C5a Receptor-Dependent Cell Activation by Physiological Concentrations of Desarginated C5a: Insights from a Novel Label-Free Cellular Assay

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The complement anaphylatoxins C3a, C5a, and desarginated C5a ($C5a^{desArg}$) play critical roles in the induction of inflammation and the modulation of innate and acquired immune responses after binding to their G protein-coupled receptors, C3a receptor and C5a receptor (C5aR). The role of $C5a^{desArg}$ in inducing cell activation has been often neglected, because the affinity of $C5a^{desArg}$ for C5aR has been reported to be much lower than that of C5a. We have used a novel label-free cellular assay to reassess the potential of $C5a^{desArg}$ to induce activation of transfected and primary immune cells. Our results indicate that physiological levels of $C5a^{desArg}$ induce significant levels of cell activation that are even higher than those achieved by stimulating cells with analogous concentrations of C5a. Such activation was strictly dependent on C5aR, because it was completely abrogated by PMX-53, a C5aR antagonist. Pharmacological inhibition of specific G proteins located downstream of C5aR indicated differential involvement of G_α proteins upon C5aR engagement by C5a or $C5a^{desArg}$. Further, mass spectrometric characterization of plasma-derived C5a and $C5a^{desArg}$ provided important insight into the posttranslational modification pattern of these anaphylatoxins, which includes glycosylation at Asn^{64} and partial cysteinylation at Cys^{27}. Although the context-specific physiological contribution of $C5a^{desArg}$ has to be further explored, our data suggest that $C5a^{desArg}$ acts as a key molecule in the triggering of local inflammation as well as the maintenance of blood surveillance and homeostatic status. *The Journal of Immunology*, **2012, 189: 4797–4805.**

Complement is increasingly recognized as an efficient surveillance system that participates in the clearance of microbes, immune complexes, and cellular debris and in the modulation of inflammatory, innate, and acquired immune responses. Under normal conditions, complement is activated in response to various pathogen- and damage-associated molecular patterns, immune complexes, and apoptotic cells, but it can also be directly induced by coagulation factors and via a tick-over mechanism that assures constant low-level complement activation in the circulation. Triggering any of the traditional activation pathways (classical, lectin, or alternative) converges in the cleavage of complement components C3 and C5 and culminates in the release of the anaphylatoxins C3a and C5a and the assembly of the terminal complement complex (1).

Anaphylatoxins exert their biological functions via the G protein-coupled receptors (GPCR) C3a receptor (C3aR) and C5a receptor (C5aR; CD88), which are present at the surface of several types of myeloid cells as well as tissue cells (2). Carboxypeptidases present in the serum and tissues quickly degrade C3a and C5a by removing their C-terminal Arg residue, producing the so-called desarginated C3a ($C3a^{desArg}$) and desarginated C5a ($C5a^{desArg}$) anaphylatoxin fragments (3). In the case of C3aR, only C3a (and not $C3a^{desArg}$) has been reported to bind and activate the receptor (4). In contrast, both C5a and $C5a^{desArg}$ have been shown to trigger activation of C5aR; however, the binding affinity of C5a toward C5aR has been reported to be ~100-fold stronger than that of $C5a^{desArg}$ for C5aR (5). Various in vitro assays have indicated a markedly lower potency of $C5a^{desArg}$ (as compared with C5a) with regard to the induction of neutrophil degranulation, chemotaxis, and release of intracellular calcium (6). Taken together, these observations over time shaped the concept of $C5a^{desArg}$ being the product of a quick deactivation of the potent C5a in circulation. Historically, however, $C5a^{desArg}$ has been found to induce robust cell responses via C5aR signaling both in vivo and in vitro, yet this activity appears to be dependent on the experimental context and readout as well on the cell type (7–10).

In addition to C5aR, a second anaphylatoxin receptor, C5a receptor-like 2 (C5L2; GPR77), has been shown to bind both C5a and $C5a^{desArg}$. Unlike C5aR, however, C5L2 does not seem to couple to G-proteins, and $C5a^{desArg}$ binds with a 20–30-fold higher affinity to C5L2 than to C5aR (5, 11). Even though the binding of C3a and $C3a^{desArg}$ to C5L2 has been proposed, their

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Abbreviations used in this article: $C5a^{desArg}$, desarginated C5a; C3aR, C3a receptor; C5aR, C5a receptor; C5L2, C5a receptor-like 2; CPNI, carboxypeptidase N inhibitor; CTX, cholera toxin; Gc-globulin, group-specific component globulin; GPCR, G protein-coupled receptor; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PC, photonic crystal; PMN, polymorphonuclear; PTX, pertussis toxin; PWV, peak wavelength value; RBL, rat basophil leukemia.

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contribution to and significance for the functional activity of this receptor are an ongoing matter of debate. Nearly 12 y after its discovery, C5L2 is still considered an enigmatic molecule. Divergent roles have been suggested for this receptor, ranging from decoy and regulatory to anti- and proinflammatory (2), and it is clear that further studies are required to fully elucidate its participation in the modulation of cellular responses. The most recent report addressing molecular mechanisms has indicated that C5L2 is predominantly intracellular in human neutrophils and that it acts by negatively modulating C5a-induced C5aR signaling through the activation of the β-arrestin signaling pathway (12).

Along with their role in promoting inflammation, anaphylatoxins have been shown to modulate the induction of $CD4^+$ T cell immunity by regulating the cytokine profile produced by APCs (13–16). They are also key determinants of the outcome of disorders such as sepsis (17), asthma (18, 19), rheumatoid arthritis (20), transplant rejection (21), cancer (22), neurodegenerative (23), and periodontal (24) disorders, among others (25). It is not surprising, therefore, that anaphylatoxin receptors are among the best-investigated targets for therapeutic intervention in complement-mediated diseases (25, 26).

For the past 25 y, the measurement of cell activation triggered by anaphylatoxins has relied on techniques such as chemotaxis, degranulation, and calcium release assays. Heterogeneous sources of anaphylatoxins (i.e., recombinantly expressed or derived from plasma-purified C3 and C5) have been explored, often with little regard for potential differences between the native and recombinant proteins, such as glycosylation patterns. Furthermore, the specific evaluation of C5a^{desArg}-induced responses has often been neglected as a consequence of the proposed lower binding affinity of C5a^{desArg} for C5aR when compared with C5a. Importantly, C5a is rapidly and efficiently converted to C5a^{desArg} by serum carboxypeptidases, and most, if not all, of the C5a present in the circulation is found in the desarginated form (27). Therefore, a more holistic and comprehensive assessment of anaphylatoxin signaling is highly desirable, with the goal of generating improved models and assays for research and drug discovery purposes.

