Complement factor C4a does not activate protease-activated receptor 1 (PAR1) or PAR4 on human platelets

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Abstract

Background: Protease-activated receptor (PAR) 1 and PAR4 are key thrombin signal mediators for human platelet activation and aggregation in response to vascular injury. They are primarily activated by thrombin cleavage of the N-terminus to expose a tethered ligand. In addition to the canonical activation by thrombin, a growing panel of proteases can also elicit PAR1- or PAR4-mediated signal transduction. Recently, complement factor C4a was reported as the first endogenous agonist for both PAR1 and PAR4. Further, it is the first endogenous nontethered ligand that activates PAR1 and PAR4. These studies were conducted with human microvascular cells; the impact of C4a on platelet PARs is unknown.

Objectives: The goal of this study was to interrogate PAR1 and PAR4 activation by C4a on human platelets.

Methods: Platelet-rich plasma (PRP) was isolated from healthy donors. PRP was stimulated with C4a, and the platelet aggregation was measured. Human embryonic kidney (HEK) 293 Flp-In T-rex cells were used to further test if C4a stimulation can initiate PAR1- or PAR4-mediated Gαq signaling, which was measured by intracellular calcium mobilization.

Results: C4a failed to elicit platelet aggregation via PAR1- or PAR4-mediated manner. In addition, no PAR1- or PAR4-mediated calcium mobilization was observed upon C4a stimulation on HEK293 cells.

Conclusions: Complement factor C4a does not activate PAR1 or PAR4 on human platelets. These data show that PAR1 and PAR4 activation by C4a on microvascular cells likely requires a cofactor, which reinforces the concept of cell type–specific regulation of protease signaling.

Keywords
complement factor C4a, PAR1, PAR4, platelet aggregation, platelets
INTRODUCTION

Protease-activated receptors (PARs) are the primary means by which proteases mediate intracellular signaling. The prototypical receptor, PAR1, was identified 30 years ago as the thrombin receptor. Subsequently, three other family members were identified (PAR2, PAR3, and PAR4) by homology screening of cDNA libraries. PARs are G-protein-coupled receptors (GPCRs) that have a unique activation mechanism in which the N-terminus is cleaved by the activating protease to generate a tethered ligand. The newly exposed ligand binds intramolecularly to the receptor, inducing a conformational change that initiates signal transduction. In addition to canonical thrombin-mediated signaling, there is a growing panel of identified proteases that also have the ability to activate PARs. PAR1 can be cleaved by activated protein C (APC), matrix metalloproteinase (MMP)-1, MMP-2, and MMP-13 at noncanonical sites to generate distinct tethered ligands. These ligands initiate diverse signaling pathways depending on the cell type and cellular cofactors. In addition to thrombin, PAR4 can be activated by trypsin, plasmin, cathepsin G, or factor Xa; however, these all cleave at the canonical thrombin site and result in the same downstream signaling.

Human platelets express PAR1 and PAR4. This dual receptor system works in concert to provide the sensitivity and duration of signaling required for hemostasis. PAR1 is highly sensitive to thrombin and results in a rapid, transient signal. In contrast, PAR4 responds to higher concentrations of thrombin and results in a prolonged signaling response. Thrombin activation of PARs initiates multiple signaling cascades in human platelets by directly coupling to Gqα and G12/13α. Gqα stimulates the formation of inositol triphosphate and diacylglycerol, leading to intracellular calcium mobilization and protein kinase C activation. G12/13α mediates Rho guanine nucleotide exchange factors and RhoA activation, which controls platelet shape change, spreading, and thrombus stability. In platelets, PAR1 and PAR4 signal indirectly through Gqα via secondary stimulation of the Gqα12 coupled P2Y12 receptor due to released ADP. However, Voss and colleagues used a pharmacologic approach to show that PAR1 signals directly through Gqα in platelets. In sum, PAR1 and PAR4 mediate several important signaling events in platelets.

The complement and coagulation systems communicate with one another. Platelets can lead to the activation of complement factors C3 and C5. Further, C4 activation can occur on platelets. Recently, Wang et al demonstrated that C4a is a novel soluble, untethered agonist for both PAR1 and PAR4 on human microvascular cells (HMEC-1). Given the importance of PAR1 and PAR4 for platelet physiology and the crosstalk between the complement system and platelets, we explored the potential of C4a as an agonist for human platelets.

