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Discovery of functionally selective C5aR2 ligands: novel modulators of C5a signalling

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABSTRACT

The complement cascade is comprised of a highly sophisticated network of innate immune proteins that are activated in response to invading pathogens or tissue injury. The complement activation peptide, C5a, binds two seven transmembrane receptors; namely the C5a receptor 1 (C5aR1) and C5a receptor 2 (C5aR2). C5aR2 is a non-G-protein-signalling receptor whose biological role remains controversial. Some of this controversy arises due to the lack of selective ligands for C5aR2. In this study, a library of 61 peptides based on the C-terminus of C5a was assayed for the ability to selectively modulate C5aR2 function. Two ligands (P32 and P59) were identified as functionally selective C5aR2-ligands, exhibiting selective recruitment of β-arrestin 2 via C5aR2, partial inhibition of C5a-induced ERK1/2 activation, and LPS-stimulated IL-6 release from human monocyte-derived macrophages (HMDM). Importantly, neither ligand could induce ERK1/2 activation or inhibit C5a-induced ERK1/2 activation via C5aR1 directly. Finally, P32 inhibited C5a-mediated neutrophil mobilization in wild-type, but not $C5aR2^{-/-}$ mice. These functionally selective ligands for C5aR2 are novel tools that can selectively modulate C5a activity *in vitro* and *in vivo*, and thus will be valuable tools to interrogate C5aR2 function.

INTRODUCTION

The complement cascade is an ancient part of our innate immune system that can be activated following tissue injury/damage and in response to foreign bodies [1]. Following activation of the cascade the complement peptides C3a, C4a and C5a are generated prior to the termination of the cascade with the formation of the membrane attack complex [1]. C5a is a potent immunomodulator, but also has emerging roles outside of immunity during embryonic development [23]. Its production is tightly controlled in order to produce a rapid response to tissue injury/pathogens yet protect against unwanted activity on host tissue [1, 4]. The disruption of this control can lead to the overproduction of C5a, which can lead to an uncontrolled inflammatory response. C5a has been associated with a number of autoimmune, inflammatory and neurodegenerative disorders [5, 6] although, interestingly, the overproduction of C5a can lead to a down regulation of the immune response in some leukocytes, but have the opposite effect on other cell types [7].

Two receptors have been shown to bind C5a: the C5a receptor 1 (C5aR1 or CD88) and the C5a receptor 2 (C5aR2 or C5L2) [8, 9]. C5aR1 and C5aR2 are known to have important functions in many inflammatory conditions [4]. C5aR1 is a classical G protein-coupled receptor (GPCR) and activation following C5a binding leads to predominantly $G\alpha_i$ activation which in turn activates multiple intracellular signalling pathways (including ERK, Akt, MAPK and PI3K) and β-arrestin recruitment for receptor desensitisation, recycling and perhaps also G-protein independent signalling [1, 4]. C5aR2 is a seven transmembrane domain receptor but is not coupled to G-proteins, partly due to key amino acid changes in the critical cytosolic DRY and NPXXY motifs where G-proteins have been shown to bind [10]. However, C5aR2 has been shown to bind to β-arrestins [11, 12]. C5aR2 was originally thought to be a simple recycling decoy receptor, similar to the chemokine receptor D6 [13]. This assumption is no longer accepted as C5aR2 has now been shown to have both pro- and anti-inflammatory activities, and also to have its own functional role, which is likely to be regulated through β-arrestin recruitment and signalling [14, 15]. We have recently published a model that potentially explains the contradictory

role of C5aR2 having both pro- and anti-inflammatory activities, through the modulation of C5a induced C5aR1 signalling via C5aR2 mediated β–arrestin recruitment [16].

One inflammatory condition that is strongly associated with the dysregulation of C5a is septicaemia [7]. The detrimental effects of C5a in sepsis have been well studied and include paralysis of neutrophil immune function, promotion of septic cardiomyopathy, driving the apoptosis of adrenal medullar cells and thymocytes, and consumptive coagulopathy which can all lead to multi-organ failure [5]. Using the caecal ligation and puncture (CLP) model of high grade sepsis in mice it was shown that C5aR1 and C5aR2 null animals had an 85% and 80% survival rate, respectively at day 3 compared to 0% in normal mice [17]. This data suggests that there is a cooperative interaction between C5aR1 and C5aR2 during the development of sepsis and that blocking of C5a, or both its receptors, could be a very effective therapeutic treatment option [18]. The pro-inflammatory cytokine IL-6 has been shown to play a key role in septicaemia and is involved in regulating the expression of C5aR1 during septicaemia [19].