In this study, we have compared the ability of native anaphylatoxins (derived from human plasma-purified C3 or C5) to induce the activation of transfected and primary cells, by using a new label-free cell assay based on photonic crystal (PC) biosensors. This method employs highly sensitive measurements of changes in binding, morphology (i.e., activation-mediated effects on the cytoskeleton), or adherence of cells and biomolecules in proximity to a titanium oxide biosensor surface. Such events usually occur downstream of several cellular activation pathways, specifically those involving GPCR-derived cell responses. By recording activation signals over time, a kinetic and cell/receptor-specific activation profile can be obtained (28–31). Our data obtained by this novel method and by orthogonal assays provide new evidence for a significant role for C5a^{desArg} in cell activation and point to a new, easy, rapid, and reliable technology for detecting GPCR-mediated cell activation.

Materials and Methods

Cell culture

Rat basophil leukemia (RBL-2H3) cells transfected with C3aR, C5aR, or C5L2 were obtained as described elsewhere (5, 32). Cells were stably transfected by electroporation, and homogeneous cell populations (confirmed by flow cytometry) were used in this study. Cells were routinely cultured at 37°C and 5% CO_2 in DMEM with GlutaMAX (Life Technologies, Carlsbad, CA) supplemented with 10% FBS (Life Technologies), 50 U/ml penicillin, 50 μg/ml streptomycin (Life Technologies) and 400 μg/ml geneticin (CellGro, Manassas, VA).

Isolation of human polymorphonuclear cells

Blood was collected from healthy donors in the presence of 50 μg/ml lepirudin as anticoagulant to maintain complement-activation capacity (33). Human polymorphonuclear (PMN) blood cells were isolated as previously described (34) using Ficoll-Paque gradient centrifugation (GE Healthcare, Pittsburg, PA) followed by a dextran-sedimentation step. After hypotonic lysis of residual RBCs, PMN cells were resuspended in PBS and immediately used in the experiments.

Anaphylatoxins source and characterization

Native human C3a, C3a^{desArg}, C5a, and C5a^{desArg} prepared from plasma-derived C3 and C5, respectively, were purchased from Complement Technology (Tyler, TX). Endotoxin levels were determined to be <0.05 EU/μg protein. Deglycosylation of anaphylatoxins was achieved by treatment with PNGase F (New England Biolabs) under nondenaturing conditions as per the manufacturer's instructions.

The identity and relative concentrations of each anaphylatoxin were determined by mass spectrometry (MS) as follows: 5 pmol sample was injected into liquid chromatograpy-MS system for MS or tandem MS (MS/MS) analysis. After injection, analytes were separated by reversed-phase liquid chromatography with an online ACQUITY UPLC (Waters, Milford, MA) system coupled to MS. The system was equipped with a 1.7-μm UPLC BEH130 C18 column (1.0 μm × 100 mm; Waters), the temperature of which was held at 40°C. Proteins/peptides were separated at a flow rate of 0.15 ml/min. A linear gradient from 5–45% B (0.1% formic acid in acetonitrile) was applied over 8 min (for proteins) or 27 min (for peptides). MS and MS/MS analysis was performed on a SYNAPT G2S mass spectrometer equipped with an electrospray ionization source controlled by MassLynx 4.1 software (Waters). The capillary voltage was set to 3.2 kV, the cone voltage to 25 V, and the source temperature to 120°C. [Glu1]-fibrinopeptide B was used for lock-mass correction with a sampling rate of 30 s. Mass spectra were acquired in positive ionization mode over a mass-to-charge ratio range of 50–1900 Da at scan rate of 1 s by using both MS and MS^E methods. Deconvoluted spectra were generated by MaxEnt1 (Waters), and MS/MS spectra were processed by the ProteinLynx Global Server 2.51.

For further characterization and identification of posttranslational modifications, peptides were generated for each anaphylatoxin by digestion with trypsin or pepsin (1:25 enzyme/protein weight ratio for 18 h at 37°C). To reduce disulfide bonds, the sample was incubated for 45 min at room temperature in the presence of 10 mM DTT followed by treatment with 20 mM iodoacetamide (45 min at room temperature) for alkylation of sulfhydryl groups. Remaining iodoacetamide was neutralized by addition of 20 mM DTT.

Cell activation assays

Label-free cell activation assays were performed on a PC biosensor instrument (BIND PROFILER; SRU Biosystems, Woburn, MA). Such technology allows for the measurement of an integrated cellular response encompassing a variety of cellular events downstream of the GPCR. Briefly, optical biosensors located at the bottom of a microtiter plate detect changes in dielectric permittivity of attached biomolecules or cells (by measuring the resonant reflection spectrum after illumination by a source of infrared light). In the case of receptor assays, signal shifts may occur due to changes in cell adhesion, cell morphology, or dynamic mass redistribution upon stimulation of the GPCR; the resulting response profile is dependent on the cell type and signaling pathway, among others (28–31). Selected wells of a 384-well BIND CA2 biosensor plate (SRU Biosystems), containing a cell attachment matrix consisting of collagen, fibronectin, and OVA (28), were hydrated with 50 μl deionized water for 30 min at room temperature. The water was tapped out, and 25 μl complete medium (DMEM + 10% FBS) was added to each well and incubated for 30 min at room temperature. Medium background was detected in each well by scanning the biosensor plate with the BIND PROFILER instrument (SRU Biosystems). RBL cells were plated at a density of 2.5×10^4 cells/well in complete medium and incubated overnight at 37°C and 5% CO_2. The plates were then equilibrated at room temperature for 30 min, and background levels of cell attachment were determined with the BIND PROFILER (SRU Biosystems). Serial dilutions of anaphylatoxins (maximum concentration 100 nM) were prepared in 25 μl complete medium (unless otherwise specified) and added to the wells. Cell activation signals, measured as peak wavelength values (PWV) in picometers, were recorded over a period of 20 min after analyte addition, and the data were processed and analyzed using EMS software (SRU Biosystems).

PMN cell assays were performed as described above, except that cells were plated at a density of 5.0×10^4 cells/well in complete medium, and cells were incubated at 37°C for only 2 h to ensure viability.

