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C5a2 can modulate ERK1/2 signaling in macrophages via heteromer formation with C5a1 and β-arrestin recruitment

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Short title: **C5a2 can modulate ERK1/2 signaling in macrophages**

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ABSTRACT

The complement system is a major component of our innate immune system, in which the complement proteins C5a and C5a-des Arg bind to two G-protein coupled receptors (GPCR); namely the C5a receptor (C5a1) and C5a receptor like-2 receptor (C5a2, formerly called C5L2). Recently, it has been demonstrated that C5a, but not C5a-des Arg, up-regulates heteromer formation between C5a1 and C5a2, leading to an increase in IL-10 release from human monocyte derived macrophages (HMDM). A bioluminescence resonance energy transfer (BRET) assay was used to assess recruitment of β-arrestins by C5a and C5a-des Arg at the C5a1 and C5a2 receptors. C5a demonstrated elevated β-arrestin 2 recruitment levels in comparison to C5a-des Arg, while no significant difference was observed at C5a2. A constitutive complex that formed between β-arrestin 2 and C5a2 accounted for half of the BRET signal observed. Interestingly, both C5a and C5a-des Arg exhibited higher potency for β-arrestin 2 recruitment via C5a2, indicating preference for C5a2 over C5a1. When C5a was tested in a functional ERK1/2 assay in HMDM, inhibition of ERK1/2 was only observed at concentrations at or above the EC_{50} for heteromer formation. This suggested that increased recruitment of the β-arrestin-C5a2 complex at these C5a concentrations might have an inhibitory role on C5a1 signaling through ERK1/2. An improved understanding of C5a2 modulation of signaling in acute inflammation could be of benefit in the development of ligands for conditions such as sepsis.

Keywords: Macrophages, Complement, Inflammation, Signal Transduction

INTRODUCTION

The complement cascade is an important part of our innate immune system that comprises more than 30 soluble and membrane bound proteins that recognize, destroy and remove foreign bodies [1]. Following activation of the complement cascade the anaphylatoxins C3a, C4a and C5a are generated prior to the termination of the cascade with the formation of the lytic membrane attack complex. C5a is one of the most potent inflammatory mediators in the body, hence its production and activity is tightly regulated. One of the main regulatory mechanisms for C5a is its rapid cleavage by carboxypeptidases to C5a-des Arg [1]. C5a-des Arg was originally reported to be 10-1000-fold less active than C5a depending on assay and cell type tested [2], but recent evidence has shown C5a-des Arg to be equipotent to C5a in some circumstances [3, 4].

There are two known G-protein coupled receptors (GPCR) to which C5a and C5a-des Arg bind: the C5a receptor (C5a1) and the C5a receptor like-2 receptor (C5a2) [5, 6]. C5a1 and C5a2 are known to have important functions in many inflammatory conditions [2], in particular sepsis [7, 8]. C5a1 activation following C5a binding leads to $G\alpha_i$ protein activation, β-arrestin recruitment and the activation of multiple intracellular signaling pathways including ERK, Akt, MAPK and PI3K [1, 2, 9]. C5a2 is known to be a non-signaling GPCR partly due to key amino acid changes in the DRY and NPXXY motifs where G-proteins are known to bind [10], but has been shown to bind to β-arrestins [11, 12]. This non-signaling nature of C5a2 has led to many postulations as to the role of this enigmatic receptor [13], with the original thought that C5a2 was a recycling decoy receptor similar to the chemokine receptor D6 [11, 14]. C5a2 has since been shown to play an important role in many inflammatory conditions and also to have its own functional role [15-17]. The simplistic view of β-arrestins merely regulating GPCR desensitization/internalization has been amended as they have been demonstrated to generate G protein-independent signals by acting as a multi-protein scaffold that can regulate multiple intracellular signaling pathways including ERK, JNK and p38 [18]. In particular, β-arrestin 2 has been implicated in a negative feedback loop that regulates the inflammatory response

during sepsis. In key in vivo experiments β-arrestin 2 knockout mice showed a significant decrease in their survival rate and a concomitant significant increase in the release of pro-inflammatory cytokines [19].