The physiological role of C5aR2 however under both pathological and normal conditions remains controversial, but it is apparent that it has an important role in C5a biology and inflammatory disease [20]. A major issue for the field has been lack of selective ligands for C5aR2, as current ligands either target C5aR1 alone or interact with both receptors. As such, all of the information generated on C5aR2-specific activity to date has been obtained using C5aR2$^{-/-}$ mice, C5aR2 genetic knockdown, or C5aR2 blocking antibodies, which each come with their own limitations. In order to better understand the contribution of C5aR2 to C5a biology, selective ligands for this receptor are required.

Recently, we designed a library of small peptides based on the C-terminus of C5a [21]. Surprisingly, none of these peptides induced or inhibited C5aR1 activity, but several were instead selective C3aR agonists, giving evidence for the promiscuity of ligands designed to target the complement activation peptide receptors. In the current study we further interrogated this library for activity at C5aR2 using a radioligand assay to assess binding to C5aR2 expressing membranes, followed by a bioluminescence resonance energy transfer (BRET) assay

to measure β-arrestin 2 recruitment. Two partial agonists were identified that could recruit β-arrestin 2, selectively inhibit the release of IL-6 and modulate C5a-induced ERK1/2 activation in human monocyte-derived macrophages (HMDMs), and inhibit C5a-induced neutrophil mobilization *in vivo* in a C5aR2 dependent manner. Here we describe the first functionally selective C5aR2 ligands, which can recruit β-arrestin 2, selectively inhibit the release of IL-6 and modulate C5a-induced ERK1/2 activation in HMDMs, and inhibit C5a-induced neutrophil mobilization *in vivo* in a C5aR2-dependent manner.

RESULTS

Binding of complement proteins to C5aR2

We initially utilised a Flp-In CHO-C5aR2 membrane preparation to test for the binding activity of the anaphylatoxins at C5aR2 using an ^{125}I-C5a displacement scintillation proximity assay (SPA). There is still some controversy in the literature as to whether C5aR2 is the receptor for C3a-des Arg/acylation-stimulating protein (ASP), with more recent data in the literature that suggests this is not the case [22]. In our studies, neither C3a nor C3a-des Arg (at concentrations up to 1 μM) showed any discernible displacement of ^{125}I-C5a from Flp-In CHO-C5aR2 membrane preparations, whilst both C5a and C5a-des Arg displayed full and potent inhibition (Fig. 1). This further confirms that C3a and C3a-des Arg (ASP) do not bind to human C5aR2 as has been previously reported [23]. Furthermore, C5a-des Arg was originally described as a ligand for C5aR2 but our data here are in agreement with recent data showing that C5a-des Arg has similar affinity/potency as C5a for both C5aR1 and C5aR2 [16, 24]. This suggests that the functional roles of C5a and C5a-des Arg are very similar at C5aR2, and the description of C5a-des Arg as a predominant ligand for C5aR2 over C5aR1 can no longer be supported.

Screening peptide library for binding to C5aR2 and counter screening hits against C5aR1

Initially the library was screened at a single concentration of 100 μM for inhibition of ^{125}I-C5a binding to C5aR2 membranes. Of the 61 peptides only two peptides (P32, Ac-RHYPYWR-OH, and P59, Ac-LIRLWR-OH) showed significant inhibition ($P < 0.05$) of ^{125}I-C5a binding to

C5aR2 membranes when compared to buffer control (Fig. 2A). P32, P59 and P48 (selected as a negative control peptide, Ac-TLLRLWR-OH) were than screened using a full concentration response up to a maximum concentration of 500 μM (maximum concentration achievable due to level of DMSO in assay). Both P32 and P59 dose-dependently inhibited ^{125}I-C5a binding to C5aR2 and C5aR1 membranes with K_i in the μM range, whilst P48 only inhibited ^{125}I-C5a binding to C5aR1 (Fig. 2B, C and Table I). P32 had a similar affinity for both C5aR2 (223 μM) and C5aR1 (234 μM) whereas P59 had ~10 fold higher affinity for C5aR2 (48 μM) over C5aR1 (466 μM). P32 and P59 were previously shown to have no activity (either as an agonist or antagonist) up to a concentration of 100 μM, when the library was previously screened for activity at C3aR and C5aR1 [21].