Where indicated, C5aR was blocked by preincubation of the cells with the C5aR antagonist PMX-53 (35). As a control, an inactive linear peptide (PMX-control) was used in which the last two amino acids (Trp-Arg) were replaced by Ala-dArg. Group-specific component-globulin (Gc-globulin) and the carboxypeptidase inhibitor DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid were obtained from Sigma-Aldrich and EMD Biosciences, respectively. G-protein inactivation was achieved by incubation of cells with 0.5 μg/ml pertussis toxin (PTX; EMD Biosciences) or 10 μg/ml cholera toxin (CTX; Sigma-Aldrich) at 37°C for 15 h.

Flow cytometry

The expression of C3aR and C5aR on human PMN cells was detected with PE-conjugated anti-C3aR (clone hC3aRZ8; BioLegend, San Diego, CA) and FITC-conjugated anti-C5aR (clone 347214; R&D Systems, Minneapolis, MN) Abs, respectively. The expression of CD11b on human neutrophils was detected by cell staining with FITC-conjugated anti-CD11b (clone CBM1/5; BioLegend). Alternatively, cells were stained with the respective isotypes. All cell preparations showed at least 95% viability, as determined by ViaProbe (BD Biosciences, San Diego, CA) staining. Samples were then analyzed using BD FACSCanto II flow cytometer software (BD Biosciences) and FCS Express (v4.0; De Novo Software).

Results

Detection of anaphylatoxin-induced cell activation using a label-free cell assay

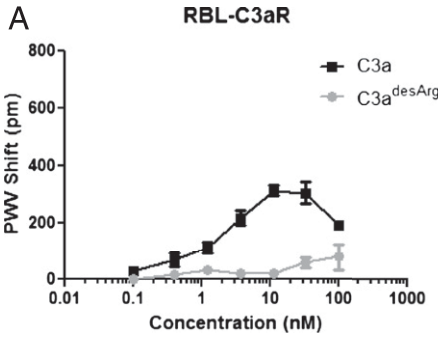
We set out to investigate the role of the anaphylatoxin receptors C3aR, C5aR, and C5L2 in triggering cell activation in response to stimulation by the complement activation fragments C3a, C3a^{desArg}, C5a, and C5a^{desArg}. We used native human serum-derived proteins to avoid potential variations related to recombinant proteins, such as folding heterogeneities or missing glycan chains. As expected, C3a, but not C3a^{desArg}, induced dose-dependent activation of C3aR-transfected RBL cells, with a maximal PWV shift of 270 ± 17 pm in response to 10 nM C3a. The calculated EC_{50} and Hill slope values of 1.8 nM and 0.37, respectively, indicated a strong stimulation of the receptor by C3a (Fig. 1A). Neither C5a nor C5a^{desArg} induced any response in C3aR-transfected cells.

In the case of the C5aR-transfected RBL cells, C5a induced strong cell activation, as evidenced by a PMV shift that reached a maximal value of 664 ± 27 pm in response to 33 nM C5a (Fig. 1B). EC_{50} and Hill slope values of 6.6 nM and 0.15, respectively, were calculated from the dose-response curve (Fig. 1B). We then determined cell activation profiles in response to the inactivated C5a form (i.e., C5a^{desArg}). Surprisingly, C5a^{desArg} triggered substantial cell activation, with a maximal value of 531 ± 23 pm and a stronger EC_{50} but higher Hill slope value than those of C5a (0.4 versus 6.6 nM and 1.6 versus 0.15, respectively) (Fig. 1B). These data suggest that low concentrations of C5a^{desArg} (up to 10 nM) have a greater ability to induce cell activation than do similar levels of C5a.

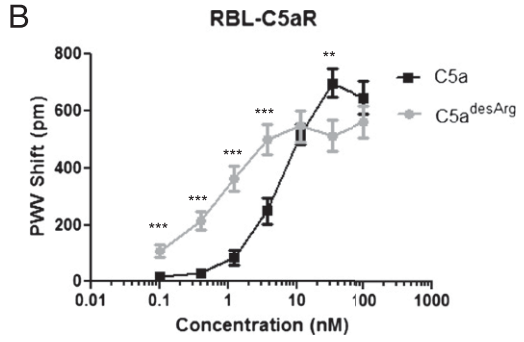
The same method was used to assess anaphylatoxin-induced activation responses mediated via the C5L2 receptor. However, none of the native anaphylatoxins (C3a, C3a^{desArg}, C5a, or C5a^{desArg}) induced significant activation signals on C5L2-transfected RBL cells (data not shown), indicating that the PC-based label-free technology may not be suitable for this particular type of non–GPCR-mediated activation event.

Mass spectrophotometric characterization of C5a and C5a^{desArg}

Our observation that C5a^{desArg} induces potent cell activation prompted us to run full MS characterization of C5a and C5a^{desArg} to certify the purity and identity of such proteins. The mass spectrum showed three main peaks with 10473, 10592, and 10708 Da for C5a (Fig. 2A, peaks on the right). Evaluation of the C5a^{desArg} sample resulted in similar spectrum of three peaks (10317, 10436, and 10582 Da), yet featuring a consistent difference of 156 Da between the C5a and C5a^{desArg} peaks that corresponds to the mass of one arginine residue (Fig. 2B, peaks on the right).



	C3a
EC_{50} (nM)	1.8 ± 0.4
Max. response (pm)	269.7 ± 16.5
Hill Slope	0.37 ± 0.1



	C5a	C5a^{desArg}
EC_{50} (nM)	6.6 ± 0.7	0.4 ± 0.07
Max. response (pm)	664 ± 27.1	531.3 ± 23.4
Hill Slope	0.15 ± 0.02	1.6 ± 0.5

FIGURE 1. Detection of anaphylatoxin-induced cell activation using a label-free cell assay. RBL-C3aR– (**A**) and RBL-C5aR–transfected (**B**) cell lines were attached to biosensor plates by incubation in complete medium (DMEM plus 10% FBS) for 16 h at 37°C, and cell activation was monitored in response to increasing concentrations of anaphylatoxins. Tables (*bottom panels*) show calculated EC_{50}, maximum response, and Hill slope values. $^{**}p < 0.01$, $^{***}p < 0.001$.