METHODS

Reagents

The VS-tag antibody conjugated with fluorescein isothiocyanate (FITC) (catalog no. R963-25) was from Invitrogen (Carlsbad, CA, USA). The Phospho-p44/42 mitogen-activated protein kinase (MAPK) (extracellular signal-regulated kinase [ERK] 1/2) (Thr202/Tyr204) (D13.14.4E) Rabbit monoclonal antibody (mAb; catalog no. 4370) and p44/42 MAPK (ERK1/2) (3A7) Mouse mAb (catalog no. 9107) were both purchased from Cell Signaling Technology, Inc (Danvers, MA, USA). Trypsin (catalog # V511A) was from Promega (Fitchburg, WI, USA). The PAR1 activation peptide, SFLLRN-NH2, and TFLLRN-NH2 were from Tocris Bioscience (Minneapolis, MN, USA). PAR4 activation peptide, AYPGKF-NH2, was from GenScript (Piscataway, NJ, USA). C4a (catalog no. A106, Lot 16) was from Complement Technology (Tyler, TX, USA). Human α-thrombin (catalog # HCT-0020, specific activity >2989 U/mg) was from Hematological Technologies (Essex Junction, VT, USA). Vorapaxar (catalog no. 1755, batch 2) was from Axon Medchem (Reston, VA, USA). All other reagents were from Thermo Fisher Scientific (Pittsburgh, PA, USA) except where noted.

Platelet isolation and aggregation

With approval by the Case Western Reserve University Institutional Review Board, human whole blood was obtained by venipuncture from healthy donors after obtaining informed consent. Whole blood was collected into sodium citrate (3.2% buffered sodium citrate). The blood was centrifuged at 200 g for 15 minutes to obtain platelet-rich plasma (PRP). Platelet concentrations were quantified using a Coulter Counter. PRP was stimulated with AYPGKF-NH2 (250 μM), SFLLRN-NH2 (30 μM), or C4a (3.2 μM). Platelet aggregation was measured under constant stirring (1200 rpm) with a Chrono-log Model 700 aggregometer using Aggrolink8 version 1.3.98 (Chrono-log Corp, Havertown, PA, USA).
2.3 | Cell culture

As reported previously, human embryonic kidney (HEK) 293 Flp-In T-REx cells were maintained in Dulbecco's modified Eagle’s medium (Invitrogen, Waltham, MA, USA) containing 5% fetal bovine serum (FBS) at 37°C with 5% CO₂. Human PAR4 containing an N-terminal V5-epitope were stably expressed using the HEK293 Flp-In T-REx cells according to the manufacturer’s protocol (Invitrogen). The concentration of tetracycline was titrated (700 ng/mL) to yield PAR4 expression at ~175 000 receptors on the cell surface at 40 hours as determined by quantitative flow cytometry, as described below.15 Some cells were processed for flow cytometry, and the remaining cells were processed for Ca²⁺ mobilization.

HMEC-1 and the immortalized endothelial cell line ea.HY926 were purchased from ATCC (Gaithersburg, MD) and were maintained as reported previously.12 Specifically, the cells were maintained in MCDB 131 (Gibco) containing 10% FBS, 1 μg/mL hydrocortisone, 2 mM glutamine, and 10 ng/mL epidermal growth factor (Fisher Scientific) at 37°C with 5% CO₂.

2.4 | Flow Cytometry

Following induction, PAR4 expressing cells were harvested in Versene solution and collected by centrifugation. The cells were then washed three times with HEPES-Tyrode’s Buffer, pH 7.4 (10 mM 4-2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 12 mM NaHCO₃, 130 mM NaCl, 5 mM D-glucose, 5 mM KCl, 0.4 mM NaHPO₄, 1 mM MgCl₂). To assess PAR4 expression before each signaling experiment, cells were incubated with FITC conjugated anti-V5 antibody for 30 minutes. Cells were analyzed using a BD LSRFortessa cell analyzer (BD Biosciences, Mississauga, ON, Canada). PAR4 expression was quantified using Quantum Simply Cellular beads from Bangs Laboratories, Inc (Fishers, IN, USA) to generate a standard curve of antibody-binding sites as previously described.13

