Partial ligand-receptor engagement yields functional bias at the human complement receptor, C5aR1

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The complement component, C5a, binds two different seven-transmembrane receptors termed C5aR1 and C5aR2. C5aR1 is a prototypical G-protein–coupled receptor that couples to the Gα subfamily of heterotrimeric G-proteins and β-arrestins (βarrs) following C5a stimulation. Peptide fragments derived from the C terminus of C5a can still interact with the receptor, albeit with lower affinity, and can act as agonists or antagonists. However, whether such fragments might display ligand bias at C5aR1 remains unexplored. Here, we compare receptor, whereas the other involves the C terminus of C5a with extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase activation at the human C5aR1. We discover that C5apep acts as a full agonist for G protein coupling as measured by cAMP response and extracellular signal-regulated kinase 1/2 phosphorylation, but it displays partial agonism for βarr recruitment and receptor endocytosis. Interestingly, C5apep exhibits full-agonist efficacy with respect to inhibiting lipopolysaccharide-induced interleukin-6 secretion in macrophages, but its ability to induce human neutrophil migration is substantially lower compared with C5a, although both these responses are sensitive to pertussis toxin treatment. Taken together, our data reveal that compared with C5a, C5apep exerts partial efficacy for βarr recruitment, receptor trafficking, and neutrophil migration. Our findings therefore uncover functional bias at C5aR1 and also provide a framework that can potentially be extended to chemokine receptors, which also typically interact with chemokines through a biphasic mechanism.

The complement peptide C5a, a potent chemotactic agent and an anaphylatoxin, is one of the most critical activation products of the human complement system (1). C5a is a 74-amino acid-long peptide that is generated upon the enzymatic cleavage of complement component C5 by C5 convertases. Abnormal levels of C5a and subsequent signaling triggered by it are crucial in a range of inflammatory disorders including sepsis, vasculitis, and trauma (1, 2). C5a exerts its effects via two seven-transmembrane receptors, namely the C5aR1 and C5aR2 (also known as C5L2) (3). Of these, C5aR1 is a prototypical G-protein–coupled receptor (GPCR) that is expressed in macrophages, neutrophils, and endothelial cells (3). Upon binding of C5a, C5aR1 couples to the Gα subfamily of heterotrimeric G proteins, resulting in inhibition of cAMP levels and mobilization of intracellular Ca2+ (3). Subsequently, C5a also triggers the phosphorylation of C5aR1 followed by recruitment of β-arrestins (βarrs) and receptor internalization (3).

Structurally, C5a harbors four different helices and connecting loops, and it is stabilized by the formation of three disulfide bonds (4). C5a interacts with C5aR1 through two distinct interfaces: one involves the core of C5a with the N terminus of the receptor, whereas the other involves the C terminus of C5a with the extracellular side of the transmembrane helices of C5aR1 (Fig. 1A) (5). It has been proposed that the structural determinants for high-affinity binding are provided by the first set of interaction, whereas the second set of interaction is responsible for driving functional responses through the receptor (5). Peptides derived from the C terminus of C5a can bind to C5aR1, albeit with much lower affinity compared with C5a, and they can also trigger functional responses (6, 7). Whether such peptides may induce differential coupling of G protein versus βarrs and exhibit biased functional responses remains completely unexplored.

Here, we focus on a modified hexapeptide, referred to as C5apep hereafter (Fig. 1B), which displays the highest binding affinity to C5aR1 among various C5a fragments (7), and char-

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4 The abbreviations used are: GPCR, G-protein–coupled receptor; βarr, β-arrestin; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; LPS, lipopolysaccharide; PTX, pertussis toxin; IL, interleukin; HMDDM, human monocye-derived macrophages; hPMN, human polymorphonuclear neutrophil; HBSS, Hank’s balanced salt solution; DMEM, Dulbecco’s modified Eagle’s medium; IMDM, Iscove’s modified Dulbecco’s medium; DPBS, Dulbecco’s PBS; FBS, fetal bovine serum; PEI, polyethylenimine; BRET, bioluminescent resonance energy transfer; MNG, maltose neopentyl glycol.
characterize it vis-à-vis C5a with respect to G\(_{\alpha_i}\) and G\(_{\beta\delta}\) coupling, functional outcomes, and cellular responses. In particular, we measure the ability of C5a and C5apep to inhibit forskolin-induced cAMP as a measure of G\(_{\alpha_i}\) coupling, inducing G\(_{\beta\delta}\) recruitment and trafficking, receptor endocytosis, ERK1/2 MAP kinase activation, IL-6 release, and neutrophil migration. We identify a significant bias in G\(_{\alpha_i}\) versus G\(_{\beta\delta}\) coupling, endocytosis versus ERK1/2 activation, and IL-6 release versus neutrophil migration between the two ligands. These findings establish a framework for investigating ligand-induced functional bias at C5aR1 and pave the way for subsequent characterization of physiological outcomes arising from such ligands.