To identify the nature of the three distinct peaks, both C5a and C5a^{desArg} were deglycosylated and further analyzed by MS. Removal of oligosaccharides by the PNGase F enzyme resulted in spectra showing two peaks each with 8269 and 8388 Da for C5a (Fig. 2A, peaks on the left) and 8113 and 8232 Da for C5a^{desArg} (Fig. 2B, peaks on the left). The composition of the oligosaccharide chain was subsequently deduced after fragmentation of electrospray-ionized N-glycopeptides at a mass-to-charge ratio of 5096.1 (Supplemental Fig. 1). The resulting spectrum was dominated by a Y-type fragmentation pattern typically observed for glycosidic linkages (36). Our analysis identified the C5a/C5a^{desArg}-associated carbohydrate moiety as a sialylated biantennary glycan with the composition $(GlcNAc)_4(Man/Gal)_5(NeuAc)_2$ that was attached to Asn^{64} of the protein (Supplemental Fig. 1). Consistent with previous findings (37), removal of the oligosaccharide chain did not account for major changes in the activity potential (data not shown).

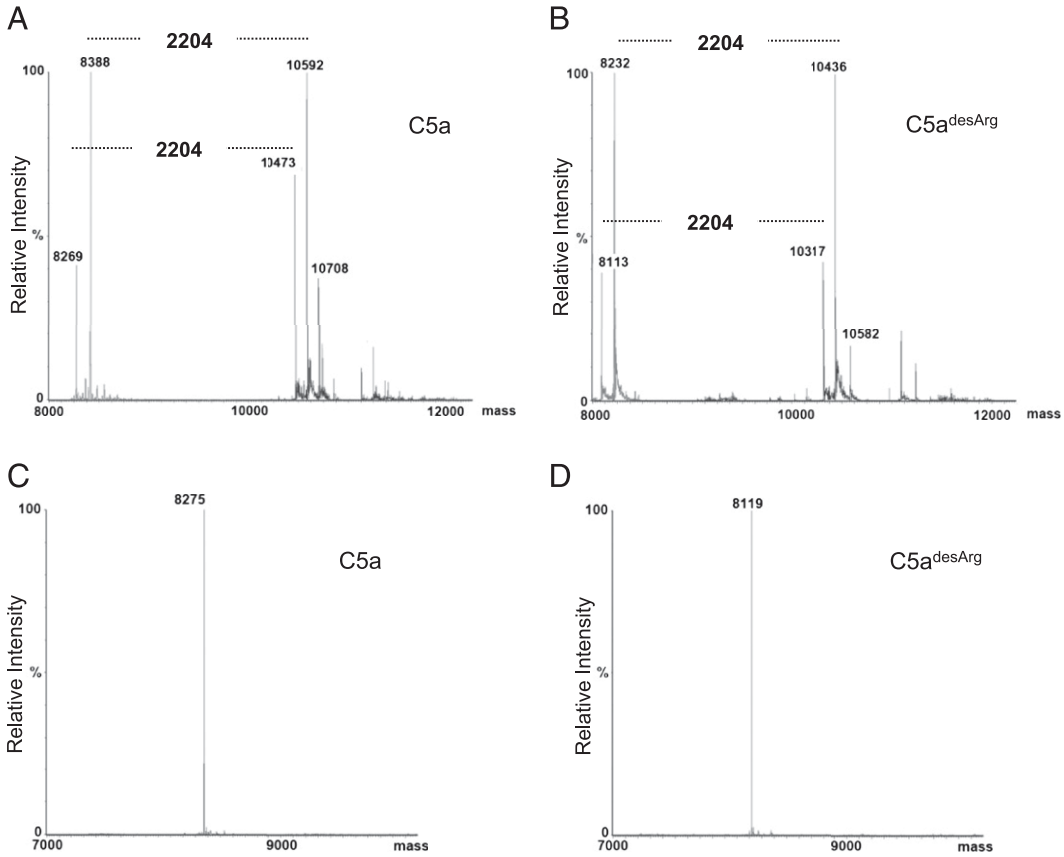


FIGURE 2. MS characterization of C5a and C5a^{desArg}. Deconvoluted data from MS analysis of native and deglycosylated C5a (**A**), C5a^{desArg} (**B**), and deglycosylated and reduced samples (**C**, **D**). Dotted line depicts mass difference between native and deglycosylated samples.

We further determined the identity of the two residual peaks after the deglycosylation of C5a and C5a^{desArg}. The minor peaks (8268 and 8113 Da, respectively) correspond to the expected mass based on the amino acid composition of these proteins (38), whereas both main peaks present a mass adduct of 119 Da. Using MS/MS analysis, the location of this modification could be pinpointed to residue Cys^{27}. Although C5a engages six of its cysteines in three disulfide bridges between residues 21–47, 22–54, and 34–55 (39), it also contains a seventh Cys residue at position 27 that is free and therefore prone to association with sulfhydryl-containing molecules in circulation (38). If the adduct involves free plasma cysteine, a phenomenon also known as cysteinylation, this results to a mass increase of 119 Da in the targeted protein (40) that should be sensitive to reductive treatment. Indeed, reduction of the disulfide bonds by DTT in deglycosylated C5a and C5a^{desArg} resulted in a spectrum with one single peak, each with mass corresponding to the amino acid sequence (Fig. 2C, 2D), thereby confirming partial cysteinylation of Cys^{27} in two out of three total C5a and C5a^{desArg}. Importantly, the peak pattern and ratios induced by these posttranslational modifications were highly comparable between C5a and C5a^{desArg}, therefore confirming that the only difference between the two proteins used in this study lies in the presence or absence of the C-terminal arginine residue.

C5a- and C5a^{desArg}-induced cell activation is entirely dependent on C5aR

As the MS results confirmed the identity of both C5a and C5a^{desArg}, we then investigated whether the potent cell activation induced by C5a^{desArg} results exclusively from C5aR signaling or may be influenced by additional, yet unidentified, mechanisms. For this purpose, we pharmacologically blocked C5aR signaling in C5aR-transfected RBL cells with the C5aR antagonist PMX-53 or an inactive control peptide (PMX-control) (35); we measured cell activation to a defined concentration of agonist (20 nM C5a^{desArg}) after addition of increasing antagonist concentrations. C5a^{desArg}-induced cell activation was inhibited by PMX-53 in a dose-dependent fashion, but not by the PMX-control peptide (Fig. 3A), indicating that the cell response triggered by C5a^{desArg} is entirely dependent on C5aR.