2.5 | Calcium mobilization in HEK295 Flp-In cells

As reported previously, parental HEK293 Flp-In or PAR4 expressing cells were incubated with 0.5 μM Fura-2 with or without 100 nM vorapaxar for 1 hour. The cells were then washed three times in HEPES-Tyrode's buffer with 200 μM CaCl₂ and diluted to 2.5 × 10⁵ cell/mL. The intracellular calcium mobilization was recorded using a fluorometer from Photon Technology International Inc (Edison, NJ, USA) for 420 seconds total. The first 50 seconds was set as the background, the response to agonist was measured for 250 seconds, and the maximum and minimum fluorescence was measured for 60 seconds each by adding 0.1% Triton X100 and 8.8 mM ethylene glycol-tetraacetic acid sequentially.13 Fluorescence values were converted to intracellular free Ca²⁺ concentration with the equations described by Grynkiewicz et al.13,14

2.6 | ERK activation in HMEC-1 cells and ea.HY926 cells

As reported previously, HMEC-1 or ea.HY926 cells were seeded into the 6-well plates and cultured at 37°C with 5% CO₂ for 48 h until confluent.12 The complete growth medium was exchanged to starving medium (Hanks' Balanced Salt Solution containing 1% bovine serum albumin), and the cells were further cultured for an additional 4 hours. The cells were treated with four different conditions: 0.3 μM of C4a for 7 minutes, 5 nM of thrombin for 10 minutes, 300 μM of AYPGKF-NH₂ for 10 minutes, or 50 μM of TFLLRN-NH₂ for 10 minutes. After stimulation, the cells were immediately washed with ice cold Dulbecco’s phosphate buffered saline and lysed in 1 × RIPA buffer containing protease inhibitors and PhosSTOP (Roche, Basel, Switzerland) on ice for 30 minutes. After a 20-minute spin at 20 000 g at 4°C, the supernatant of each sample was collected and the loading buffer was added. The samples were then boiled for 5 minutes, separated by 10% SDS-PAGE, and analyzed using western blot. The levels of ERK1/2 and total ERK1/2 were detected by anti-phospho-ERK1/2 and anti-ERK1/2 antibodies. Immunoblots were imaged by the Odyssey infrared imaging system and quantified by Image Studio software (LI-COR Biosciences, Lincoln, NE, USA). The level of ERK1/2 activation was presented as the ratio of densitometry of phospho-ERK1/2 of total ERK1/2.

3 | RESULTS AND DISCUSSION

Wang et al used a cell-based β-arrestin reporter assay to demonstrate that C4a dose-dependently activated both PAR1 and PAR4 in HMEC-1.12 The half maximal effective concentration (EC₅₀) of C4a activation of PAR1 and PAR4 was 0.8 μM and 0.6 μM, respectively.12 Since PAR1 and PAR4 have primary roles in platelet activation, we investigated the potential of C4a to serve as an agonist for human platelets. PRP was isolated from whole blood of four independent donors on different days for aggregation studies. To increase the rigor of the study, platelet aggregation experiments were performed by two individuals. The four donors were consisted of different races, ages, and genders. In all four cases, platelets responded to 30 μM PAR1-AP (SFLLRN) or 250 μM PAR4-AP (AYPGKF). In contrast, platelets did not respond to 3.2 μM C4a. Figure 1 shows the representative data from two donors. We extended the recording time to 15 minutes, and still no aggregation was triggered by 3.2 μM C4a (Figure 1B).

The EC₅₀ values of C4a reported by Wang et al12 is 0.8 μM for PAR1 and 0.6 μM for PAR4. The dose of C4a we used in our platelet aggregation assay is 4 times of the EC₅₀ of PAR1 measured by Wang et al. Theoretically, this should give us the maximum activation of PAR1 if C4a is truly a platelet PAR1 agonist. Further, the plasma concentration of C4a varies from 68.91 ± 33.23 ng/mL (7.97 nM) measured by a cytometric bead assay to 2398 ng/mL (277 nM) using an ELISA.15,16 Nonetheless, in our experiments, platelets do not respond to C4a at a concentration 12- to 400-fold...
higher than is found in plasma. Given that platelets are exquisitely sensitive to agonists due to their many feed-forward activation mechanisms and the concentration of C4a in our experiment (3.2 μM) is beyond physiologically relevant levels, we did not attempt higher concentrations of C4a. Our observation is in agreement with the conclusions from other reports that C4a does not activate platelets.17-21