P32 and P59 recruit β-arrestin 2 via C5aR2, but not C5aR1, and are unable to up-regulate heteromer formation between C5aR1 and C5aR2

Due to the non G-protein signalling nature of C5aR2, a BRET C5aR2-β-arrestin 2 recruitment assay, previously developed in our lab [16], was used to test peptides for functional activity at C5aR2. Both, P32 and P59 were identified as partial agonists for the recruitment of β-arrestin 2 via C5aR2 (Table 1). Neither ligand could recruit β-arrestin 2 to the same level as C5a, but they could recruit β-arrestin 2 at a similar rate to C5a (Fig. 3A). P32 and P59 dose dependently recruited β-arrestin 2 via C5aR2 with significantly higher efficacy than their binding affinity for C5aR2 (Fig. 3B, Table 1), in contrast to the reduced potency exhibited by C5a. It is now accepted that GPCRs act as prototypic allosteric proteins that do not necessarily have a stoichiometric relationship between ligand affinity and efficacy with interacting intracellular partners [25]. Neither, P32 or P59 could recruit β-arrestin 2 via C5aR1 (Fig. 3C), giving further evidence for the selectivity of the ligands for C5aR2. In addition, the complete library was tested for ligand induced β-arrestin 2 recruitment at C5aR1 and C5aR2 using DiscoverX PathHunter kits. Only P32 and P59 showed activity at C5aR2 and no peptide had any activity at C5aR1 (data not shown) [26]. We have previously shown that high concentrations of C5a can up-

regulate heteromer formation between C5aR1-C5aR2 [27], but neither P32 or P59 were able to up-regulate heteromer formation between the C5a receptors (Fig. 3D).

P32 and P59 inhibit C5a-induced ERK1/2 activation in HMDM but have no effect on C5a-induced ERK1/2 activation in CHO-C5aR1 cell line

It is well known that C5a binding to C5aR1 induces rapid and robust ERK1/2 activation; we have recently shown that C5a binding to C5aR2 has no such effect on ERK1/2 activation [16]. Although C5aR2 has been shown to modulate C5aR1 ERK1/2 activation, this has only been tested to date using C5aR2 antibodies [12, 16]. Therefore, P32, P59 and P48 were tested for their ability to either directly stimulate ERK1/2 activation, or inhibit C5a-induced ERK1/2 activation via C5aR1, in HMDMd and a CHO-C5aR1 cell line (Fig. 4A, B). Neither P32 nor P59 could induce ERK1/2 activation alone in either cell type, but both could significantly inhibit C5a-induced ERK1/2 activation in HMDMs, with no effect in CHO-C5aR1 cells. P48 had no direct activity, or effect on C5a-induced ERK1/2 activation, in either cell type. To further confirm that P32 and P59 were acting selectively via C5aR2, and not C5aR1, C5a-induced ERK1/2 activation was quantified in the presence of the potent, selective C5aR1 antagonist, PMX53 [28]. PMX53, unlike P32 and P59, was able to almost completely inhibit ERK1/2 activation in both cell types, again further suggesting that P32 and P59 do not exert effects at C5aR1 (Fig. 4A, B and Table I). These data support the theory that C5aR2 can modulate C5aR1 induced ERK1/2 activation most likely through a β-arrestin dependent mechanism, and demonstrates the C5aR2-functional selectivity of P32 and P59.