All of the assays described thus far were performed in the presence of medium containing 10% inactivated FBS to render the assay conditions more physiologic; similar results were obtained in the presence of 10% inactivated normal human serum (data not shown). To exclude the possibility that carboxypeptidases present in the serum were influencing our results by rapidly converting C5a into C5a^{desArg}, we repeated the assay in the presence of a potent carboxypeptidase inhibitor (2-mercaptomethyl-3-guanidinoethylthiopropionic acid; carboxypeptidase N inhibitor [CPNI]). Importantly, C5a induced similar levels of cell activation in the presence or absence of CPNI (Fig. 3B), thereby indicating that the inactivated FBS is devoid of active carboxypeptidases.

Serum potentiates C5a- and C5a^{desArg}-induced cell activation

To determine whether anaphylatoxin-induced cell signaling is affected by other factors present in the serum, we assessed cell activation after 2 h of serum starvation. Serum starvation resulted in markedly reduced levels of cell activation induced by either C5a or C5a^{desArg}, which produced maximal values of only 390 $\pm$ 26 and 381 $\pm$ 20 pm, respectively (Fig. 4A), as compared with 664 $\pm$ 27 and 531 $\pm$ 23 pm in the presence of serum (Fig. 1B). Notably, C5a and C5a^{desArg} induced similar maximal cell responses in the absence of serum, yet C5a^{desArg}-induced cell activation was associated with lower EC_{50} and higher Hill slope values than was C5a (0.5

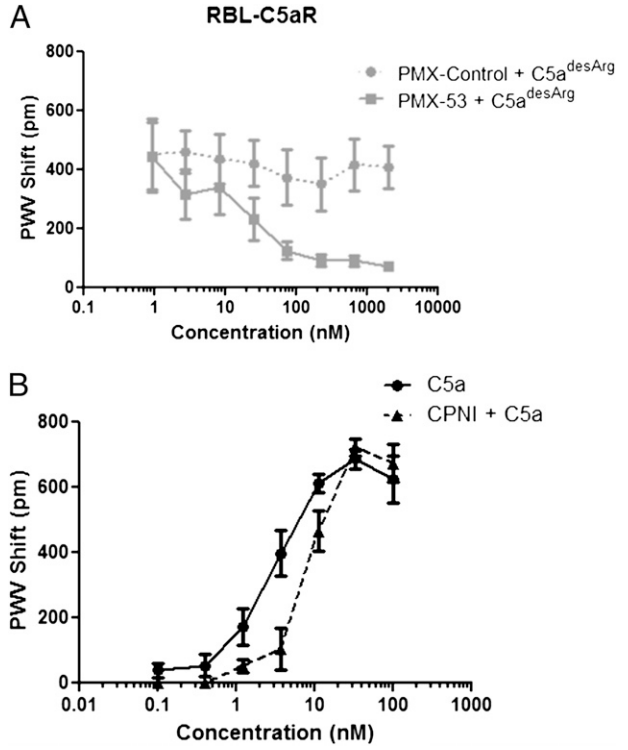


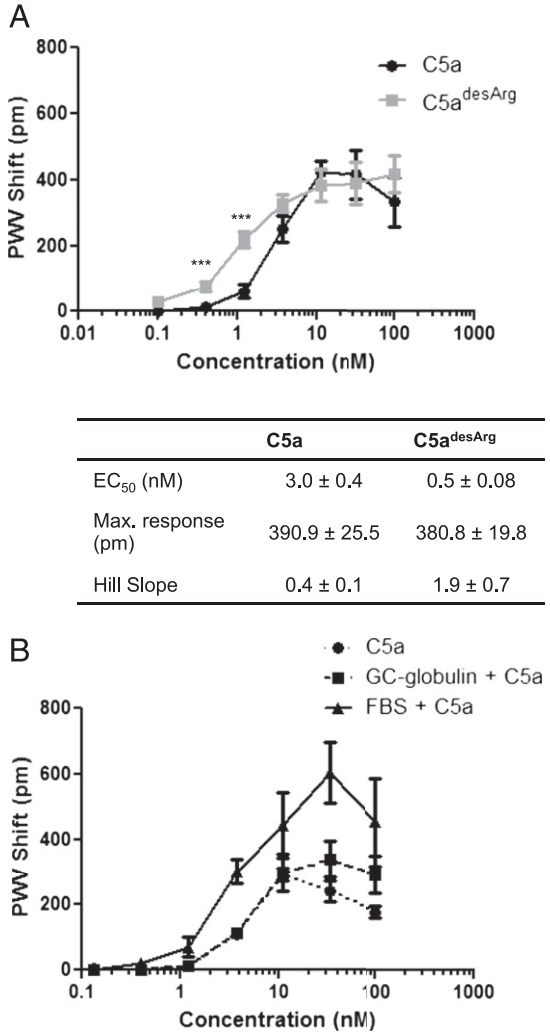
FIGURE 3. C5a- and $C5a^{desArg}$-induced cell activation is entirely dependent on C5aR. (**A**) RBL-C5aR cells attached to biosensor plates were preincubated for 1 h with various concentrations of the C5aR antagonist PMX-53 or a control peptide (PMX-Control), and cell activation was assessed after the addition of 20 nM $C5a^{desArg}$. (**B**) C5a-induced cell activation was assessed in cells that were preincubated (for 1 h) with or without 1 mM CPNI.

versus 3 nM and 1.9 versus 0.4, respectively) (Fig. 4A). These data indicate that factors present in the serum likely potentiate anaphylatoxin-induced cell activation in in vitro assays, either directly or by maintaining cell viability. Importantly, the 2 h of serum starvation did not change the expression levels of C5aR on the transfected RBL cells (data not shown).

Correlations between increased C5a-dependent chemotactic activity and the presence of Gc-globulin (also known as vitamin D-binding protein) in serum have been reported (41). We therefore asked whether Gc-globulin might have an impact on C5a-induced cell activation in our model. Preincubation of C5aR-transfected RBL cells with 50 nM of Gc-globulin in serum-free medium did not enhance C5a-induced cell activation to the extent we observed in the presence of serum (Fig. 4B), indicating that Gc-globulin is not responsible for the observed differences in C5a signaling in the presence and absence of serum.