We next tested the ability of C4a to activate calcium signaling via PAR1 in HEK293 Flp-in T-rex cells, which express PAR1 endogenously. Thrombin or the PAR1-specific activation peptide, TFLLRN-NH₂, induced transient intracellular Ca²⁺ mobilization (Figure 2A and B, red traces). In both cases, Ca²⁺ signaling was completely abolished when the cells were preincubated for 1 hour with 100 nM of PAR1 antagonist, vorapaxar (Figure 2A and B, orange traces). As expected, there was no calcium flux induced by the stimulation of 250 μM of PAR4-activation peptide (AYPGKF-NH₂) since HEK293 Flp-in cells do not express PAR4 endogenously (Figure 2B). We next used this experimental design to specifically test PAR1 response to C4a. Similar to our platelet aggregation experiments, C4a did not activate PAR1-mediated Gαq signaling at 3.2 μM (Figure 2C), which is four times the reported EC₅₀ to activate PAR1 in Chinese hamster ovary (CHO)-K1 cells.12
To evaluate C4a-mediated PAR4 activation, we used inducible cell lines stably expressing wild-type PAR4 with an N-terminal V5-epitope tag. To isolate the thrombin signaling to the exogenously expressed PAR4, cells were pretreated for 1 hour with 100 nM vorapaxar as above. PAR4 elicited the expected sustained Ca\textsuperscript{2+} mobilization compared to PAR1 stimulation (Figure 3A).\textsuperscript{11,22} Vorapaxar specifically ablated the response to PAR1, but left PAR4 response unaltered as determined by sequential activation by 50 μM of PAR1-AP stimulation (for endogenous PAR1) and 250 μM of PAR4-AP stimulation (for exogenous PAR4) (Figure 3B). As we observed in platelets, 2.4 μM C4a failed to induce Ca\textsuperscript{2+} flux in vorapaxar treated cells expressing PAR4 (Figure 3C), which is four times the reported EC\textsubscript{50} to activate PAR4 in CHO-K1 cells.\textsuperscript{12} Typically, treatment of an agonist at a dose of four times its EC\textsubscript{50} should lead to the maximum receptor response in a dose-response curve.

To further evaluate the C4a-mediated PAR activation on HMEC-1 cells and ea.HY926 cells, we measured the level of the ERK1/2 activation upon C4a stimulation.\textsuperscript{12} The activation of PAR1 and PAR4, either by thrombin via proteolysis or by their activation peptides, leads to a series downstream signaling mediated by G-proteins, which includes the phosphorylation of ERK1/2.\textsuperscript{23,24} We stimulated the HMEC-1 with four different conditions: 0.3 μM of C4a for 7 minutes, 5 nM of thrombin for 10 minutes, 300 μM of AYPGKF-NH\textsubscript{2} for 10 minutes, or 50 μM of TFLLRN-NH\textsubscript{2} for 10 minutes, and the level of ERK1/2 activation was measured by western blot. Compared to the untreated group, stimulation with 5 nM of thrombin induced an increase of phospho-ERK/total-ERK by two-fold. In addition, both 300 μM of PAR4-AP (for endogenous PAR4) or 50 μM of PAR1-AP (for endogenous PAR1) activated ERK1/2 in HMEC-1 cells. Interestingly, upon 0.3 μM of C4a stimulation, we observed a variable signal of ERK1/2 activation over the course of five replicates (Figure 4A). A representative blot is shown in Figure 4B. We detected an increase of ERK1/2 activation in only one of the replicates. The ea.HY926 cells responded to thrombin, but not to C4a stimulation (data not shown). Taken together, our data suggest that C4a is not an endogenous agonist for PAR1 or PAR4.

The PAR family members share the same proteolytic activation mechanism; however, the sequence homology between family members is low and not significantly higher than other GPCRs. It is not surprising that agonist peptides derived from the tethered ligand and small-molecule antagonist generally do not cross react between PARs. The notable exception is the cross reactivity of the PAR1 agonist peptide (SFLLRN) with PAR2. Specific activation of PAR1 is achieved with the synthetic peptide TFLLRN. PAR1 and PAR4 are both activated by thrombin cleaving the N-terminus. However, the exodomains of PAR1 and PAR4 are dramatically different, which results in unique tethered ligands. The tethered ligand sequence for PAR1 is SFLLRNPNDKYE… compared to GYPGQVCANDSD… for PAR4. Since the canonical ligands for PAR1 and PAR4 are distinct and do not cross react, it implies that the ligand binding sites are also unique. Our recent hydrogen deuterium exchange data support this hypothesis.\textsuperscript{13} Wang and colleagues\textsuperscript{12} show that both C4a and its inactive metabolite, C4a desArg, act as soluble activation ligands for both PAR1 and PAR4. These observations are exciting for two reasons. First, there have been no physiological soluble agonist for PAR1 or PAR4 previously described. Second, it strongly suggested that PAR1 and PAR4 have an additional ligand binding site that is conserved between PAR1 and PAR4 and regulates receptor function allosterically. Wang and colleagues screened C4a against a panel of GPCRs and identified C4a as a ligand for both PAR1 and PAR4 using a cell-based reporter assay. In human endothelial cells, their comprehensive