GPCRs are now known to form oligomers, but the functional relevance and existence of these oligomers in native tissues is still under debate [20, 21]. It has been shown that C5a1 can form homomers [22] and heteromers with C5a2 and CCR5 [23, 24]. Recent evidence has shown C5a, but not C5a-des Arg can up-regulate the heteromer formation between C5a1 and C5a2, leading to an increase in the release of the anti-inflammatory cytokine IL-10 from human monocyte derived macrophages (HMDM) [25]. In the current study, we aimed to delineate and understand the signaling mechanism C5a1-C5a2 hetreomer formation uses to regulate the release of IL-10. Furthermore, an understanding of whether the heteromer formation regulates the release of the inflammatory mediators G-CSF and high mobility group box-1 protein (HMGB1) from HMDM was sought. Based on the current understanding surrounding C5a2, we hypothesized that due to the non G-protein induced signaling nature of C5a2, β-arrestins would play a role in heteromer formation and signaling. The role of β-arrestins was investigated using a widely used BRET based assay to look at the recruitment of β-arrestin 2 by C5a1 and C5a2 [26]. In this study, it is proposed that C5a1-C5a2 heteromer formation modulates ERK signaling through the increased recruitment of β-arrestin 2 via C5a2.

RESULTS

β-arrestins, C5a1 and C5a2 expression in HMDM

To understand which of the two β-arrestin isoforms (1 and 2) associated with GPCRs had the highest expression in HMDM, multiple HMDM donor lots were tested by qRT-PCR for the expression level of β-arrestin 1 and 2. This revealed that β-arrestin 2 had significantly higher (17-fold) expression than β-arrestin 1 (Fig. 1A). This guided the β-arrestin isoform of focus for BRET studies of the C5a receptors that best represented the environment within HMDM.

To further understand the relationship between the C5a receptors and β-arrestin expression within HMDM, qRT-PCR was also performed to look at the expression levels of the C5a1 and C5a2 receptors. It is widely reported that the C5a receptors are quite often co-expressed in many cell types especially of the myeloid linage [1], but usually the C5a1 receptor is expressed at a higher level than C5a2 [2]. Interestingly, as shown in figure 1B, within HMDM used in this study the C5a2 receptor looks to have slightly higher mRNA expression levels than that of the C5a1 receptor. The qRT-PCR data for the expression of the C5a receptors corresponds with previously published microscopy data on HMDM, which also showed that C5a1 is expressed predominantly on the cell surface whilst C5a2 is predominantly expressed intracellulary [25].

C5a1 and C5a2 heteromer formation regulates G-CSF release, but not HMGB1 release by HMDM

It has been shown that C5a, but not C5a-des Arg, up-regulates heteromer formation between C5a1-C5a2 at C5a concentrations of $\geq$ 100 nM, as a mechanism to regulate anti-inflammatory cytokine release [25]. Further support for this observation was demonstrated when differences in G-CSF release induced by C5a and C5a-des Arg at 100 nM from mouse peritoneal elicited macrophages was reported by Bosmann et al. [27]. To further validate the role of C5a up-regulated C5a1-C5a2 heteromer formation, G-CSF release from HMDM was quantified using various concentrations of C5a and C5a-des Arg (1, 100 and 200 nM) in the presence of LPS (10 ng/ml). As shown in figure 2A, C5a at 100 and 200 nM induces a significantly elevated release of G-CSF relative to C5a-des Arg, supporting the notion that up-regulation of heteromer formation by C5a modulates anti-inflammatory cytokine release.

It was also hypothesized that C5a1-C5a2 heteromer formation might regulate the release of HMGB1, a known late mediator of acute inflammatory conditions like sepsis, as it has been shown that C5a2 is critical for the release of HMGB1 during acute inflammation [7]. HMGB1 release was quantified from HMDM with and without LPS (10 ng/ml) at various concentrations of C5a and C5a-des Arg, 24 h after stimulation of the cells. Neither C5a nor

C5a-des Arg alone was able to induce a significant increase in the amount of HMGB1 released from HMDM. Whilst in the presence of LPS there is a trend of higher release of HMGB1 the result is not significant and there is no observable difference between C5a and C5a-des Arg (Fig. 3B). This data suggests that C5a1-C5a2 heteromer formation is not responsible for the modulation of HMGB1 release from macrophages during acute inflammation.

β-arrestin 2 recruitment by C5a1 and C5a2 receptors using BRET assay

Initial assays calculating BRET ratio for C5a1 and C5a2 recruitment of β-arrestin 2 suggested constitutive formation of a C5a2-β-arrestin 2 complex, but not for a C5a1-β-arrestin 2 complex (Fig. 3A, B). The C5a2-β-arrestin 2 complex had a BRET ratio of ~0.1 without the presence of ligand (Fig. 3B) whilst there was no discernable BRET ratio for C5a1-β-arrestin 2 in the absence of ligand (Fig. 3A). C5a is able to recruit β-arrestin 2 to a similar maximal BRET ratio of 0.25 through both C5a1 and C5a2.