### Results and discussion

**C5apep is a full agonist for G\(_{\alpha_i}\) coupling**

Although C5apep exhibits the highest binding affinity for C5aR1 among the peptides derived from and modified based on the C terminus of C5a, its binding affinity for C5aR1 is still significantly lower than C5a (IC\(_{50}\) \(~70\) nM for C5apep and 1 nM for C5a) (7). We therefore first measured the ability of C5apep to trigger G\(_{\alpha_i}\) coupling to C5aR1 in HEK-293 cells using the GloSensor assay (8). The cells were stimulated with forskolin to generate cAMP followed by incubation with various doses of C5a and C5apep. We observed that both C5a and C5apep inhibited cAMP level to a similar extent at saturating concentrations (Fig. 1C). As expected, based on their binding affinities for the receptor, C5apep was \(~100\)-fold less potent in cAMP inhibition compared with C5a (IC\(_{50}\) \(~0.26\) nM for C5a and IC\(_{50}\) \(~16\) nM for C5apep). We also measured the efficacy of C5apep in C5aR1 expressing CHO cells using LANCE cAMP assay (9) and observed a pattern of efficacy and potency very similar to that in HEK-293 cells (Fig. 1D).

### C5apep is a partial agonist for G\(_{\beta\delta}\) coupling

Upon C5a stimulation, C5aR1 undergoes phosphorylation and recruits \(\beta\)arrs, which is important for receptor desensitization and internalization, similar to other prototypical GPCRs (10, 11). Thus, we next measured the ability of C5apep to induce \(\beta\)arr coupling using a standard co-immunoprecipitation assay. There are two isoforms of \(\beta\)arrs known as \(\beta\)arr1 and \(\beta\)arr2 that exhibit a significant functional divergence despite a high level of sequence and structural similarity (12). We expressed either \(\beta\)arr1 or \(\beta\)arr2 with FLAG-tagged C5aR1 in HEK-293 cells and then measured their interaction upon ligand stimulation. As presented in Fig. 2 (A and B), we observed a robust recruitment of both isoforms of \(\beta\)arrs upon stimulation of cells with C5a.
Interestingly, the levels of barr recruitment induced by C5a<sub> pep</sub> were significantly lower compared with C5a, even at saturating ligand concentrations. As a control, we stimulated cells with W54011, an antagonist of C5aR1 (13), and as expected, it did not elicit any significant levels of barr recruitment. To probe whether there may be a temporal difference in C5aR1-barr interaction pattern for C5a versus C5a<sub> pep</sub>, we further carried out a time-course experiment for barr recruitment. However, C5a<sub> pep</sub>-induced barr recruitment was significantly lower than C5a (Fig. 2, C and D). Taken together with the cAMP data presented above, these findings suggest that C5a<sub> pep</sub> is a full-agonist for Go<sub>i</sub>-coupling but a partial agonist for barr recruitment.

**C5a<sub> pep</sub> triggers slower endosomal trafficking of barrs**

barrs are normally distributed in the cytoplasm and upon agonist stimulation; they traffic to the membrane and interact with receptors (14). Consequently, upon prolonged agonist stimulation, barrs either dissociate from the receptor (class A pattern of barr recruitment) or co-internalize with activated receptors in endosomal vesicles (class B pattern of barr recruitment) (14). To probe whether C5a<sub> pep</sub> might differ from C5a with respect to barr trafficking patterns, we co-expressed C5aR1 with either barr1-YFP or barr2-YFP and visualized the trafficking of barrs using confocal microscopy. We observed that C5a<sub> pep</sub> was capable of promoting surface translocation of barrs at similar levels as C5a during the early phase of agonist stimulation (Fig. 3, second column). However, we observed that C5a<sub> pep</sub> was significantly slower in promoting the appearance of barrs in endosomal punctae and vesicles compared with C5a (Fig. 3, third column), although ultimately it did induce robust endosomal localization of barrs (Fig. 3, fourth column).

**C5a<sub> pep</sub> exhibits a bias between ERK1/2 MAP kinase activation and receptor endocytosis**

To probe whether C5a<sub> pep</sub> may also exhibit differential efficacy compared with C5a in functional assays, we next measured the ability of C5a<sub> pep</sub> to induce receptor endocytosis and ERK1/2 MAP kinase activation in HEK-293 cells. In agreement with our data on barr recruitment and trafficking, we observed lower levels of receptor endocytosis induced by C5a<sub> pep</sub> compared with C5a (Fig. 4A). This observation hints that barrs may play a crucial role in endocytosis of C5aR1, and therefore, weaker barr recruitment by C5a<sub> pep</sub> translates into lower endocytosis. Interestingly however, C5a<sub> pep</sub> was as efficacious as C5a in stimulating phosphorylation of ERK1/2 MAP kinase in HEK-293 cells, at least at the time points that were tested in this experiment (Fig. 4, B and C). Thus, correlation of maximal levels of endocytosis triggered by C5a and C5a<sub> pep</sub> with the ERK1/2 phosphorylation reveals a bias of C5a<sub> pep</sub> in these two functional responses. Full efficacy of C5a<sub> pep</sub> for ERK1/2 phosphorylation, similar to that in cAMP assay, suggests that this response may be primarily driven by Go<sub>i</sub>. To probe this possibility, we measured ERK1/2 phosphorylation after pretreatment of cells with