Human PMN cells are activated by C5a and $C5a^{desArg}$, but not C3a or $C3a^{desArg}$

We next examined the ability of anaphylatoxins to induce the activation of human primary cells, more specifically blood PMN cells that express high levels of anaphylatoxin receptors under physiological conditions (Supplemental Fig. 2A). Despite their high expression of C3aR, human PMN cells did not respond to either C3a or $C3a^{desArg}$ (Fig. 5A). In contrast, C5a induced strong cell activation, leading to a maximal PWV shift of 1094 ± 94 pm and EC_{50} and Hill slope values of 10.3 nM and 0.1, respectively. Interestingly, the $C5a^{desArg}$-dependent cell response only reached a maximal PWV value of 612 ± 53 pm, ~2-fold lower than that induced by C5a (Fig. 5B). Yet despite the lower absolute response, the dose-dependent activation was actually stronger in the case of

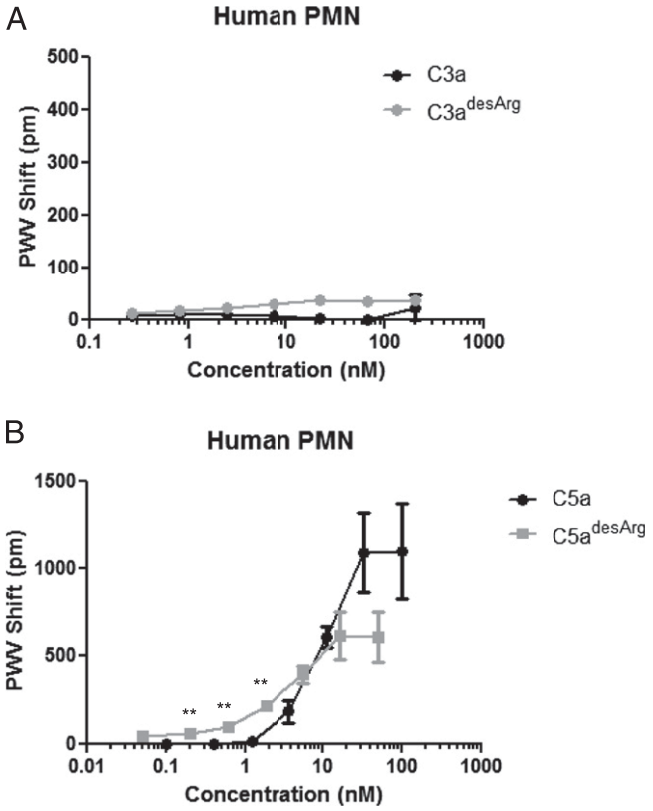


	C5a	$C5a^{desArg}$
EC_{50} (nM)	3.0 ± 0.4	0.5 ± 0.08
Max. response (pm)	390.9 ± 25.5	380.8 ± 19.8
Hill Slope	0.4 ± 0.1	1.9 ± 0.7

FIGURE 4. The presence of serum potentiates C5a- and $C5a^{desArg}$-induced cell activation. (**A**) RBL-C5aR cells were attached to biosensor plates by incubation in complete medium (DMEM plus 10% FBS) for 16 h at 37°C. The medium was replaced by serum-free medium, and cell activation in response to increasing concentrations of C5a or $C5a^{desArg}$ was monitored after 2 h of serum starvation. Calculated EC_{50}, maximum response, and Hill slope values are shown in the table (*bottom panel*). (**B**) C5a-induced cell activation was assessed in the presence and absence of 10% FBS (FBS + C5a) and 50 nM Gc-globulin (GC-globulin + C5a). $^{***}p < 0.001$.

$C5a^{desArg}$ with EC_{50} and Hill slope values of 4.0 nM and 0.2, respectively. Again, and as we had observed for the C5aR-transfected RBL cell line, low concentrations of $C5a^{desArg}$ (up to 5 nM) were more potent inducers of cell activation than analogous concentrations of C5a. This finding is of particular importance as physiologic plasma concentrations of $C5a^{desArg}$ range from 5–10 ng/ml (~1 nM) (42). Therefore, our data point to a constant activation of blood cells by $C5a^{desArg}$ that may be important for the maintenance of basal activation and blood surveillance.

The label-free cell assay detects the immediate response induced by the triggering of GPCRs by anaphylatoxins. In the case of PMN cells, activation signals were detected as early as 1 s upon addition of 100 nM of either C5a or $C5a^{desArg}$, with a maximal response obtained after 3 to 4 min (Supplemental Fig. 2B, *left panel*). Similarly, cell activation in response to lower concentrations (1 nM) of C5a or $C5a^{desArg}$ could be detected as early as 1 s, with a maximal response detected after 1 min upon ligand addition (Supplemental Fig. 2B, *right panel*). Notably, $C5a^{desArg}$-induced response is



	C5a	C5a^{desArg}
EC_{50} (nM)	10.3 ± 1.5	4 ± 0.9
Max. response (pm)	1094 ± 94.3	611.8 ± 52.8
Hill Slope	0.1 ± 0.06	0.2 ± 0.06

FIGURE 5. Human neutrophils are activated by C5a and C5a^{desArg}, but not C3a or C3a^{desArg}. Human PMN cells were isolated from blood and analyzed for anaphylatoxin receptor activation in response to anaphylatoxin stimulation. PMN cells in complete medium (DMEM plus 10% FBS) were attached to biosensors, and cell activation was monitored in response to increasing concentrations of C3a (**A**) or C5a (**B**). $^{**}p < 0.05$.

stronger and sustained for longer when compared with the response induced by same concentration (1 nM) of C5a (Supplemental Fig. 2B, *right panel*). To compare the magnitude of the anaphylatoxin-induced cell activation achieved at later time points, we evaluated the expression levels of CD11b in human PMN cells that had been incubated for 1 h with either C5a or C5a^{desArg}. Physiologic concentrations (1 nM) of both anaphylatoxins were able to induce similar upregulation of CD11b (Supplemental Fig. 2C). A similar trend was seen in response to higher concentrations (Supplemental Fig. 2C), indicating that C5a^{desArg}, especially at physiologic concentrations, induces substantial activation of PMN cells.

We also verified that C5a- and C5a^{desArg}-induced PMN cell activation was specifically inhibited in the presence of the C5aR antagonist PMX-53, but not in the presence of the control peptide (Fig. 6), thereby confirming the specificity of C5a and C5a^{desArg} signaling via C5aR.