P32 and P59 inhibit IL-6 release from HMDM

It is widely established that C5a receptors are important for the control of LPS-stimulated cytokine release from macrophages [29]. C5a dose-dependently inhibits the release of the pro-inflammatory cytokines IL-6 and TNFα, whilst increasing the release of the anti-inflammatory cytokine IL-10 from LPS-stimulated HMDMs [30]. We thus tested the ability of P32, P59 and P48 to directly modulate LPS-induced cytokine release from HMDMs. We found that P32 and P59

significantly inhibited the release of IL-6 (Fig. 5A), but had no effect on TNFα and IL-10 from LPS-stimulated HMDMs (Fig. 5C, E), whereas P48 again had no effect on any of the cytokines tested (only IL-6 data shown). Interestingly, both P32 and P59 had a similar maximum level of inhibition of IL-6 to C5a. To further demonstrate that P32 and P59 are acting via C5aR2, LPS stimulated IL-6 release from HMDMs was quantified in the presence of the C5aR1 antagonist, PMX53 (Fig. 5B, D, F). In the presence of PMX53 the effects of C5a on IL-6, TNFα and IL-10 were completely blocked, whereas the inhibition of IL-6 release by P32 and P59 was unchanged. This result gives further evidence for the functional selectivity of P32 and P59 for C5aR2.

P32 inhibits C5a-induced neutrophil mobilization in wild-type, but not C5aR2$^{-/-}$ mice

Finally, we were interested in assessing whether these C5aR2-selective ligands could be used to probe for the role of C5aR2 *in vivo*. We thus tested the effects of P32 in an acute mouse model of C5a-induced neutrophil mobilization. Systemic administration of C5a induces rapid neutrophil mobilization (neutrophilia) [31], and C5aR2 is known to affect leukocyte migration and adhesion [12, 32]. In our hands, intravenous injection of C5a (50 µg/kg) induced a rapid 6-fold increase in circulating neutrophils within 30 min (Fig. 6A). When P32 (3 mg/kg i.v.) was administered immediately prior to C5a, a significant 50% inhibition of neutrophil mobilization was observed (Fig. 6A). Assuming a mouse blood volume of 1.8 mL for a 25g mouse [33], our concentration of 3 mg/kg would result in C_{max} blood concentrations of ~50 uM, a dose within the active range of this compound from our *in vitro* studies. To confirm this effect of P32 *in vivo* was due to activity at C5aR2, the same experiment was repeated in C5aR2$^{-/-}$ mice. Notably, the inhibitory effect of P32 was completely attenuated in C5aR2$^{-/-}$ mice (Fig. 6B), demonstrating selectivity of P32 for C5aR2 *in vivo* at the concentration used.

DISCUSSION

We have utilised a novel computationally-designed library of 61 hexa-, hepta- and octapeptides [21] based on the C-terminal sequence of C5a (C-terminal octapeptide sequence = HKDMQLGR-OH) to discover the first functionally selective agonists for C5aR2 (C5L2/GPR77).

Subsequently, we showed that these ligands can recruit β-arrestin 2 via C5aR2, modulate C5a-induced ERK1/2 activation and specifically inhibit IL-6 release from human macrophages. C5aR2 was first thought to be solely a recycling decoy receptor, expressed by cells to remove excess C5a from the extracellular fluid, in order to avoid the detrimental C5aR1-mediated effects of high C5a concentrations [13]. It has now been shown that C5aR2 has additional roles in regulating the release of some cytokines and in a number of inflammatory conditions [14, 17, 29] One of the major impediments to a better understanding of the role of C5aR2 is the dearth of selective ligands [20].

Previously, it has been shown that C5aR2 can recruit and form a complex with β-arrestins, which can modulate ERK1/2 signalling in macrophages and neutrophils [12, 16, 34]. The simplistic view of β-arrestins as only regulators of GPCR desensitisation/internalisation has been updated in recent years. β-arrestins are now known to have important signalling roles both independent of and in competition with G-protein signalling in addition to their role in GPCR recycling and chemotaxis [35]. For example, β-arrestin 2 has been shown to act as part of a negative feedback loop that regulates the inflammatory response during sepsis [36]. With the C5a receptors, IL-6 and β-arrestin 2 being shown to be key components in the progression of sepsis [17, 19, 36], the ability to now selectively modulate each of these components should further our understanding of this difficult to treat condition. Sepsis currently accounts for ~350 000 deaths/year with a mortality rate of up to 50% and is responsible for 40% of ICU admissions, costing >$25 billion worldwide annually [37].