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**Figure 2. C5a<sub> pep</sub> is a partial agonist for barr recruitment.** A, HEK-293 cells expressing FLAG-C5aR1 and either barr1 or 2 were stimulated with the indicated concentrations of different ligands (W54011, 0.1 μM C5a, 1 μM; C5a<sub> pep</sub>, 10 μM) followed by cross-linking using DSP. Subsequently, FLAG-C5aR1 was immunoprecipitated using anti-FLAG antibody agarose, and co-elution of barrs was visualized using Western blotting. B, densitometry-based quantification of data presented in A (averages ± S.E.; n = 2) normalized with respect to signal for C5a-barr1 condition (treated as 100%) and analyzed using two-way ANOVA. *, p < 0.05. C, a time-course co-immunoprecipitation experiment to measure the interaction of C5aR1 with barrs. The experiment was performed following the protocol as indicated except that cells were stimulated for different time points. D, densitometry-based quantification of the data presented in C (average ± S.E.; n = 2) normalized with respect to signal for C5a at 30 min condition (treated as 100%) and analyzed using two-way ANOVA. ***, p < 0.001.
pertussis toxin (PTX) and observed a robust inhibition of ERK1/2 phosphorylation for both C5a and C5a\textsuperscript{pep} (Fig. 5, A–D).

Previous studies on several G\textsubscript{i}/H9251-coupled receptors have documented an interplay of βarss and G\textsubscript{i}/H9251 in agonist-induced ERK1/2 phosphorylation. For example, nicotinic acid induced ERK1/2 phosphorylation downstream of GPR109A can be inhibited by either PTX pretreatment or βarr knockdown (15). Similarly, carvedilol-induced ERK1/2 phosphorylation downstream of β1-adrenergic receptor requires a contribution from both G\textsubscript{i} and βarss (16, 17). This interesting aspect of GPCR signaling is being actively explored and discussed in the literature (18–22). Although our data shows nearly complete inhibition of C5a/C5a\textsuperscript{pep}-induced ERK1/2 phosphorylation for C5aR1, evaluating the contribution of βarss in this process would be an interesting avenue for future studies.

**C5a\textsuperscript{pep} exhibits βarr isof orm bias at a chimeric C5aR1**

The interaction of βarss with GPCRs is a biphasic process, which involves the receptor tail (\textit{i.e.} phosphorylated C terminus) and the receptor core (cytoplasmic surface of the transmembrane bundle) (11, 23). Based on the stability of their interaction with βarss, GPCRs are categorized as class A and B, which represent transient and stable interactions, respectively (14). Receptors having clusters of phosphorylatable residues in their C terminus (such as the vasopressin receptor, V2R) typically interact stably with βarss. Because C5aR1 does not harbor...
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We next measured the interaction of βarrs with C5a-V2R and expectedly observed an enhanced βarr recruitment for C5a-V2R compared with C5aR1 (Fig. 7, A and B). Interestingly, however, unlike C5aR1, we observed an equal recruitment of βarr1 by C5apep and C5a with C5a-V2R (Fig. 7, C and D). On the other hand, similar to C5aR1, βarr2 recruitment induced by C5apep was still significantly weaker than C5a at this chimeric receptor (Fig. 7, A and B). These findings reveal a βarr isomor bias at the chimeric receptor, a pattern that has significant implications if applicable to other GPCRs as well. For example, several high-throughput screening assays of βarr recruitment, such as the Tango assay, utilize chimeric GPCRs with V2R tail (24). This may result in miscalculation of ligand bias, and therefore, the patterns observed using chimeric receptors in primary screening should be reconfirmed using WT receptors for calculating bias profile.

C5apep is a partial agonist for βarr recruitment at C5aR2

C5a interacts with two distinct seven-transmembrane receptors, C5aR1 and C5aR2. Of these, C5aR2 does not exhibit any detectable G-protein coupling as measured in functional assays, although it robustly recruits βarrs upon agonist-stimulation (9, 25, 26). In line with previous reports in the literature, comparison of the primary sequences of C5aR1 and C5aR2 identifies mutations in highly conserved DRY and NPXY motifs (Fig. 8A). We observed that C5apep acts as a partial agonist for βarr2 recruitment at C5aR2, similar to C5aR1 (Fig. 8B). Interestingly, however, unlike C5aR1, we did not observe agonist-induced phosphorylation of ERK1/2 downstream of C5aR2 in HEK-293 cells (Fig. 8, C and D). Although the mutations in the DRY and NPXY motifs may be the primary determinants for the lack of functional G-protein coupling, it is also plausible C5aR2 adopts an active conformation upon agonist binding that is distinct from that of C5aR1. Such a conformation may preclude functional G-protein coupling but allow receptor phosphorylation and βarr interaction. However, future studies designed to investigate agonist-dependent conformational changes in C5aR1 and C5aR2 are required to probe such a possibility.