C5a and C5a^{desArg} trigger differential activation of G proteins

The data obtained in both transfected and primary immune cells demonstrated that plasma-derived C5a^{desArg} acts as a potent inducer of C5aR-mediated cell activation at physiologic concentrations. However, they also revealed a distinct concentration-dependent signaling pattern, which prompted us to further investigate the molecular events downstream of C5aR in response to C5a and C5a^{desArg}. Previous studies have shown that in cells such as neutrophils and mast cells, C5aR couples primarily to the PTX-sensitive G protein $G_{\alpha i2}$ (43, 44). We therefore determined the impact of $G_{\alpha i}$ inhibition on the promotion of cell activation by C5a and C5a^{desArg}. Indeed, PTX negatively affected C5a- and C5a^{desArg}-induced responses (Fig. 7A). Though not statistically significant, C5a-induced cell responses seemed to be less affected by PTX, whereas 60–85% of C5a^{desArg}-induced responses were inhibited by PTX, and C5a-induced cell responses were inhibited by only 45–65% (Fig. 7A). To determine if other pathways may be responsible for the residual C5a-induced response, the influence of $G_{\alpha s}$ was investigated via inactivation with CTX. C5a^{desArg}-induced responses were only marginally affected by CTX, and the effect was independent of the ligand concentration (Fig. 7B). In contrast, however, CTX affected the cell responses promoted by C5a in a highly distinct manner. Although the responses induced by 10–100 nM C5a were slightly decreased by CTX (to a similar extent as for C5a^{desArg}), the responses triggered by low concentrations of C5a (0.1–1.0 nM) were conversely magnified by CTX (Fig. 7B). As such, these data not only indicate that C5a and C5a^{desArg} promote differential activation of G proteins but also point to the involvement of different classes of G proteins depending on the concentrations of the ligands.

Discussion

Anaphylatoxin-induced inflammatory responses play a crucial role in many physiological and pathophysiological processes, and their therapeutic modulation has moved into the spotlight of complement-related drug discovery efforts (2, 25). Yet, although the triggering of cell responses by anaphylatoxin receptors is well appreciated, their quantitation so far has mostly been assessed indirectly by means of calcium release, cell degranulation, oxygen burst, cytokine production, and chemotaxis (5). Although widely employed, such assays have significant disadvantages: they can be time-consuming, require high cell numbers, are restricted by low throughput, and do not reflect integrated cellular responses. We present in this study the use of a new high-throughput label-free cell assay based on PC biosensors that is able to directly detect anaphylatoxin-induced cell activation over time, in a specific and rapid manner, using as few as 2.5×10^4 cells. Although the detection principle shares similarity with that of surface plasmon resonance, the limited lateral propagation of light and lack of microfluidics render PC biosensors amendable to the analysis of whole cells. Importantly, compared with the orthogonal cell activation assays mentioned above, PC biosensors are able to simultaneously detect a broad range of cellular events downstream of GPCR signaling that lead to changes in cell morphology or adhesion properties. As a consequence, such technology may display a more holistic cellular response that is usually not detected by other conventional assays (28–30).

In the case of C3-derived anaphylatoxins, we detected C3a-induced activation of C3aR-transfected RBL cells (EC_{50} ~2 nM), but not human PMN cells. Furthermore, no cell activation was induced in response to C3a^{desArg} triggering, neither in RBL or PMN cells nor in the neutrophil migration model. The release of β-glucosaminidase by C3aR-RBL cells in response to C3a stimulation has been reported previously (EC_{50} 14 nM). Consistent with our data and the effectiveness of the label-free assay, C3a, but not C3a^{desArg}, has been previously described as a potent chemotactic factor for eosinophils but not neutrophils. Despite the presence of

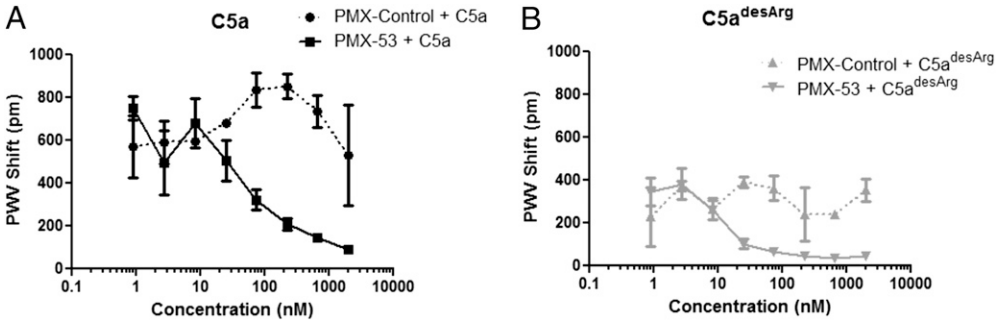


FIGURE 6. C5aR antagonist abrogates C5a- and $C5a^{desArg}$-induced PMN activation. Human PMN cells in complete medium (DMEM plus 10% FBS) were attached to biosensors. Cells were preincubated for 1 h with different concentrations of the C5aR antagonist PMX-53 or the control peptide (PMX-Control), and cell activation was assessed after the addition of 20 nM C5a (**A**) or $C5a^{desArg}$ (**B**).

C3aR on human neutrophils, no degranulation or oxygen burst has previously been observed in response to C3a (4, 45). Although these results support and validate our label-free assay approach, further investigation is clearly required to address the lack of C3aR-related signaling in human neutrophils.

C5L2 stimulation by C3a, $C3a^{desArg}$, C5a, and $C5a^{desArg}$ did not trigger any response in RBL-C5L2 cells that could be detected by the label-free cell assay. In line with these results, no β-hexosaminidase secretion or intracellular Ca^{2+} release was detected when RBL-C5L2 cells were activated with 100 nM of C5a, $C5a^{desArg}$, C3a, or C4a in previous studies (5). Despite the high expression of C5L2 in human neutrophils (12), the presence of these receptors does not account for C5a- or $C5a^{desArg}$-induced neutrophil activation in our assay, because the cell response was completely abrogated by the C5aR antagonist PMX-53 (35). Because C5L2 has been implicated in the activation of the β-arrestin signaling pathway (12), more suitable assays should be considered to address potential stimulation of cells via C5L2 activation. However, it cannot be excluded that C5L2 may be involved in the lower absolute signal response for $C5a^{desArg}$ on PMN, either by acting as a coreceptor or by internalizing the ligand, as C5L2-induced responses could not be detected by the label-free cellular assay.