The 61-peptide library used in this study was originally designed for binding at C5aR1, but we have recently shown the library to contain C3aR-selective agonists with no activity at C5aR1[21]. We next decided to test the library for C5aR2 activity due to the similarities between C3a and C5a anaphylatoxin receptors. Using a ^{125}I-C5a displacement assay on C5aR2 expressing membranes, we were able to discover two ligands (P32 and P59) that showed significant displacement of ^{125}I-C5a. Follow-up testing showed both ligands to have high micromolar affinity for C5aR2 and C5aR1, but more importantly neither displayed any

functional activity at C3aR or C5aR1 when previously tested at concentrations up to 100 μM [21]. Having previously established a BRET C5aR2 β-arrestin 2 recruitment assay [16] the two hit peptides were tested for the ability to recruit β-arrestin 2 via C5aR2. Both, P32 and P59 were able to recruit β-arrestin 2 with ~37- and 6-fold higher efficacy respectively, than the affinity for C5aR2. Even though the peptides had significantly higher efficacy for β-arrestin 2 recruitment, neither was a full agonist when compared to native C5a. P32 and P59 were also both able to inhibit C5a-induced ERK1/2 activation in HMDMs, but had no effect on C5a-induced ERK1/2 activation in CHO-C5aR1 cells. In addition P32 and P59 could selectively inhibit LPS-induced IL-6 release from HMDM even in the presence of PMX53. This data suggests that despite the fact that P32 and P59 could inhibit ^{125}I-C5a binding to C5aR1 membranes, we were unable to identify any functional effect of this binding, giving further credence that these ligands are functionally selective for C5aR2. Interestingly, our data also demonstrates that C5aR2 can selectively modulate LPS-induced IL-6 release in human macrophages, revealing a selective functional role for C5aR2 in inflammatory cytokine production. These results are in accord with the reported role for C5aR2 in the regulation of IL-6 generation from data obtained using C5aR2$^{-/-}$ mice and anti-C5aR2 antibodies [15, 17].

P32 and P59 both have a C-terminal arginine, which is believed to be a key residue for interaction of C5a with the transmembrane region of C5aR2 [38]. Both peptides replace the adjacent glycine residue found in C5a with tryptophan and have a hydrophobic residue (Leu or Tyr) at the next position (Leu in C5a), but otherwise differ from C5a. Interestingly, the original library of 61 peptides contained some closely related peptides (differing at one or two positions from P32 and P59) that did not show significant activity in the original radioligand displacement screening assay. In order to begin to establish structure-activity relationships (SAR), the ligand displacement K_i and/or β-arrestin 2 recruitment EC_{50} of these compounds could be determined, along with a set of analogues based on the P32/P59 sequences. There is precedent for converting peptide ligands of complement receptors into potent small-molecules [39], and peptidomimetic approaches could also be investigated.

In summary, here we report the discovery of two functionally selective ligands (P32, P59) for C5aR2, which are partial agonists (when compared to C5a) for β-arrestin 2 recruitment. Importantly, our current and previous [21] data demonstrate that P32 and P59 are devoid of activity (either as agonists or antagonists) when screened against C3aR and C5aR1 in a range of assays, and can hence be considered as functionally selective ligands for C5aR2. We acknowledge that the utility of these ligands will be as tool compounds, rather than direct therapeutic candidates, due to their low affinity for C5aR2. Nevertheless these tool ligands provide a good starting point to investigate the biological role of C5aR2. This is verified by our demonstration that P32 could inhibit C5a-induced neutrophil mobilization in mice, in a C5aR2-dependent manner. The discovery of these functionally selective C5aR2 ligands will therefore now allow complement researchers to selectively probe the functional role of C5aR2 *in vitro* and *in vivo*. We plan to use these ligands as a medicinal chemistry template for development of higher affinity selective ligands for C5aR2. Indeed, the ability of P32 to inhibit C5a-mediated ERK1/2 activation and selectively inhibit IL-6 secretion from human macrophages, and block C5a-mediated neutrophil mobilization *in vivo*, indicates that potent and selective C5aR2 agonists may have therapeutic potential as treatments for inflammatory disease.