C5apep elicits biased cellular responses

C5aR1 is endogenously expressed at high levels in macrophages and neutrophils, where it modulates multiple inflammatory responses (3). Stimulation of C5aR1 in human macrophages reduces LPS-induced release of IL-6 (2), whereas neutrophil C5aR1 activation induces rapid chemotaxis (3). To assess whether C5apep might exhibit a bias at the level of these cellular responses, we utilized primary human monocyte-derived macrophages (HMDMs) and human polymorphonuclear neutrophils (HPNs) to measure IL-6 release and migration, respectively. Measuring G-protein-mediated cAMP responses in these primary cells is technically challenging, and therefore, we measured Ca^{2+} mobilization in HMDMs upon stimulation by C5a and C5apep. We observed a full-agonist profile of C5apep similar to that observed in cAMP assays in HEK-293 and CHO cells, and the potency of these two agonists for Ca^{2+} mobilization was also similar to that observed in HEK-293 and CHO cells (Fig. 9A). Quantification and comparison of C5a and C5apep for

Figure 4. C5apep exhibits a bias between receptor endocytosis and ERK1/2 MAP kinase phosphorylation. A, HEK-293 cells expressing C5aR1 were stimulated with C5a (1 μM) and C5apep (10 μM) for indicated time points followed by the assessment of surface receptor levels using a whole-cell ELISA assay. C5apep displays a weaker efficacy in promoting C5aR1 endocytosis compared with C5a. The data represent averages ± S.E. from five independent experiments. B, C5apep induces robust phosphorylation of ERK1/2 MAP kinase at levels similar to C5a. HEK-293 cells expressing C5aR1 were stimulated with C5a (1 μM) and C5apep (10 μM) for indicated time points followed by measurement of ERK1/2 phosphorylation using Western blotting. C, densitometry-based quantification of the ERK1/2 phosphorylation data presented in B (averages ± S.E.) of five independent experiments.

Figure 5. C5apep is a partial agonist for βarr recruitment at C5aR2. A, HEK-293 cells expressing C5aR1 and C5aR2 were stimulated with C5a-V2R (20 μg/mL) and C5apep (100 μg/mL) for indicated time points followed by the assessment of surface receptor levels using a whole-cell ELISA assay. B, C5apep induces robust phosphorylation of ERK1/2 MAP kinase at levels similar to C5a. HEK-293 cells expressing C5aR1 were stimulated with C5a (1 μM) and C5apep (10 μM) for indicated time points followed by measurement of ERK1/2 phosphorylation using Western blotting. C, densitometry-based quantification of the ERK1/2 phosphorylation data presented in B (averages ± S.E.) of five independent experiments.
inducing βarr recruitment in HMDMs and hPMNs at endogenous level of the receptor are technically very challenging and require development of novel sensors and assays going forward. Interestingly, however, C5apep stimulation resulted in only a submaximal phosphorylation of ERK1/2 in HMDMs compared with C5a (Fig. 9B). This is in contrast with our observation in HEK-293 cells in which C5apep stimulated ERK1/2 phosphorylation at levels similar to C5a (Fig. 4, B and C). We also found that ERK1/2 phosphorylation in HMDMs was sensitive to PTX pretreatment as in HEK-293 cells (Fig. 9C). This is particularly interesting considering that C5apep behaves as full agonist in Ca²⁺ mobilization experiments in HMDMs. There is growing evidence for context-specific effector coupling and functional responses downstream of several GPCRs (27, 28). These emerging findings have refined our current understanding of biased signaling by providing substantial evidence of additional levels of complexities in GPCR signaling. Our data with C5apep, especially in the context of ERK1/2 activation in HMDMs, further add to this important paradigm of GPCR signaling.

We next compared the ability of C5a and C5apep to inhibit LPS-induced IL-6 release in HMDMs and to stimulate chemotaxis in hPMNs. We observed that the inhibition of LPS-induced IL-6 release in HMDMs was comparable for both C5a and C5apep (Fig. 10A) and was sensitive to PTX pretreatment (Fig. 10B). Notably, however, we observed that C5apep displayed a significantly blunted response in neutrophil chemotaxis compared with C5a even at saturating doses (Fig. 10C), although similar to IL-6 release, chemotaxis was also sensitive to PTX. These observations therefore uncover that C5apep exhibits bias at the level of cellular responses when compared with C5a in primary cells expressing endogenous levels of C5aR1. It is interesting to note here that both LPS-induced IL-6 release and hPMN chemotaxis are sensitive to PTX pretreatment, suggesting that these processes are driven primarily by Goαi coupling to C5aR1. However, because βarr knockdown or knockout studies in HMDMs and hPMNs are technically challenging and typically have suboptimal efficiency, we cannot rule out either a direct contribution of βarrs or an interplay of βarrs and Goαi in these processes, and it remains to be explored in future studies (16).