Monitoring ligand induced BRET ratio controls for any constitutive association between the two proteins, as only a ligand-induced change in the BRET ratio is observed. Interestingly, C5a-des Arg recruited β-arrestin 2 to a similar level as C5a through C5a2, but could only recruit β-arrestin 2 to ~40% of the level of C5a through C5a1 (Fig. 3C, D). This result is in agreement to a previous study suggesting C5a-des Arg is a partial agonist for β-arrestin 2 recruitment through C5a1 [12]. Interestingly the time-course for recruitment was also different for C5a and C5a-des Arg through C5a1 but not C5a2. C5a caused a faster rate of recruitment to C5a1 with maximal signal being reached within 5 min of ligand addition whilst C5a-des Arg took closer to 20 min. In addition there was a difference in the time-course for recruitment for C5a between the receptors with C5a1 having a fast recruitment of 5 min to max whilst C5a2 had a much slower recruitment time not reaching max till ~20 min after ligand addition. The EC_{50} values for the recruitment of β-arrestin 2 through C5a1 for C5a and C5a-des Arg was 74 ± 3 nM and 98 ± 7 nM, respectively (Fig. 3E). Both ligands had a higher

affinity for β-arrestin 2 recruitment through C5a2, with EC_{50} values for C5a and C5a-des Arg of 50 ± 5 nM and 32 ± 3 nM, respectively (Fig. 3F). This suggests that even though C5a2 had low surface expression [11, 25, 28] there was a clear preference for C5a and C5a-des Arg to recruit β-arrestin 2 through C5a2 over C5a1.

C5a2 modulates C5a1 receptor recruitment of β-arrestin 2

To further investigate the ability of C5a and C5a-des Arg to recruit β-arrestin 2 through both C5a1 and C5a2, competition BRET assays were performed. This involved co-transfection of an equal amount of either unlabeled C5a1 or C5a2 with the C5a2-Venus/β-arrestin 2-Rluc8 or C5a1-Rluc8/β-arrestin 2-Venus BRET pairs, respectively. To assist with control of expression, the unlabeled receptor constructs were contained in the same expression plasmid as the labeled receptors, which are under the control of the same promoter. When unlabelled C5a2 was co-transfected with the C5a1-Rluc8/β-arrestin 2-Venus BRET constructs there was > 45% reduction in the maximal amount of β-arrestin 2 recruited by both ligands (Fig. 4A). The C5a2-Venus/β-arrestin 2-Rluc8 BRET constructs in the presence of unlabelled C5a1 showed a significantly smaller reduction (< 20%) in the maximal amount of β-arrestin 2 recruited by C5a2 (Fig. 4B).

The effect on the EC_{50} values for the C5a1-Rluc8/β-arrestin 2-Venus in the presence of the unlabelled C5a2 showed a reduction in potency of ~20 nM and 30 nM for C5a and C5a-des Arg, respectively (Fig. 4C). However, the C5a2-Venus/β-arrestin 2-Rluc8 with unlabelled C5a1 showed the opposite effect with a small increase in potency for C5a and C5a-des Arg of ~ 23 nM and 9 nM, respectively (Fig. 4D). This result further suggests that when C5a2 is present on the cell surface with C5a1, there may be a preference to recruit β-arrestin 2 through C5a2, which would in turn lead to modulation of C5a1 signaling or alternatively C5a2 could act as a better ligand-scavenger than C5a1 thereby reducing the effective amount of ligand present.

C5a2 modulates ERK1/2 activation of C5a1 in HMDM

One common signaling pathway for both G-proteins and β-arrestins is the ERK1/2 pathway. It has been previously suggested that C5a2 may modulate ERK1/2 activation by C5a1 in human polymorphonuclear leukocytes [28]. Previous testing of ERK1/2 activation in HMDM has been undertaken, but not at concentrations at or above 100 nM, which is the BRET EC_{50} for C5a1-C5a2 heteromer formation [3, 25]. In figure 5A a significant reduction in the amount of ERK1/2 activation by C5a at concentrations at and above 100 nM are depicted, but no change for C5a-des Arg was observed. To eliminate the possibility of technical errors in the dynamic range of the kit, HMDM lysate was diluted and the assay repeated, yielding the same result (data not shown). To exclude any effects due to the use of recombinant C5a versus isolated C5a-des Arg, ERK1/2 assays were performed on HMDM and a C5aR stable cell line using isolated C5a and no difference was observed between isolated and recombinant C5a (data not shown). This suggested that at the concentrations required to increase heteromer formation between C5a1 and C5a2 ERK1/2 activation is reduced, possibly through a C5a2-β-arrestin mediated mechanism.