METHODS

Materials

β-arrestin 2-Rluc8 and β-arrestin 2-Venus clones were kindly supplied by Associate Prof. Kevin Pfleger (WAIMR, UWA, Perth, Australia). C5aR2 cDNA was supplied by Missouri S&T cDNA Resource Center (www.cdna.org). C5aR1-Rluc8 and C5aR2-Venus clones were generated previously [27] and kind permission for use of the RLuc8 and Venus constructs was provided 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 C3a, C3a-des Arg and C5a-des Arg were purchased from Merck Biosciences (Darmstadt, Germany), recombinant mouse

C5a was purchased from Hycult Biotech (Uden, The Netherlands). Glycerol, *E. coli*-derived LPS, BSA, $MgCl_2$, $CaCl_2$, Tris-HCL, DMSO and HEPES were purchased from Sigma-Aldrich (St Louis, MO, USA). DMEM, phenol-red free DMEM, IMDM, FBS, streptomycin, penicillin, hygromycin B, G418, trypsin-EDTA and PBS were purchased from Life Technologies (Carlsbad, CA, USA). Recombinant human macrophage-CSF (M-CSF) was purchased from Miltenyi Biotec (Macquarie Park, NSW, Australia).

Design of the peptide library

The peptide library consisted of 61 octa-, hepta- and hexapeptides designed around the C-terminal sequence and structure of C5a. A two-stage de novo protein design computational framework was used to construct the peptide library, based on flexible structural templates of C5a from molecular dynamics simulations, as previously described [21]. The full list of sequences of the peptide library is in Halai et al, 2014 [21]. The computational framework, its evolution and applications in peptide design are described in [21, 40, 41]

Peptide synthesis

All initial peptides were synthesized by GenScript (Piscataway, NJ) with acetylated N-termini at >95% purity. The C-termini of all peptides was unblocked. Purity was confirmed by analytical HPLC. The 61 peptides of the library were dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 10 mM and stored at -20^{o}C till needed for assay. Lead peptides were then dissolved in DMSO at a stock concentration of 100 mM. Resyntheses of P32 and P59 were conducted by Mimotopes Pty Ltd (Clayton, Vic, Australia) to >95% purity, confirmed by analytical HPLC and LCMS analysis.

Cell Culture and transfection

HEK293 cells were maintained in DMEM containing 10% FBS, 50 IU mL^{-1} penicillin, 50 mg mL^{-1} streptomycin (Life technologies) at 37 ^{o}C and 5% CO_2. Flp-In™ CHO cells stably expressing human C5aR2 were generated in-house using the Flp-In™ system (Life

Technologies). The Flp-In™ CHO-C5aR2 were cultured in a buffer of Ham's F12, 10% FBS, 50 IU mL^{-1} penicillin, 50 µg mL^{-1} streptomycin and 1000 µg mL^{-1} hygromycin B (Life Technologies). CHO cells stably expressing C5aR1 (Perkin Elmer, Waltham, MA, USA) were maintained were cultured in Ham's F12, 10% FBS, 100 IU mL^{-1} penicillin, 100 µg mL^{-1} streptomycin and 400 µg mL^{-1} G418 (Life Technologies). Cells were passaged at 80% confluence using 0.05% trypsin: EDTA in phosphate-buffered saline (PBS), pH 7.4. All cells in culture, once seeded, were incubated at 37 °C with 5% CO_2, unless stated otherwise. Transient transfections of HEK cells were performed 24 h after seeding cells at 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 manufacturer's instructions. Cells were collected 24 h after transfection with 0.05% trypsin-EDTA in PBS (Life Technologies). To generate HMDMs, 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 (Miltenyi Biotec) [42]. Isolated $CD14^+$ monocytes were differentiated in culture for 7 days with 10 ng ml^{-1} human M-CSF (Miltenyi Biotec) into HMDMs in the presence of IMDM containing L-glutamine supplemented with 10 % FBS, 50 IU ml^{-1} penicillin and 50 mg ml^{-1} streptomycin (Life Technologies) [43].