It is important to note that chemokines, like complement C5a, also interact with their cognate GPCRs through a biphasic mechanism. Thus, it is tempting to speculate that fragments derived from the C terminus of chemokines may also exhibit biased signaling through their receptors. Our findings also suggest that a more comprehensive analysis of C5a fragments might yield additional βarr-biased ligands at C5aR1. Although crystal structures of C5aR1 bound to small molecule antagonists have been determined recently (29, 30), a C5a-bound structure is still not available. Future high-resolution data of C5a-bound C5aR1 may provide structural insights into differential engagement of C5apep compared with C5a and how these differential interactions in the ligand-binding pocket yield transducer-coupling bias.

In summary, we discover C5apep as a biased C5aR1 agonist at the levels of Goαi versus βarr coupling, functional outcomes, and cellular responses. Going forward, an interesting avenue might be to evaluate the physiological responses elicited by C5apep in vivo. Given that C5a attenuates LPS-mediated cytokine production from macrophages (31) (Fig. 10), a biased ligand such as C5apep that retains this beneficial activity, although diminishing the more pro-inflammatory activities of neutrophil migra-

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Figure 5. C5a/C5apep-induced ERK1/2 phosphorylation is sensitive to PTX treatment. A, HEK-293 cells stably expressing C5aR1 were incubated with 100 ng/ml PTX for 12–16 h followed by serum starvation and ligand stimulation. Subsequently, ERK1/2 phosphorylation was measured by Western blotting. B, densitometry-based quantification of data presented in A from three independent experiments normalized with respect to maximal response (treated as 100%) and analyzed using one-way ANOVA. *** p < 0.001. C, effect of PTX treatment on C5apep-induced ERK1/2 phosphorylation measured as in A above. D, densitometry-based quantification of data presented in C, normalized, and analyzed as in B.
tion, may be a novel therapeutic approach for treating inflammatory disorders. Furthermore, as we have demonstrated the recruitment of both isoforms of \( \beta \text{arr} \) (i.e. \( \beta \text{arr}1 \) and \( \beta \text{arr}2 \)) to \( \text{C5aR1} \), it would be interesting to evaluate the shared and distinct roles of these \( \beta \text{arr} \) isoforms in regulation of \( \text{C5aR1} \). It is also notable that the second receptor activated by \( \text{C5a} \) (\( \text{C5aR2} \)) does not exhibit any detectable G-protein coupling but displays robust \( \beta \text{arr} \) recruitment (25). Although we did not observe agonist-induced \( \text{ERK1/2} \) phosphorylation downstream of \( \text{C5aR2} \) upon \( \text{C5a} \) stimulation, it is plausible that it may activate other signaling pathways, and a comprehensive study is required to probe this possibility in future.

Materials and methods

General reagents, constructs, and cell culture

Most of the reagents were purchased from Sigma unless mentioned otherwise. The coding region of human \( \text{C5aR1} \) was cloned in pcDNA3.1 vector with the N-terminal signal sequence and a FLAG tag. Coding regions of bovine \( \beta \text{arr}1 \) and \( \beta \text{arr}2 \) were cloned in pCMV vector. \( \text{C5a}^{\text{pop}} \) was synthesized from Genscript. Recombinant human \( \text{C5a} \) was either purchased from Sino Biological or purified following a previously published protocol (4). Ultrapure lipopolysaccharide from \( \text{Escherichia coli} \) K12 strain was purchased from Invivogen. BSA was purchased from Sigma. For cell culture, trypsin-EDTA, Hanks’ balanced salt solution (HBSS), HEPES, Dulbecco’s modified Eagle’s medium (DMEM), phenol-red free DMEM, Ham’s F-12 medium, Iscove’s modified Dulbecco’s medium (IMDM), and penicillin/streptomycin were purchased from Thermo Fisher Scientific. Dulbecco’s PBS (DPBS) was purchased from Lonza.

The following cell lines were cultured as previously described (9). Chinese hamster ovary cells stably expressing the human \( \text{C5aR1} \) (CHO-\( \text{C5aR1} \)) were maintained in Ham’s F-12 medium containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 \( \mu \text{g/ml streptomycin} \), and 400 \( \mu \text{g/ml G418} \) (Invivogen). Human embryonic kidney-293 (HEK-293, ATCC) cells were maintained in DMEM containing 10% FBS, 100 IU/ml penicillin, and 100 \( \mu \text{g/ml streptomycin} \). All cell lines were maintained in T175 flasks (37 °C, 5% \( \text{CO}_2 \)) and subcultured at 80–90% con-
fluency using 0.05% trypsin-EDTA in DPBS. To ensure the consistency of cell function, cell morphology was continually monitored, and neither cell line was used beyond passage 20.