C5a and C5a-des Arg possessed similar EC_{50} values for ERK1/2 activation of 1.99 ± 0.33 nM and 0.93 ± 0.15 nM, respectively (Fig. 5B), hence selective ERK1/2 activation is driven by ligand affinity. To further test if C5a2 is responsible for the change in ERK1/2 activation we used a C5a2 Ab (5 µg/ml) to attempt to block C5a2. As is shown in Fig. 5C at a C5a concentration of 10 nM there was a significant increase in ERK1/2 activation, but there was no change in ERK1/2 activation at 100 nM in the presence of the C5a2 Ab. C5a-des Arg had the opposite effect where there was a small, but not statistically significant reduction in the amount of ERK1/2 activation at all concentrations tested was observed (Fig. 5C). A possible explanation for why there is an effect at the 10 nM, but not the 100 nM, concentration of C5a is that the C5a2 Ab is unable to inhibit heteromer formation between C5a1 and C5a2.

ERK1/2 activation in RBL cells stably expressing C5a1, C5a2 or co-expressing C5a1/C5a2

To further test the effect of ERK1/2 activation following C5a and C5a-des Arg binding to the C5a receptors, we used RBL cells that stably express C5a1, C5a2 or C5a1/C5a2. RBL cells transfected with empty vectors and the RBL-C5a2 cells showed no ERK1/2 activation in the presence of C5a or C5a-des Arg at concentrations up to 500 nM with a 10 min stimulation time (the same as used for HMDM) (Fig. 6A, B). The RBL-C5a1 cells showed a robust ERK1/2 activation for both C5a and C5a-des Arg with EC_{50} values of 5.2 ± 1 nM and 3 ± 0.5 nM, respectively (Fig. 6C). The co-transfected RBL-C5a1/C5a2 also had robust ERK1/2 activation for C5a and C5a-des Arg with a ~5 fold reduction in potency with EC_{50} values of 23 ± 4 nM and 15 ± 2 nM respectively (Fig. 6D). Interestingly, there was no inhibition of C5a induced ERK1/2 activation at concentrations of $\geq$ 100 nM as was seen in the HMDM. This may be attributable to different intracellular G-protein or arrestin concentrations between RBL being rat cells in comparison to HMDM that are human cells. Furthermore, differences in the responses observed in human and rodent cells for the C5a receptors have already been documented [1]. It appeared that in the RBL cell line that when the C5a1 and C5a2 receptors are co-expressed C5a2 has more of an influence on ligand efficacy as evidenced by the ~5 fold reduction in potency.

DISCUSSION

Having recently shown that increased C5a receptor heteromer formation modulates the release of the anti-inflammatory cytokine IL-10 from HMDM [25], we wanted to understand the possible signaling mechanism that controlled this process. To further test whether C5a1-C5a2 heteromer formation controls the release of other anti-inflammatory cytokines, the release of G-CSF from HMDM was quantified using both C5a and C5a-des Arg. G-CSF was released from HMDM at significantly higher levels by C5a at concentrations at or above those required for heteromer formation. The increased G-CSF release gives further evidence to the theory that C5a2 acts to modulate C5a1 activity depending on the concentration of C5a

present. C5a2 has been shown to be critical for the release of HMGB1 during acute inflammation [7] and it was postulated that C5a1-C5a2 heteromer formation could be a mechanism to control the release of HMGB1. HMGB1 release from HMDM was quantified by ELISA, and there was no observable differences in HMGB1 release initiated by C5a or C5a-des Arg with or without LPS present, suggesting that C5a1-C5a2 heteromer formation was not a regulatory mechanism.

As C5a2 is known to be a non-signaling GPCR [10], which still binds to β-arrestins [11, 12], we hypothesized that the possible regulation of C5a1 signaling may occur through β-arrestins, which have already been shown to have a regulatory role in sepsis-induced inflammation [19]. Using HMDM as the model system, qRT-PCR analysis was performed to first quantitate the levels of expression of the two main β-arrestin isoforms (1 and 2) known to associate with non-visual GPCR [18]. Analysis revealed that β-arrestin 2 was expressed at significantly higher levels than β-arrestin 1 in HMDM, so β-arrestin 2 was chosen as the focus for this study. A BRET based assay was developed to test β-arrestin 2 recruitment by C5a and C5a-des Arg through C5a1 and C5a2. Initial data showed that C5a2 had a constitutive interaction with β-arrestin 2 that accounted for ~50% of the total BRET signal generated while there was no such evidence for C5a1. When looking at the ability of C5a and C5a-des Arg to recruit β-arrestin 2 through C5a1 it was evident that C5a-des Arg was only a partial agonist, being able to generate only ~40% of the BRET signal of C5a. However there was no such difference in β-arrestin 2 recruitment by C5a2. The partial agonism observed for C5a-des Arg is in agreement with a previous study [12] and could, in part, explain why C5a-des Arg is unable to up-regulate heteromer formation as was previously shown [25]. Interestingly both C5a and C5a-des Arg had higher potency at C5a2 over C5a1 for the recruitment of β-arrestin 2 with the greatest difference being a three-fold increase in potency for C5a-des Arg. Also C5a showed a difference in the time-course for β-arrestin 2 recruitment by C5a1 and C5a2 where C5a-des Arg did not. The time-course for β-arrestin 2 recruitment by C5a2 corresponded well with the time-course observed for heteromer formation between C5a1 and C5a2 in a previous