C5aR2/C5aR1 membrane preparation

Membranes were prepared from Flp-In CHO-C5aR2 and CHO-C5aR1 cells by a previously described method [44]. Briefly, cells were washed with PBS, pH 7.4, and harvested into buffer (50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM $CaCl_2$) before homogenizing with a Polytron homogenizer for 3 min on setting 22. Cells were centrifuged at 150 x g for 5 min at 4 °C. The supernatant was recovered and centrifuged at 22 000 x g for 1 h at 4 °C. Membrane pellets were then resuspended in 0.4 mL of ice-cold buffer (50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM $CaCl_2$, containing 10% glycerol) with aliquots stored at −80 °C. The protein concentration of the membrane preparation was determined by the Bradford method [45].

C5aR2/C5aR1 receptor binding studies

C5aR2/C5aR1 receptor binding studies were performed using a modification of a previously described scintillation proximity assay (SPA) method [46]. Briefly, SPA studies were performed in 96-well white isoplates with clear flat bottoms (Perkin Elmer). SPA beads (200 µg/well) were added, following the addition of various concentrations of competing ligands or buffer (6 pM to 500 µM) in a total volume of 80 µL of assay buffer (50 mM Tris-HCl, pH 7.4, 0.5% BSA, 5 mM $MgCl_2$, 1 mM $CaCl_2$). ^{125}I-C5a (~20 pM) (specific activity, 2200 Ci $mmol^{-1}$) (Perkin Elmer) was added to membrane preparations (~2 µg/well). The final reaction volume per well comprised 20 µL of ligand/buffer, 20 µL of SPA beads, 20 µl of membrane and the assay was initiated by the addition of 20 µL of ^{125}I-C5a. The plate was then sealed using TopSeal-A (Perkin Elmer) sealing film and incubated with shaking for 1 h at room temperature. Radioligand binding was then assessed for 30 s/well using a 1450 Microbeta scintillation counter (Perkin Elmer).

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

Assays were performed as a ligand-induced BRET experiment similar to previously described [16, 27, 47]. In brief, HEK293 cells were transiently transfected with either C5aR1-Rluc8 and β-arrestin 2-Venus or C5aR2-Venus and β-arrestin 2-Rluc8 or C5aR1-Rluc8 and C5aR2-Venus in a 100-mm dish. 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 M1000Pro plate reader (Tecan, Männedorf, Switzerland). 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 520–550 nm emission over the 460–490 nm emission for a cell sample containing the C5aR1-Rluc8 or β-arrestin 2-Rluc8 construct from a cell sample containing both the C5aR1-Rluc8 and β-arrestin 2-Venus or C5aR2-Venus and β-arrestin 2-Rluc8 or C5aR1-Rluc8 and C5aR2-Venus constructs. Peptides were also tested for ligand-induced β-arrestin 2 recruitment at 100μM using DiscoveRx PathHunter kits for human C5aR1 and C5aR2 (DiscoveRx Corporation, Birmingham, UK), according to the manufacturer's instructions.

ERK1/2 Phosphorylation Assays

ERK1/2 phosphorylation assays were performed using the Alphascreen Surefire ERK1/2 kit (Perkin Elmer) as per manufacturers' instructions and previously described [44]. Briefly, HMDMs or CHO-C5aR1 cells were seeded at 50 000 cells/well in 96-well tissue culture plates (Nunc, Waltham, MA, USA) and serum starved overnight. Ligands were prepared in serum-free media. C5aR2 ligands were pre-incubated with cells for 30 min at 37 °C and 5% CO_2, prior to the addition of C5a (5 nM) for 10 min at 37 °C and 5% CO_2. 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 M1000Pro plate reader (Tecan).

Measurement of cytokine release from HMDM

Briefly, HMDMs were seeded in 96 well tissue culture plates (Nunc) at 100 000 cells/well and allowed to adhere overnight at 37 °C and 5% CO_2. The next day HMDMs were stimulated with *E.coli* derived LPS (10 ng ml^{-1}) only or LPS (10 ng ml^{-1}) and either C5a or C5aR2 ligands at

various concentrations (1-100 nM (C5a) or 0.1-100 µM (C5aR2 ligands)). For testing cytokine release in the presence of C5aR1 antagonist (PMX53), cells were pre-incubated with PMX53 (10 µM) for 30 min prior to addition of C5a or C5aR2 ligands. Following ligand addition HMDMs were returned to 37 °C and 5% CO_2 incubator and 24 h after stimulation supernatant was removed from cells and transferred to 96 well polypropylene microplate (Nunc) and stored at -20 ^{o}C till needed for assay. Quantification of cytokine release was performed using ELISA kits (BD OptEIA™) as per manufacturers' instructions (BD Biosciences, San Jose, CA, USA).