To generate HMDM, human buffy coat blood from anonymous healthy donors was obtained through the Australian Red Cross Blood Service. Human CD14 \(^{+}\)/H11001 monocytes were isolated from blood using Lymphoprep density centrifugation (STEMCELL) followed by CD14 \(^{+}\)/H11001 MACS magnetic bead separation (Miltenyi Biotec). The isolated monocytes were differentiated for 7 days in IMDM supplemented with 10% FBS, 100 units/ml penicillin, 100 \(\mu\)g/ml streptomycin, and 15 ng/ml recombinant human macrophage colony stimulating factor (Peprotech) on 10-mm square dishes (Sterilin). Nonadherent cells were removed by washing with DPBS, and the adherent differentiated HMDMs were harvested by gentle scraping.

hPMNs were obtained from venous whole blood (20 ml) collected from healthy volunteers under informed consent. The samples were collected using venepuncture into BD K2EDTA Vacutainer \(^{\text{TM}}\) blood collection tubes and processed within 5 h. For neutrophil isolation, the anticoagulated blood was first layered over a Lymphoprep (STEMCELL) density gradient and centrifuged (800 \(\times\) g for 30 min at 22 °C), followed by residual erythrocytes removal using hypotonic lysis. Isolated PMNs were counted and resuspended in a HBSS-based migration buffer (containing calcium and magnesium, supplemented with 20 mM HEPES and 0.5% BSA).

**Preparation of C5aR1, C5a-V2R, and C5aR2 expressing stable HEK-293 cell line**

50–60% confluent HEK-293 cells were transfected with 7 \(\mu\)g of FLAG-tagged C5aR1/C5a-V2R/C5aR2 DNA complexed with 21 \(\mu\)g of polyethylenimine (PEI). Next day, stable selection was started with optimal dose of G418 along with untransfected cells kept as negative control. After completion of stable selection, clonal population was prepared by the limited dilution method. The highest expressing clones were propagated further and kept under G418 selection throughout the course of experiments. Surface expression of C5aR1, C5a-V2R, and C5aR2 was measured using a previously described whole-cell surface ELISA protocol (32).

**ERK1/2 phosphorylation assay**

Agonist-induced ERK1/2 phosphorylation was measured primarily using a previously described Western blotting–based protocol (33). C5aR1 +, C5a-V2R +, or C5aR2-expressing stable cell lines were seeded into 6-well plate at a density of 1 million cells/well. The cells were serum-starved for 12 h followed by stimulation with 1 \(\mu\)M of C5a and 10 \(\mu\)M of C5a-pep, respectively, at selected time points. After the completion of time course, the
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A

B

C

D

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medium was aspirated, and the cells were lysed in 100 μl of 2× SDS dye/well. The cells were heated at 95 °C for 15 min followed by centrifugation at 15,000 rpm for 10 min. 10 μl of lysate was loaded per well and separated on SDS-PAGE followed by Western blotting. The blots were blocked in 5% BSA (in TBST) for 1 h and incubated overnight with rabbit phospho-ERK (catalog no. 9102/CST) primary antibody at 1:5000 dilution. The blots were washed thrice with TBST for 10 min each and incubated with anti-rabbit HRP-coupled secondary antibody (1:10,000, catalog no. A00098/Genscript) for 1 h. The blots were washed again with TBST for three times and developed with Promega ECL solution on chemidisc (Bio-Rad). The blots were stripped with low pH stripping buffer and then reprobed for total ERK using rabbit total ERK (catalog no. 9102/CST) primary antibody at 1:5000 dilution. To measure the effect of PTX on ERK activation, HEK-293 cells stably expressing C5aR1 were seeded in 6-well plate. The cells were treated with 100 ng/ml PTX for 12–16 h followed by serum starvation. The cells were seeded in 6-well plate. The cells were treated with 100 ng/ml PTX for 12–16 h followed by serum starvation. The cells were then incubated with luciferase-substrate for 2 h. Subsequently, the cells were stimulated with respective ligands, and BRET signals were monitored using Surefire Ultra (PerkinElmer) following the manufacturer’s protocol. Briefly, HMDMs were seeded (50,000/well) in tissue culture-treated 96-well plates (Corning) for 24 h and serum-starved overnight. All ligand dilutions were prepared in serum-free IMDM containing 200 ng/ml PTX or vehicle (1% FBS) followed by incubation for 96-well plate already containing 100 μl of 1 M H2SO4. Absorbance was read at 450 nm in a multiplate reader (Victor X4). For normalization, cell density was measured using janus green. Briefly, 3,3′,5,5′-tetramethylbenzidine was removed, and the cells were washed twice with 1× TBS followed by incubation with 0.2% (w/v) janus green for 15 min. Destaining was done with three washes of 1 ml ofdistilled water. Stain was eluted by adding 800 μl of 0.5 N HCl per well. 200 μl of this solution was transferred to a 96-well plate, and absorbance was read at 595 nm. Data normalization was done by dividing A150 by A595 values.

GloSensor assay for cAMP measurement

50 – 60% confluent HEK-293 cells were co-transfected with 3.5 μg each of C5aR1 or C5a-V2R and 22F (Promega) plasmids. 24 h post-transfection, the cells were trypsinized and harvested by centrifugation at 1500 rpm for 10 min. The medium was aspirated, and cells were resuspended in luciferin sodium solution (0.5 mg/ml) (Gold Biotech) prepared in 1× HBSS (Gibco) and 20 mm HEPES, pH 7.4. The cells were then seeded in a 96-well plate at a density of 0.4 million cells/well and kept at 37 °C for 1.5 h in the CO2 incubator followed by incubation at room temperature for 30 min. Basal reading was read on luminescence mode of multplate reader (Victor X4), and cycles were adjusted until basal values were stabilized. The cells were then incubated with 1 μM forskolin, and readings were recorded until maximum luminescence values were obtained. This was followed by stimulation of cells with specified concentrations of C5a and C5aapop, and values were recorded for 1 h. The data were normalized with respect to minimal stimulation dose of ligand after basal correction.