study [25] suggesting that C5a2 β-arrestin 2 recruitment might control the rate of heteromer formation.

Interestingly, when the BRET experiments were performed in the presence of the corresponding unlabelled receptor (e.g. unlabelled C5a2 co-transfected with C5a1-Rluc8 and β-arrestin 2-Venus), there was a significant reduction (> 45%) in the maximal amount of β-arrestin 2 able to be recruited through C5a1 for both C5a and C5a-des Arg. Concomitant with the decrease in the maximal signal there was also a reduction in the potency of C5a and C5a-des Arg by 20 nM and 30nM, respectively for β-arrestin 2 recruitment through C5a1. While there was an effect on the ability of C5a and C5a-des Arg to recruit β-arrestin 2 through C5a2 in the presence of C5a1 it was to a much lower level (< 20%) than seen for C5a1 in the presence of C5a2. Also there was the opposite effect on the potency of C5a and C5a-des Arg with there being an increase in potency for both C5a and C5a-des Arg of 23 nM and 9 nM. This data supports the evidence for the clear preference of C5a and C5a-des Arg to recruit β-arrestin 2 through C5a2. This increased preference to recruit β-arrestin 2 through C5a2 may be the mechanism that modulates C5a1 signaling.

One of the intracellular signaling pathways known to interact with both G-proteins and β-arrestins is the ERK1/2 pathway [18, 29]. Our group and many others have shown that following C5a binding to C5a1 there is a rapid robust activation of the ERK pathway [3, 28, 30-33]. Looking at ERK1/2 activation in HMDM following stimulation with C5a and C5a-des Arg this rapid robust activation of ERK1/2 was observed. However at C5a concentrations (≥ 100 nM) known to up-regulate heteromer formation between C5a1 and C5a2 there was a significant inhibition of ERK1/2 activation that was not observed with C5a-des Arg. This data leads to the suggestion that the formation of the C5a1-C5a2 heteromer might be suppressing the activation of ERK1/2. As was shown, C5a2 forms a constitutive complex with β-arrestin 2 similar to that seen for another non-signaling GPCR the chemokine decoy receptor D6 [14] although C5a2 doesn't have the constant surface expression observed for D6. A previous study with human neutrophils observed an increase in ERK1/2 activation in the presence of a

C5a2 neutralizing antibody [28]. Testing ERK1/2 activation of HMDM in the presence of a C5a2 neutralizing antibody did significantly increase ERK1/2 activation at C5a concentrations below those required to up-regulate heteromer formation, but did not reverse the suppression of ERK1/2 activation observed at C5a concentrations required to up-regulate heteromer formation. We theorized that this was most likely due to the inability of the C5a2 Ab to block the heteromer formation between C5a1 and C5a2. This data gives further evidence that C5a2 is more than just a recycling decoy receptor [11] and has a function in regulating intracellular signaling events. Using RBL cell lines stably expressing C5a1, C5a2 and co-expressing C5a1 and C5a2 we tested the ability of C5a1 and C5a2 to activate ERK1/2. C5a2 was unable to activate ERK1/2 at any of the concentrations of C5a and C5a-des Arg tested, while robust ERK1/2 activation was observed in the C5a1 and dual C5a1-C5a2 cell lines. There was no observed inhibition of ERK1/2 activation for C5a in the dual transfected cell line. This may be due to the RBL cell line hosting the receptors, as there would presumably be higher expression of the receptors on the transfected cell line compared to HMDM that would effect how the cells respond to activation by ligand.