Triggering of C5aR by either C5a or $C5a^{desArg}$ induced the activation of both transfected RBL-C5aR cells and human PMN cells. Such responses were specifically inhibited in a dose-dependent fashion by the C5aR antagonist PMX-53 (35), confirming the requirement for C5aR for C5a- and $C5a^{desArg}$-induced cell responses. Most importantly, we found that physiologic levels of $C5a^{desArg}$ (5–10 ng/ml; ~1 nM) induce substantial cell responses in relation to similar concentrations of C5a, a possibility that might have been overlooked in previous cases due to the lack of assays able to determine integrated cellular responses, or the comparison between C5a and $C5a^{desArg}$ is not even considered in the experimental planning. Of note, it has previously been shown that C5a is entirely converted to $C5a^{desArg}$ within 5 min in the presence of plasma (27), highlighting the importance of $C5a^{desArg}$ over C5a in the circulation. Although $C5a^{desArg}$ is often considered an inactivation product of C5a, a natural variation in responses to C5a and $C5a^{desArg}$ has been observed in the past. Both C5a and $C5a^{desArg}$ bear similar efficacy at inducing histamine and leukotriene C4 release by primed basophils (8), calcium mobilization in mouse peritoneal macrophages (46), and neutrophil-mediated platelet aggregation (47). Further, both proteins are able to prime macrophages and enhance their susceptibility to HIV infection (48). Therefore, $C5a^{desArg}$-induced cell responses are critically dependent on the type of cell and response studied (7–10).

Our data also point to the requirement for serum, mimicking physiological conditions, to obtain maximal cell response and reliable activation pattern to anaphylatoxin stimulation. This is of particular importance for designing anaphylatoxin assays to ensure comparability, as many assays described in literature have been performed in a variety of buffer/media conditions. C5a- and $C5a^{desArg}$-induced cell activation is greatly decreased by serum starvation, highlighting the importance of close-to-physiological conditions for proper cell stimulation. The presence of plasma Gc-globulin has previously been found to correlate with a higher potential for C5a-induced chemotaxis (41). However, the presence of Gc-globulin did not affect the C5a-induced responses in our

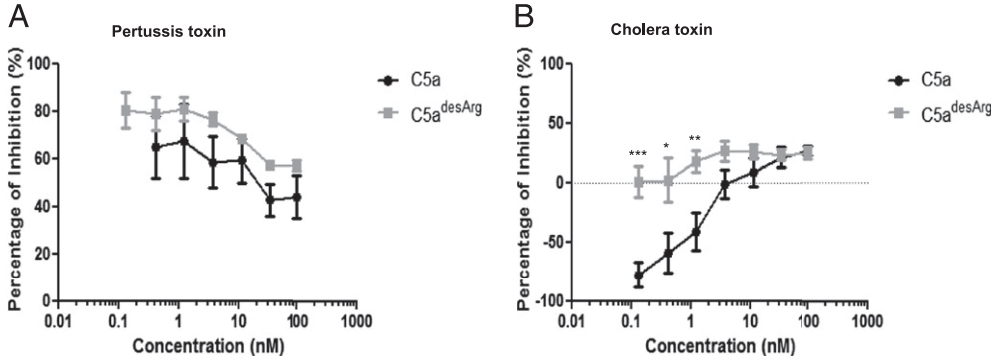


FIGURE 7. C5a and $C5a^{desArg}$ trigger differential activation of G-proteins. C5aR-transfected RBL cells were preincubated for 15 h with PTX (0.5 μg/ml) (**A**) or CTX (10 μg/ml) (**B**), and cell activation was assessed after the addition of increasing concentrations of C5a or $C5a^{desArg}$. The results are shown as percentage of inhibition (%) calculated in relation to the maximum activation achieve in response to each individual concentration in the absence of G protein inhibition. Graphs depict mean ± SEM from four (A) or seven (B) independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

model, indicating that other serum factors may also contribute to C5aR activity, either by acting as cofactors or by generally enhancing the fitness of the cells.

C5a- and C5a^{desArg}-induced responses were inhibited by PTX treatment consistent with a previous observation that C5aR signaling relies on heterotrimeric G-proteins and is mainly achieved by the PTX-sensitive α unit $G_{\alpha i2}$ (43, 44). Further, our data shed new light on the signaling pathways triggered downstream C5aR by showing that not only $G_{\alpha i}$ but also $G_{\alpha s}$ proteins may likely be involved in C5a- and C5a^{desArg}-induced cellular responses. The apparent distinct engagement of G proteins induced by C5a and C5a^{desArg} may reflect differential binding of these proteins to the C5aR. Indeed, the interaction between the residues Lys^{68} in the ligand and Glu^{199} in the C5aR has been shown to be essential for C5a^{desArg}- but not C5a-induced receptor activation (49). The $G_{\alpha s}$ protein has been previously implicated in the C5a-dependent release of 5-hydroxy [^{3}H]tryptamine by transfected RBL cells (32). Notably, it is well appreciated that the activation of $G_{\alpha s}$ proteins counteracts the effects induced by $G_{\alpha i}$ proteins and vice versa, especially when it comes to the regulation of intracellular levels of cAMP (50). C5a has been previously shown to modulate the intracellular levels of cAMP in negative and positive manners (46, 51). Such apparent controversy may rely on the different cell types (which express different classes of G_{α} proteins) and ligand concentrations used in these studies. Indeed, ligand concentration has been previously shown to determine preferential activation of $G_{\alpha s}$ versus $G_{\alpha i}$ proteins (52). Although further investigation is required to determine the biological consequence of such differential involvement of $G_{\alpha i}$ and $G_{\alpha s}$ proteins upon C5aR engagement by C5a or C5a^{desArg} and whether differential molecular mechanisms are triggered by C5a and C5a^{desArg} during the course of inflammation and homeostasis, our data suggest that constant activation of blood cells by physiologic concentrations of C5a^{desArg} may play a role in blood surveillance as well as the maintenance of blood homeostasis.

Finally, the full MS characterization of C5a and C5a^{desArg} provided additional insight into the posttranslational modification pattern of these anaphylatoxins, which includes glycosylation at Asn^{64} and partial cysteinylation at Cys^{27}. Although the 2204 kDa oligosaccharide chain deduced as $(GlcNAc)_4(Man/Gal)_5(NeuAc)_2$ does not influence the anaphylatoxic activity of C5a and C5a^{desArg} (37), the impact of cysteinylation on the activity of such molecules has not been addressed so far. Increased plasma levels of cysteinylated proteins has been previously associated with aging and irreversible oxidation of plasma proteins (53). Additional studies are required to assess whether the ratio of free versus cysteinylated Cys^{27} on C5a and C5a^{desArg} correlates with changes in activity and pathologies.

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Disclosures

The authors have no financial conflicts of interest.

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