Cross-linking and co-immunoprecipitation

50 – 60% confluent HEK-293 cells were co-transfected with C5aR1 or C5a-V2R and βarr1/βarr2 plasmids by PEI (as mentioned earlier). 48 h post-transfection, the cells were serum-starved for 6 h and stimulated with respective doses of C5a/ C5aapop, harvested, and proceeded for cross-linking experiment. The cells were lysed by Dounce homogenization in 20 mm

Figure 8. Sequence alignment of C5aR2 with C5aR1, βarr2 recruitment, and ERK1/2 phosphorylation. A, the sequences of human C5aR1 and C5aR2 were retrieved from Uniprot and aligned on M-coffee server with default parameters. Alignment reliability was assessed by core/TCS and generated alignment was visualized using Espript 3. Specific mutations in the DRY and NPXY motif are highlighted. B, HEK-293 cells expressing C5aR2-Venus and βarr2-Rlu8 constructs were first incubated with luciferase-substrate for 2 h. Subsequently, the cells were stimulated with respective ligands, and BRET signals were monitored using a dose-response curve. The data represent averages ± S.E. of three independent experiments, and the EC50 values are compared using unpaired t test, *** , p < 0.001. C, HEK-293 cells expressing C5aR1 or C5aR2 were stimulated with C5a (100 nM) for indicated time points followed by detection of ERK1/2 phosphorylation using Western blotting. D, densitometry-based quantification of data presented in C normalized with C5a response for C5aR1 (treated as 100%) and analyzed using two-way ANOVA. ***, p < 0.001.
**Figure 9.** C5aR2 exhibits bias at the level of Ca²⁺ mobilization and ERK1/2 phosphorylation in HMDMs. A, C5aR2 is a full agonist in Ca²⁺ mobilization assay in HMDM cells. HMDMs were first loaded with Fluo-4 calcium indicator followed by the addition of respective ligands and subsequent measurement of fluorescence intensity. The data were normalized with maximal response obtained for C5a and represent means ± S.E. of triplicate experiments performed from three independent donors, and the EC₅₀ values of C5a and C5aR2 were analyzed using unpaired t test. ***, p < 0.001. B, HMDMs were stimulated with respective ligands for 10 min at room temperature before being lysed. The phospho-ERK1/2 content in the lysate was detected using Alphalisa SureFire Ultra p-ERK1/2 kit. The data were normalized with maximal response obtained for C5a and represent the means ± S.E. of triplicate experiments performed from five independent donors and analyzed using unpaired t test. ***, p < 0.001. C, C5a/C5aR2-induced ERK1/2 phosphorylation in HMDMs is sensitive to PTX. HMDMs seeded in tissue culture-treated 96-well plates were preincubated with 200 ng/ml PTX or vehicle in serum-free IMDM overnight. Subsequently, the cells were stimulated with C5a/C5aR2 and ERK1/2 phosphorylation was measured using Alphalisa SureFire Ultra p-ERK1/2 kit. The data are normalized with maximal response obtained for C5a and represent the means ± S.E. of triplicate experiments performed from three independent donors and analyzed using paired two-way ANOVA. ***, p < 0.001; ****, p < 0.0001. CTL, control.

HEPES, pH 7.4, 100 mM NaCl, 1× phosphatase inhibitor mixture (Roche), 2 mM benzamidine hydrochloride, and 1 mM phenylmethylsulfonyl fluoride. This was followed by the addition of 1 mM dithiobis(succinimidyl-propionate) from a freshly prepared 100 mM stock in DMSO. Lysate was tumbled at room temperature for 40 min, and the reaction was quenched by adding 1× Tris, pH 8.5. Lysates were solubilized in 1% (v/v) MNG for 1 h at room temperature followed by centrifugation at 15,000 rpm for 15 min. Cleared supernatant was transferred to a separate tube already containing pre-equilibrated M1-FLAG beads supplemented with 2 mM CaCl₂. The solution was tumbled for 2 h at 4°C and washed alternately with low salt buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 0.01% MNG, 2 mM CaCl₂) and high salt buffer (20 mM HEPES, pH 7.4, 350 mM NaCl, 0.01% MNG, 2 mM CaCl₂), respectively. The bound proteins were eluted in FLAG-elution buffer containing 20 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.01% MNG, and 250 µg/ml FLAG peptide. Co-immunoprecipitated bArr was detected by Western blotting using rabbit anti-bArr mAb (1:5000, CST catalog no. D24H9). The blots were stripped and reprobed for receptor with HRP-conjugated anti-FLAG M2 antibody (1:5000). The blots were developed on Chemidoc (Bio-Rad) and quantified using ImageLab software (Bio-Rad).