In conclusion, we propose a possible working model for C5a1-C5a2 heteromer formation by C5a and modulation of ERK1/2 activation via C5a2 mediated β-arrestin recruitment. It is likely that the ERK1/2 activation seen at C5a concentrations that up-regulate heteromer formation might be due to a combination of C5a1-induced activation and formation of a C5a1-C5a2 heteromer β-arrestin inhibitory complex. In this study, we have shown that C5a2 forms a constitutive complex with β-arrestin 2 and that both C5a and C5a-des Arg have a preference to recruit β-arrestin 2 through C5a2. We also have evidence for the ability of the C5a1-C5a2 heteromer to modulate ERK1/2 activation in HMDM. This modulation of ERK1/2 activation may be the mechanism by which C5a receptor heteromers regulate cytokine release. Interestingly it was shown that C5a1-C5a2 heteromer formation might regulate ERK1/2 activation, G-CSF release, but not HMGB1 release from HMDM. It should be noted that this proposed model may only be relevant to human macrophages, as there is an

increased understanding within the complement C5a receptor field, that there are signaling and expression differences for the C5a receptors which are both species and cell type specific [1, 2]. A better understanding of the important role β-arrestins play in relation to C5a1-C5a2 heteromer formation and the modulation of C5a1 signaling should aid further studies of the role of this complex during acute inflammation.

METHODS

Materials

β-arrestin 2-Rluc8 and β-arrestin 2-Venus clones were kindly supplied by Associate Prof. Kevin Pfleger (WAIMR, UWA, Perth, Australia). C5a1-Rluc8 and C5a2-Venus clones were generated previously [25] and kind permission for use of the RLuc8 and Venus constructs was given by Sanjiv Gambhir (Rluc8, Stanford University, CA, USA) and Atsushi Miyawaki (Venus, RIKEN Brain Science Institute, Wako-city, Japan). Human recombinant C5a was purchased from Sino Biologicals (Beijing, China), human isolated C5a-des Arg was purchased from Merck Biosciences (Darmstadt, Germany). Glycine, E. coli-derived LPS, saponin, BSA, $MgCl_2$, $CaCl_2$, Tris-HCL, HEPES, were purchased from Sigma-Aldrich (St Louis, MO, USA). DMEM, phenol-red free DMEM, IMDM, FBS, streptomycin, G418, penicillin, puromycin, HBSS and PBS were purchased from Life Technologies (Carlsbad, USA). Recombinant human M-CSF was purchased from PeproTech. C5a2 receptor polyclonal Ab was purchased from Imgenex (San Diego, USA).

Cell culture and transfection

HEK293 cells were maintained in DMEM containing 10% FBS, 50 IU per ml penicillin, 50 mg/ml streptomycin (Life technologies) at 37 ^{o}C and 5% CO_2. RBL-2H3 cells stably expressing either empty vector, -C5a1, -C5a2 or -C5a1/C5a2 were cultured in DMEM, 10% FBS, 50 IU per ml penicillin, 50 mg/ml streptomycin, 800 µg/ml G418 and 1.5 µg/ml puromycin at 37 ^{o}C and 5% CO_2. Transient transfections were performed 24 h after seeding

cells at 3 x 10^5 cells per well in a six-well plate or 1 x 10^6 cells per well in a 100-mm dish. X-tremeGENE 9 transfection reagent (Roche, Basel, Switzerland) was used according to the manufacturers instructions. Cells were collected 24 h after transfection with 0.05% trypsin-EDTA in PBS (Life Technologies).

To generate HMDM, human monocytes were isolated from buffy coat blood supplied by unknown donors by the Australian Red Cross Blood Service (Kelvin Grove, Australia) using Ficoll-Plaque Plus (GE Healthcare, Rydalmere, NSW, Australia) and density centrifugation. $CD14^+$ cell selection was performed using MACS magnetic beads (Miltenyl Biotec, Macquarie Park, NSW, Australia) [34]. Isolated monocytes were differentiated in culture for 7 days with 10 ng/ml human M-CSF (Peprotech, Rocky Hill, NJ, USA) into HMDM in the presence of IMDM containing L-glutamine supplemented with 10 % FBS, 50 IU per ml penicillin and 50 mg/ml streptomycin (Life Technologies) [25, 32].

ERK1/2 Phosphorylation Assay

ERK1/2 phosphorylation assays were performed using the Alphascreen Surefire ERK1/2 kit (Perkin Elmer) as per manufacturers' instructions. Briefly, HMDM and RBL-C5a1, -C5a2, -C5a1/C5a2 cells were serum starved and incubated in 96-well tissue culture plates (Nunc) overnight at 60,000 and 50,000 cells/well, respectively. Ligands were prepared in serum-free media. Ligands were incubated with cells at room temperature for 10 min. Media was then removed and cells lysed with lysis buffer for 10 min on a shaker, 4 µL of lysate was transferred to a white 384-well proxiplate plate (Perkin Elmer) and incubated in the presence of 7 µL of reaction mix. The plate was sealed with TopSeal-A, incubated for 2 h at 37 °C and measured on an Envision plate reader (Perkin Elmer).