**FIGURE 3** C4a does not induce protease-activated receptor 4 (PAR4)-mediated intracellular calcium mobilization. (A) Human embryonic kidney (HEK) 293 cells expressing wild-type PAR4 were stimulated with 100 nM thrombin in the presence of 100 nM vorapaxar, and calcium mobilization was recorded. (B) The endogenous PAR1 on HEK293 was activated by 50 μM TFLLRN (PAR1-AP; green trace), which was inhibited by 100 nM vorapaxar (blue trace). The exogenously expressed PAR4 was activated by 250 μM AYPGKF (PAR4-AP). (C) No PAR4-mediated calcium mobilization was triggered by 2.4 μM C4a.
FIGURE 4  C4a does not activate extracellular signal-regulated kinase (ERK) 1/2 in human microvascular endothelial cells (HMEC-1). (A) Thrombin, protease-activated receptor 4 (PAR4)-AP (AYPGKF), and PAR1-AP (TFLLRN) activated ERK on HMEC-1 cells compared to the untreated group. The experiment was repeated 5 times indicated as scattered dots. (B) A representative blotting is shown.

experiments of ERK activation and calcium mobilization indicated that C4a-mediated cell activation is a PAR1- or PAR4-dependent manner, which suggested that C4a can act as a nontraditional free ligand that regulates both PAR1 and PAR4. In addition, the inactive metabolite of C4a, C4a desArg, was also a soluble agonist for PAR1 and PAR4, although less potent compared to C4a. The report from Wang et al also added to the complement field. C4a has been described as an anaphylatoxin with low activity. The functional profile of C4a is extremely low, and it was initially thought to be inactive. Furthermore, C4a is converted to its inactive form, C4a desArg, by the action of the plasma enzyme carboxypeptidase N within minutes. This has made studying the role of C4a difficult. Moreover, to date, there has been no C4a receptor identified and C4a cannot signal through C3a and C5a receptors. The report by Wang et al showed the potential of C4a signaling through PAR1 and PAR4, which indicates the C4a may have an unrecognized role in complement system.

In agreement with several previous reports, we did not see activation platelets in response to C4a stimulation. Our data also show that C4a does not activate platelet PAR1 or PAR4. C4a was also unable to activate PAR1- or PAR4-mediated Gq activation on HEK293, which was measured by intracellular calcium mobilization. C4a concentration in plasma can be very different depending on the context, the disease condition, and sensitivity of the measurement methods. For example, the C4a concentration in the healthy control group is 68.91 ± 33.23 ng/mL (7.97 nM) compared to the 108.48 ± 83.83 ng/mL or 12.54 nM in the participants with neovascular age-related macular degeneration measured by a cytometric bead assay. In another study using an ELISA, the concentration of C4a is 2398 ng/mL (277 nM) in the healthy controls compared to 2304 ng/mL (266 nM) in patients with chronic hepatitis B infection but without liver failure and 1811 ng/mL (209 nM) is the patient with hepatitis B infection-related acute-on-chronic liver failure. However, in any case, treating human platelets with 3.2 μM of C4a (4- to 12-fold higher than physiological levels) should elicit platelet aggregation if C4a is an agonist for platelet PARs. The contradiction to the published results by Wang et al may further demonstrate the importance of cellular context for receptor activation. For example, PARs signal indirectly through Gq in platelets via feedback to P2Y12. C4a may be allosterically activating PAR1 or PAR4 in microvascular cells to trigger Gq signaling. The mechanism by which cell selective activation of PARs by C4a is mediated is not known but may involve specific membrane localization, cofactors, or both. Cell-specific regulation of protease signaling by soluble mediators offers intriguing new lines of research to identify cofactors or membrane environments required. As the field moves forward, we can draw on the knowledge gained though investigating the APC-PAR1 axis, which requires membrane localization and endothelial protein C receptor.