**Sequence alignment of C5aR1 and C5aR2 sequences**

Reviewed sequences of human C5aR1 and C5aR2 (also referred to as C5L2) were retrieved from Uniprot. Sequences were aligned on M-coffee server with default parameters. Alignment reliability was assessed by core/TCS, and generated alignment was visualized using Espript 3.

**BRET assay for measuring the interaction of bArr with C5aR2**

C5a-mediated bArr2 recruitment to C5aR2 was measured using bioluminescent resonance energy transfer (BRET) as previously described (9). Briefly, HEK-293 cells were transiently transfected with C5aR2-Venus and bArr2-Rluc8 constructs using XTG9 (Roche). At 24 h post-transfection, the cells were gently detached using 0.05% trypsin-EDTA and seeded (100,000/well) onto white 96-well TC plates (Corning) in phenol-red free DMEM containing 5% FBS. On the following day, the cells were firstly incubated with the substrate EnduRen (30 µl/ml) supplemented with 2 mM CaCl₂. The solution was tumbled at room temperature for 1 h, and the reaction was quenched by adding 1× Tris, pH 8.5. Lysates were solubilized in 1% (v/v) MNG, pH 7.4, 150 mM NaCl, 0.01% MNG, and 250 µg/ml FLAG peptide. Co-immunoprecipitated bArr was detected by Western blotting using rabbit anti-bArr mAb (1:5000, CST catalog no. D24H9). The blots were stripped and reprobed for receptor with HRP-conjugated anti-FLAG M2 antibody (1:5000). The blots were developed on Chemidoc (Bio-Rad) and quantified using ImageLab software (Bio-Rad).

**Confocal microscopy**

For visualization of ligand-induced bArr recruitment, HEK-293 cells were co-transfected with C5aR1 and bArr1-YFP or bArr2-YFP plasmids in 1:1 ratio (total 7 µg) by PEI. 24 h post-transfection, 1 million cells were seeded in 35-mm glass bottom dish precoated with 0.01% poly-D-lysine. After 24 h, the cells were serum-starved for 6 h and stimulated with respective doses of C5a (1 µM) and C5aR2 (10 µM). For live-cell imaging, images were acquired using Carl Zeiss LSM780NLO confocal microscope for specified time intervals, and image processing was done in ZEN lite (Zen-blue/ZEN-black) software from Zeiss. Confocal microscopy experiments were performed on
two independent set of cells (i.e. independent transfections), and multiple cells were imaged and analyzed.

**Intracellular calcium mobilization assays**

Ligand-induced intracellular calcium mobilization was assessed using Fluo-4 NW Calcium Assay kit (Thermo Fisher Scientific) following the manufacturer’s instructions. Briefly, HMDMs were seeded (50,000/well) in black clear-bottom 96-well TC plates (Corning) for 24 h before the assay. The cells were first stained with the Fluo-4 dye in assay buffer (1× HBSS, 20 mM HEPES) for 45 min (37 °C, 5% CO2). Respective ligands were prepared in assay buffer containing 0.5% BSA. On a Flexstation 3 platform, the fluorescence (excitation/emission, 494/516 nm) was continually monitored for a total of 100 s with ligand addition performed at 16 s.

**Chemotaxis assays**

Ligand-induced hPMN migration was assessed using 6.5-mm Transwell polycarbonate membrane inserts with 3.0-μm pore (Corning) to create a modified Boyden chamber (31). Freshly isolated hPMNs were seeded onto inserts (500,000/well) for 20 min (37 °C, 5% CO2) in a HBSS-based migration buffer as described in previous section. To initiate cell migration, respective ligands prepared in migration buffer were added to the receiver wells and then allowed to migrate for 1 h. The number of migrated cells was recorded and normalized to the maximal C5a-induced migration. The data were presented, normalized, and analyzed as in C above.

**Measurement of cytokines release using ELISA**

The immunomodulatory effect of respective C5aR1 ligands on LPS-induced cytokine release was assessed in primary human macrophages as previously described (34). HMDMs were seeded in 96-well TC plates (100,000/well) for 24 h before...
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Treatment. All ligands were prepared in serum-free IMDM containing 0.1% BSA. For stimulation, the cells were co-treated with LPS and respective C5aR1 ligands for 24 h (37 °C, 5% CO2). The supernatant was collected and stored at −20 °C till use. IL-6 levels in the supernatant were quantified using respective human ELISA kits (BD OptEIA) as per the manufacturer’s protocol.

Data collection, processing, and analysis

All experiments were conducted in triplicate and repeated on at least three separate days (for cell lines) or using cells from at least three donors (for HMDMs) unless otherwise specified. The data were analyzed using GraphPad software ( Prism 8.0) and expressed as means ± S.E. The data from each repeat were normalized accordingly before being combined. For all dose-response assays, logarithmic concentration-response curves were plotted using combined data and analyzed to determine the respective potency values.

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