Measurement of G-CSF and HMGB1 release from HMDM

HMDM were stimulated with LPS (10 ng/ml) only or LPS (10 ng/ml) and either C5a or C5a-des Arg at various concentrations and 24 h after stimulation supernatant was removed from

cells and transferred to a 96-well polypropylene microplate (Perkin Elmer) and stored at -20 ^{o}C. Quantification of the release of the G-CSF was performed using the human G-CSF AlphaLISA cytokine kit (Perkin Elmer) while quantification of HMGB1 release was performed by ELISA (IBL International) as per the manufacturers' instructions.

RNA extraction and qRT-PCR analysis on HMDM

HMDM were lysed and the RNA reverse transcribed with the TaqMan® Gene Expression Cells-to-CT™ Kit (Life Technologies) according to the manufacturers instructions. The following TaqMan® Gene Expression Assay probes (Life Technologies) were used for the relative mRNA level determination by qRT-PCR: POLR2A, Hs00172187_m1; C5a2 (GRP77), Hs00218495_m1; C5a11, Hs00383718_m1; ARRB1, Hs00244527_m1 and ARRB2, Hs01034135_m1. All probes span an exon-exon junction. The cDNA was amplified with a ViiA 7 from Applied Biosystems based on the following conditions: 2 min at 50 °C, 10 min at 95 °C for enzyme activation and 40 cycles of 15 sec at 95 °C and 1 min at 60 °C for the amplification. The ViiA 7 RUO software was used for the analysis of the qRT-PCR data.

BRET assays for the recruitment of β-arrestin 2 to C5a receptors

Assay was performed as a ligand-induced BRET experiment similar to previously described [26]. In brief, HEK293 cells were transiently transfected with either C5a1-Rluc8 and β-arrestin 2-Venus or C5a2-Venus and β-arrestin 2-Rluc8 in a 100-mm dish, competition BRET assays were performed by co-transfecting HEK293 cells with an equal amount of unlabeled C5a1 or C5a2 receptor plasmid. 24 h after transfection cells were collected and transferred to a 96-well white microplate (Corning, NY, USA) in phenol-red free DMEM containing 5% FBS and 25 mM HEPES (Life Technologies). Cells were allowed to settle for 24 h and then were incubated with 30 µM Enduren (Promega, Madison, WI, USA) for 2 h at 37 ^{o}C and 5% CO_2 to ensure that the substrate had reached equilibrium. Following incubation BRET baseline measurements were taken for 15 min then ligands were added to plate and BRET

measurements were taken for a further 1 h at 37 ^{o}C using a POLARstar optima microplate reader (BMG Labtech, Ortenberg, Germany). Filtered light emissions were sequentially measured for 1 s in each of the following windows 460–490 nm and 520–550 nm, both before and following ligand addition. The ligand-induced BRET ratio was calculated by subtracting the ratio of 520–550 nm emission over the 460–490 nm emission for a vehicle-treated sample from a ligand-treated sample of the same aliquot of cells in triplicate. The BRET ratio was calculated by subtracting the ratio of 520–550nm emission over the 460–490nm emission for a cell sample containing the C5a1-Rluc8 or β-arrestin 2-Rluc8 construct from a cell sample containing both the C5a1-Rluc8 and β-arrestin 2-Venus or C5a2-Venus and β-arrestin 2-Rluc8 constructs.

Data and statistical analysis

The Graphpad Prism version 6 software was used for all statistical and graphical analysis (Graphpad Software, La Jolla, CA, USA). Sigmoidal curves were fitted to the dose-response curves using non-linear regression. Data from at least three separate experiments are expressed as mean, and error bars represent SEM. Statistical analysis was performed using either a Student's t-test or by one-way ANOVA with Sidak correction, for multiple comparisons, differences were considered significant if $p < 0.05$.

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AUTHORSHIP CONTRIBUTIONS

Daniel Croker proposed, designed, performed and analyzed the experiments. Reena Halai contributed to the design of the experiments. Geraldine Kaeslin designed and performed qRT-PCR experiments. Elisabeth Wende, Beate Fehlhaber, Andreas Klos created and supplied RBL cell lines and assisted with data analysis, Peter N. Monk assisted with data analysis. Matthew Cooper contributed to the design of the experiments and obtained funding for the research. All authors participated in writing the manuscript.

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FIGURE LEGENDS

Figure 1 qRT-PCR analysis of β-arrestins and C5a receptors gene expression in HMDM. (A) Gene expression of β-arrestin 1 and 2 was measured in HMDM. **(B)** Gene expression of C5a1 and C5a2 was measured in HMDM. Genes were detected by qRT-PCR and normalized against POLR2A expression. Data represents testing of at least three independent HMDM donors ($n \geq 3$). Error bars represent SEM. *** $p < 0.001$ by Student t test.