C4a has been studied for 40 years and its role is still undefined. To date, a C4a-specific receptor has not been identified. Although C4a binds to C3a receptor (C3aR) at micromolar concentrations, this binding does not trigger calcium mobilization. In addition, as Lienknlaus et al showed in 1998, C4a could potentially be an agonist of the guinea pig but not human C3a receptor. In this report, they expressed and purified C4a in Escherichia coli to avoid contamination of C3a and C5a that could occur from purifying C4a from human serum. They used HEK293 cells expressing either guinea pig C3aR or human C3aR, and showed that their purified C4a triggered calcium mobilization in HEK293 cells expressing guinea pig C3aR but not HEK293 cells expressing human C3aR. It is important to note that HEK293 cells express endogenous expressing PAR1, we also used HEK293 cells in our study and did not see a response to C4a. The lack of response of human C3aR expressing HEK cells with up to 10^6 M C4a is in agreement with our HEK293 data (Figure 2). Finally, several studies have used HEK293 cell lines to study C4a binding to C3aR or C5aR. If C4a stimulation triggered any cellular response of HEK293, which has PAR1 on its surface, it would have been observed in these studies. Taken together, a careful reading of the literature and our platelet data are in agreement that C4a is not a physiological agonist for PAR1 or PAR4.

AUTHOR CONTRIBUTIONS

XH, MD, and MN conceived the study, designed the experiments, performed the experiments, and analyzed the data. XH and MN wrote the manuscript. MD critically read and edited the manuscript.
The authors declare no conflicts of interest.

REFERENCES

1. Vu TK, Hung DT, Wheaton VI, Coughlin SR. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell. 1991;64(6):1057–68.

2. Nieman MT. Protease-activated receptors in hemostasis. Blood. 2001;108(2):169–77.

3. Han X, Bouck EG, Zunica ER, Arachiche A, Nieman M. Protease-activated receptors in hemostasis. Blood. 2016;128(2):169–77.

4. Zhao P, Metcalf M, Bunnett NW. Biased signaling of protease-activated receptors. Front Endocrinol (Lausanne). 2014;5:67.

5. Cottrell GS, Coelho AM, Bunnett NW. Protease-activated receptors: the role of cell-surface proteolysis in signalling. Essays Biochem. 2002;38:169–83.

6. Camerer E, Kataoka H, Kahn M, Lease K, Coughlin SR. Genetic evidence that protease-activated receptors mediate factor Xa signaling in endothelial cells. J Biol Chem. 2002;277(18):16081–7.

7. Sveshnikova AN, Balatsky AV, Demianova AS, Shepelyuk TO, Shakhidzhanov SS, Balatsky MN, et al. Systems biology insights into the meaning of the platelet’s dual-receptor thrombin signaling. J Thromb Haemost. 2016;14(10):2045–57.

8. Woulfe DS. Platelet G protein-coupled receptors in hemostasis and thrombosis. J Thromb Haemost. 2005;3(10):2193–200.

9. Voss B, McLaughlin JN, Holinstat M, Zent R, Hamn HE. PAR1, but not PAR4, activates human platelets through a Gi/o/phosphoinositide-3 kinase signaling pathway. Mol Pharmacol. 2007;71(5):1399–406.

10. Peerschke EI, Yin W, Ghebrehiwet B. Complement activation on platelets: implications for vascular inflammation and thrombosis. Mol Immunol. 2010;47(3):2170–91.

11. Peerschke EI, Yin W, Ghebrehiwet B. Complement activation on platelets: implications for vascular inflammation and thrombosis. Mol Immunol. 2001;47(3):2170–91.

12. Wang H, Ricklin D, Lambris JD. Complement-activation fragment C4a mediates effector functions by binding as unbound agonist to protease-activated receptors 1 and 4. Proc Natl Acad Sci U S A. 2017;114(41):10948–53.

13. Han XU, Hofmann L, de la Fuente M, Alexander N, Palczewski K, Nieman MT. PAR4 activation involves extracellular loop 3 and transmembrane residue Thr153. Blood. 2020;136(19):2217–28.

14. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca2+ indicators with greatly improved fluorescence properties. J Biol Chem. 1985;260(6):3440–50.

15. Lechner J, Chen M, Hogg RE, Toth L, Silvestri G, Chakravarthy U, et al. Higher plasma levels of complement C3a, C4a and C5a increase the risk of subretinal fibrosis in neovascular age-related macular degeneration: complement activation in AMD. Immun Ageing. 2016;13:4.

16. Li Q, Lu Q, Zhu MQ, Huang C, Yu KK, Huang YX, et al. Lower level of complement component C3 and C3a in the plasma means poor outcome in the patients with hepatitis B virus related acute-on-chronic liver failure. BMC Gastroenterol. 2020;20(1):106.

17. Barnum SR. C4a: an anaphylatoxin in name only. J Innate Immun. 2015;7(4):333–9.

18. Klos A, Tenner AJ, Johnkewitz KO, Ager RR, Reis ES, Kohl J. The role of the anaphylatoxins in health and disease. Mol Immunol. 2009;46(14):2753–66.

19. Klos A, Wende E, Wareham KJ, Monk PN. International Union of Basic and Clinical Pharmacology. [corrected]. LXXXVII. Complement peptide C5a, C4a, and C3a receptors. Pharmacol Rev. 2013;65(1):500–43.

20. Meuer S, Ecker U, Hadding U, Bitter-Suermann D. Platelet-serotonin release by C3a and C5a: two independent pathways of activation. J Immunol. 1981;126(4):1506–9.

21. Meuer S, Hugli TE, Andreotta RH, Hadding U, Bitter-Suermann D. Comparative study on biological activities of various anaphylatoxins (C4a, C3a, C5a). Inflammation. 1981;5(4):263–73.

22. Covic L, Gresser AL, Kulis H. Biphasic kinetics of activation and signaling for PAR1 and PAR4 thrombin receptors in platelets. Biochemistry. 2000;39(18):5458–67.

23. Coughlin SR. Thrombin signalling and protease-activated receptors. Nature. 2000;407(6801):258–64.

24. Elshingrello H, Kongsamut S. Minireview: targeting GPCR activated ERK pathways for drug discovery. Curr Chem Genom Transl Med. 2013;7:9–15.

25. Gorski JP, Hugli TE, Muller-Eberhard HJ. C4a: the third anaphylatoxin of the human complement system. Proc Natl Acad Sci U S A. 1979;76(10):5299–302.

26. Mueller-Ortiz SL, Wang D, Morales JE, Li L, Chang YJ, Wetzel RA. Targeted disruption of the gene encoding the murine small subunit of carboxypeptidase N (CPN1) causes susceptibility to C5a anaphylatoxin-mediated shock. J Immunol. 2009;182(10):6533–9.

27. Tsuruta T, Yamamoto T, Matsubara S, Nagasawa S, Tanase S, Tanaka J, et al. Novel function of C4a anaphylatoxin. Release from monocytes of protein which inhibits monocyte chemotaxis. Am J Pathol. 1993;142(6):1848–57.

28. Ames RS, Tornetta MA, Foley JJ, Hugli TE, Sarau HM. Evidence that the receptor for C4a is distinct from the C3a receptor. Immunopharmacology. 1997;38(1–2):87–92.

29. Lienenklaus S, Ames RS, Tornetta MA, Sarau HM, Foley JJ, Crass T, et al. Human anaphylatoxin C4a is a potent agonist of the guinea pig but not the human C3a receptor. J Immunol. 1998;161(5):2089–93.

30. Kalant D, Cain SA, Smolarska M, Sniderman AD, Cianflone K, Monk PN. The chemoattractant receptor-like protein C5L2 binds the C3a-des-Arg77/acylation-stimulating protein. J Biol Chem. 2003;278(13):11123–9.

31. Kalant D, MacLaren R, Cui W, Samanta R, Monk PN, Laporte SA, et al. C5L2 is a functional receptor for acylation-stimulating protein. J Biol Chem. 2005;280(25):23936–44.

32. Okinaga S, Slattery D, Humbles A, Zsengeller Z, Morteau O, Kinrade MB, et al. C5L2, a nonsignaling C5a binding protein. Biochemistry. 2003;42(31):9406–15.

33. Zhang T, Garstka MA, Li K. The controversial C5a receptor C5aR2: its role in health and disease. J Immunol Res. 2017;2017:8193932.