dale GL, Remenyi G, Friese P. Quantitation of microparticles released from coated-platelets. *J Thromb Haemost* 2005;3:2081–2088. [PubMed: 16102115]
 30. Alberio L, Safa O, Clemetson KJ, Esmon CT, Dale GL. Surface expression and functional characterization of alpha-granule factor V in human platelets: Effects of ionophore A23187, thrombin, collagen, and convulxin. *Blood* 2000;95:1694–1702. [PubMed: 10688826]
 31. Markiewski MM, Nilsson B, Ekdahl KN, Mollnes TE, Lambris JD. Complement and coagulation: Strangers or partners in crime? *Trends Immunol* 2007;28:184–192. [PubMed: 17336159]
 32. Huber-Lang M, Sarma JV, Zetoune FS, Rittirsch D, Neff TA, McGuire SR, Lambris JD, Warner RL, Flierl MA, et al. Generation of C5a in the absence of C3: A new complement activation pathway. *Nat Med* 2006;12:682–687. [PubMed: 16715088]
 33. Flaumenhaft R. Formation and fate of platelet microparticles. *Blood Cells Mol Dis* 2006;36:182–187. [PubMed: 16466949]
 34. Nilsson G, Johnell M, Hammer CH, Tiffany HL, Nilsson K, Metcalfe DD, Siegbahn A, Murphy PM. C3a and C5a are chemotaxins for human mast cells and act through distinct receptors via a pertussis toxin-sensitive signal transduction pathway. *J Immunol* 1996;157:1693–1698. [PubMed: 8759757]
 35. Monsinjon T, Gasque P, Chan P, Ischenko A, Brady JJ, Fontaine MC. Regulation by complement C3a and C5a anaphylatoxins of cytokine production in human umbilical vein endothelial cells. *FASEB J* 2003;17:1003–1014. [PubMed: 12773483]
 36. Mause SF, von Hundelshausen P, Zernecke A, Koenen RR, Weber C. Platelet microparticles: A transcellular delivery system for RANTES promoting monocyte recruitment on endothelium. *Arterioscler Thromb Vasc Biol* 2005;25:1512–1518. [PubMed: 15890969]
 37. Tschopp J, Jenne DE, Hertig S, Preissner KT, Morgenstern H, Sapino AP, French L. Human megakaryocytes express clusterin and package it without apolipoprotein A-1 into alpha granules. *Blood* 1993;82:118–125. [PubMed: 8324215]
 38. Gnatenko DV, Dunn JJ, McCorkel SR, Weissmann D, Oeritta PL, Bahou WF. Transcript profiling of human platelets using microarray and serial analysis of gene expression. *Blood* 2003;101:2285–2293. [PubMed: 12433680]
 39. Perez-Pujol S, Martinez MB, Higgins LA, Lesestuen GL, Key NS. Proteomic analysis of platelets and platelet-derived microparticles by iTRAQ Laser Spectrometry. *J Thromb Haemost* 2005;3(S1): 1–9. [PubMed: 15634257]
 40. Schmaier AH, Amenta S, Xiong T, Heda GD, Gewirtz AM. Expression of platelet C1 inhibitor. *Blood* 1993;82:465–474. [PubMed: 8329703]
 41. Cai S, Davis AE III. Complement regulatory protein C1 inhibitor binds to selectins and interferes with endothe-lial-leukocyte adhesion. *J Immunol* 2003;171:4786–4791. [PubMed: 14568956]
 42. Kilgore KS, Flory CM, Miller BF, Evans VM, Warren JS. The membrane attack complex of complement induces interleukin-8 and monocyte chemoattractant protein-1 secretion from human umbilical vein endothelial cells. *Am J Pathol* 1996;149:953–961. [PubMed: 8780399]
 43. Beek J, Elward K, Gasque P. Activation of complement in the central nervous system: Roles in neurodegeneration and neuroprotection. *Ann N Y Acad Sci* 2003;992:56–71. [PubMed: 12794047]
 44. Niculescu F, Rus H. The role of complement activation in atherosclerosis. *Immunol Res* 2004;30:73–80. [PubMed: 15258311]
 45. Rinder CS, Rinder HM, Smith BR, Fitch JC, Smith MJ, Tracey JB, Matis LA, Squinto SP, Rollins SA. Blockade of C5a and C5b-9 generation inhibits leukocyte and platelet activation during extracorporeal circulation. *J Clin Invest* 1995;96:1564–1572. [PubMed: 7657827]
 46. Arumugam TV, Shiels IA, Woodruff TM, Granger DN, Taylor SM. The role of the complement system in ischemia-reperfusion injury. *Shock* 2004;21:401–409. [PubMed: 15087815]

47. Makrides SC. Therapeutic inhibition of the complement system. *Pharmacol Rev* 1998;50:59–87. [PubMed: 9549758]
48. Niculescu F, Rus H. Complement activation and atherosclerosis. *Mol Immunol* 1999;36:949–955. [PubMed: 10698349]
49. Vlaicu R, Niculescu F, Rus HG, Cristea A. Immunohistochemical localization of the terminal C5b-9 complement complex in human aortic fibrous plaque. *Atherosclerosis* 1985;57:163–177. [PubMed: 3910058]
50. Laine P, Pentikainen MO, Wurzner R, Penttila A, Paavonen T, Meri S, Kovanen PT. Evidence for complement activation in ruptured coronary plaques in acute myocardial infarction. *Am J Cardiol* 2002;90:404–408. [PubMed: 12161231]
51. Speidl WS, Exner M, Amighi J, Kastl SP, Zorn G, Maurer G, Wagner O, Huber K, Minar E, Wojta J, et al. Complement component C5a predicts future cardiovascular events in patients with advanced atherosclerosis. *Eur Heart J* 2005;26:2294–2299. [PubMed: 15917276]

Figure 1.