Figure 2. Quantification of G-CSF and HMGB1 release from HMDM. (A) Measurement of G-CSF release from HMDM, 24 h after costimulation with LPS (10 ng/ml) and C5a or C5a-des Arg at concentrations of 1 nM, 100 nM or 200 nM. G-CSF was quantified by AlphaLISA, data are representative of at least six independent experiments performed in triplicate (n = 6). **(B)** Measurement of HMGB1 release from HMDM, 24 h after stimulation with C5a or C5a-des Arg (1 nM, 100 nM, 200 nM) with and without LPS (10 ng/ml) present. HMGB1 was quantified by ELISA, data are representative of at least four independent HMDM donors performed in duplicate (n = 4). CTRL represents non-stimulated HMDM. Error bars represent SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ by one-way ANOVA followed by Sidak's post hoc test.

Figure 3. BRET assay testing for β-arrestin 2 recruitment by C5a1 and C5a2. (A) BRET ratio calculated for β-arrestin 2 recruitment by C5a1 for C5a (500 nM) and vehicle minus donor only (C5a1-Rluc8) control. **(B)** BRET ratio calculated for β-arrestin 2 recruitment by C5a2 for C5a (500 nM) and vehicle minus donor only (β-arrestin 2-Rluc8) control. **(C, D)** Ligand-induced BRET ratio for β-arrestin 2 recruitment by C5a1 **(C)** or C5a2 **(D)** for C5a or C5a-des Arg (200 nM). **(E, F)** Sigmoidal dose response curves for C5a or C5a-des Arg for β-arrestin 2 recruitment by C5a1 **(E)** or C5a2 **(F)** calculated at ~30 min after ligand addition. Data are representative of three independent experiments performed in triplicate (n = 3). Error bars represent SEM.

Figure 4. Competition BRET assays showing C5a2 modulation of β-arrestin 2 recruitment by C5a1. (A) Competition BRET assay for C5a1 recruitment of β-arrestin 2 +/- co-transfection of an equal concentration of unlabelled C5a2 for C5a or C5a-des Arg (200 nM). **(B)** Competition BRET assay for C5a2 recruitment of β-arrestin 2 +/- co-transfection of an equal concentration of unlabelled C5a1 for C5a or C5a-des Arg (200 nM). **(C)** Sigmoidal

dose response curves for C5a or C5a-des Arg for C5a1 recruitment of β-arrestin 2 by BRET assay +/- co-transfection of an equal concentration of unlabelled C5a2 calculated at ~30 min after ligand addition. **(D)** Sigmoidal dose response curves for C5a or C5a-des Arg for C5a2 recruitment of β-arrestin 2 by BRET assay +/- co-transfection of an equal concentration of unlabelled C5a1 calculated at ~30 min after ligand addition. Data are representative of three independent experiments performed in triplicate (n = 3). Error bars represent SEM.

Figure 5. Analysis of ERK1/2 activation in HMDM for C5a or C5a-des Arg. (A) ERK1/2 activation in HMDM after stimulation for 10 min with C5a or C5a-des Arg at multiple concentrations. **(B)** Sigmoidal dose response analysis of ERK1/2 activation in HMDM following stimulation with C5a or C5a-des Arg for 10 min excluding C5a concentrations 100 nM and 500 nM. **(C)** ERK1/2 activation in HMDM with or without pre-treatment with a C5a2 neutralizing Ab (5 µg/ml) for 30 min prior to stimulation for 10 min with C5a or C5a-des Arg (1 nM, 10 nM, 100 nM). ERK activation measured by AlphaScreen Surefire assays. Data are representative of four to six independent experiments performed in triplicate (n = 4-6). Error bars represent SEM. * $p < 0.05$, **** $p < 0.0001$ by one-way ANOVA followed by Sidak's post hoc test.

Figure 6. Analysis of ERK1/2 activation in RBL cell lines transfected with C5a1, C5a2 and C5a1/C5a2. (A, B) AlphaScreen signal generated by stimulation of untransfected RBL-2H3 **(A)** or RBL-C5a2 **(B)** cells following 10 min stimulation with C5a or C5a-des Arg. **(C, D)** Sigmoidal dose response curve for ERK1/2 activation of RBL-C5a1 **(C)** or RBL-C5a1/C5a2 **(D)** cells following 10 min stimulation with C5a or C5a-des Arg. ERK1/2 activation measured by AlphaScreen Surefire assays. Data are representative of three independent experiments performed in triplicate (n = 3). Error bars represent SEM.