Characterization of A23187-activated platelets and PMP by flow cytometry. Panels A and B depict typical dot plots of the light scattering properties of PMP relative to unstimulated (Panel A) and A23187 activated (Panel B) platelets. A platelet gate was established using unstimulated platelets, as shown in Panel A. The microparticle gate was defined as events smaller than intact single platelets (Panel B). Typical reactivity of resting and activated platelets (panels C-E) and A23187-generated PMP (panels F-H) with antibodies to CD62P and CD41, as well as Annexin V binding, is shown. MOPC served as species matched, non-immune control IgG.

Figure 2.

Expression of complement activators and regulators on PMP. A PMP gate was established based on PMP size and light scattering properties using flow cytometry. Panels A–E represent typical histograms depicting reactivity of PMP within this gate with antibodies against gC1qR (A), IgG (B), CI- INH (C), CD55 (D), and CD59 (E). Appropriate species matched nonimmune IgG served as controls.

Figure 3.

Deposition of complement components on PMP. A PMP gate was established based on PMP size and light scattering properties using flow cytometry. Analyses were performed on particles within this gate. Panel A depicts a representative histogram demonstrating C4 deposition on PMP incubated (45 min, 37°C) with purified C1 and C4 in combination. PMP reactivity with antibodies to C4 in the absence of C1, and baseline fluorescence in the presence of buffer, are shown for comparison. Panels B–D demonstrate typical surface deposition of activated complement components, C4d (B), C3b (C) and C5b-9 (D) on PMP, observed following incubation (45 min, 37°C) with plasma (1/10). Complement components were visualized by flow cytometry using antibodies to C4d, iC3b and SC5b-9. Baseline antibody binding in the absence of plasma is shown for comparison.

Table I

Expression of complement activators and regulators on PMP.

Surface expression of:	% Positive (mean $\pm$ S.D.)
gC1qR	30 $\pm$ 20 [★] (n = 6)
IgG	35 $\pm$ 6 [★] (n = 3)
C1-INH	18 $\pm$ 5 [★] (n = 7)
CD55	45 $\pm$ 9 [★] (n = 3)
CD59	52 $\pm$ 8 [★] (n = 3)

Surface expression of complement activators and regulators was evaluated by flow cytometry using specific polyclonal or monoclonal antibodies. PMP were distinguished from platelets based on their size and light scattering properties. A PMP gate was established based on these properties, and analyses were performed on particles within this gate.

[★] statistically significant increases in % positive PMP relative to species matched control antibodies, using the paired Student's *t*-test.

Table II

Complement activation on PMP.

PMP incubated with	Surface expression of complement components (% positive PMP)			
	C4	C4d	iC3b	SC5b-9
C1 ± C4	22 ± 10 [★] (n = 6)	N.A.	N.A.	N.A.
C4, only	5 ± 3 (n = 4)	N.A.	N.A.	N.A.
Plasma (1/10)		14 ± 5 [★] (n = 9)	8 ± 2 [★] (n = 7)	6 ± 2 [★] (n = 4)

Surface expression of complement components was evaluated by flow cytometry. A23187-activated platelet suspensions were incubated (45 min, 37°C) with plasma or purified complement components, washed, and probed with antibodies to stable complement components. PMP were distinguished from platelets based on their size and light scattering properties. A PMP gate was established based on these properties, and analyses were performed on particles within this gate.

[★] statistically significant increases in % positive PMP relative to species matched control antibodies using the paired Student's *t*-test; N.A., not applicable.

Table III

Comparison of complement activation and C1-INH expression on A23187 activated platelets and PMP.

CELLS	Surface expression of complement components (Mean Fluorescence Intensity)			
	C4d	iC3b	SC5b-9	C1-INH
Platelets	242 ± 334 (<i>n</i> = 9)	226 ± 159 (<i>n</i> = 7)	103 ± 95 (<i>n</i> = 5)	41 ± 16 (<i>n</i> = 7)
PMP	51 ± 63 (<i>n</i> = 9)	25 ± 13 (<i>n</i> = 7)	10 ± 1.48 (<i>n</i> = 3)	19 ± 5 (<i>n</i> = 7)
PMP (estimated results corrected for surface area) [#]	~5100	~2500	~1000	~1900

Surface expression of complement components and C1 INH was evaluated by flow cytometry. A23187 activated platelet suspensions were incubated (45 min, 37°C) with plasma (1/10), washed and probed with antibodies to stable complement components or C1-INH. Platelets and PMP were distinguished based on size and light scattering properties. Separate platelet and PMP gates were established. Fluorescence data was collected for particles falling within either the platelet or PMP gates.

[#] Mean fluorescence intensity of PMP was normalized based on surface area relative to platelets. The surface area of PMP has been estimated to be approximately two-orders of magnitude smaller than that of activated platelets [4].