Neutrophil mobilization experimental design

The University of Queensland Animal Ethics Committee approved all mouse experiments. C57BL/6J wild-type mice or C5L2$^{-/-}$ mice on a C57BL/6J background were given the vehicle (sterile saline) or C5L2 agonist (P32) (3 mg/kg) intravenously into the femoral vein. Following this injection a further intravenous injection of either vehicle (sterile saline) or recombinant mouse C5a (50 µg/kg) was administered to the opposite femoral vein. After 30 minutes blood samples (collected in 1 mg/mL EDTA and 0.1 mg/mL FUT-175) were collected from the abdominal aorta. Total circulating leukocyte counts were determined from blood smears and total white blood cell counts using a hemocytometer.

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 a one-way ANOVA with Dunnett's or Sidak's 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. Peter Monk proposed, designed, performed and analyzed experiments. Reena Halai contributed to the design of the experiments and data analysis. Geraldine Kaeslin, Zoe Schofield and Mike Wu designed and performed experiments. Dimitrios Morikis, Christodoulos Floudas contributed the design of the peptide library. Richard Clark, Mark Blaskovich analysed data and arranged peptide resynthesis. Matthew Cooper contributed to the design of the experiments and obtained funding for the research. Trent Woodruff proposed, designed, performed and analysed experiments. All authors participated in writing the manuscript.

REFERENCES

1. Klos A, Wende E, Wareham KJ, Monk PN. International Union of Pharmacology. LXXXVII. Complement Peptide C5a, C4a, and C3a Receptors. *Pharmacological Reviews* 2013; **65**(1)**:** 500-543.

2. Denny KJ, Coulthard LG, Jeanes A, Lisgo S, Simmons DG, Callaway LK *et al.* C5a receptor signaling prevents folate deficiency-induced neural tube defects in mice. *J Immunol* 2013; **190**(7)**:** 3493-9.

3. Hawksworth OA, Coulthard LG, Taylor SM, Wolvetang EJ, Woodruff TM. Brief report: complement C5a promotes human embryonic stem cell pluripotency in the absence of FGF2. *Stem Cells* 2014; **32**(12)**:** 3278-84.

4. Monk PN, Scola AM, Madala P, Fairlie DP. Function, structure and therapeutic potential of complement C5a receptors. *Br J Pharmacol* 2007; **152**(4)**:** 429-448.

5. Klos A, Tenner AJ, Johswich K-O, Ager RR, Reis ES, Köhl J. The role of the anaphylatoxins in health and disease. *Mol Immunol* 2009; **46**(14)**:** 2753-2766.

6. Woodruff TM, Ager RR, Tenner AJ, Noakes PG, Taylor SM. The role of the complement system and the activation fragment C5a in the central nervous system. *Neuromolecular Med* 2010; **12**(2)**:** 179-92.

7. Ward PA. The dark side of C5a in sepsis. *Nat Rev Immunol* 2004; **4**(2)**:** 133-142.

8. Gerard NP, Gerard C. The chemotactic receptor for human C5a anaphylatoxin. *Nature* 1991; **349:** 614-617.

9. Cain SA, Monk PN. The Orphan Receptor C5L2 Has High Affinity Binding Sites for Complement Fragments C5a and C5a des-Arg74. *J Biol Chem* 2002; **277**(9)**:** 7165-7169.

10. Okinaga S, Slattery D, Humbles A, Zsengeller Z, Morteau O, Kinrade MB *et al.* C5L2, a nonsignaling C5A binding protein. *Biochemistry* 2003; **42**(31)**:** 9406-15.

11. Van Lith LH, Oosterom J, Van Elsas A, Zaman GJ. C5a-stimulated recruitment of β-arrestin2 to the nonsignaling 7-transmembrane decoy receptor C5L2. *J Biomol Screen* 2009; **14**(9)**:** 1067-75.

12. Bamberg CE, Mackay CR, Lee H, Zahra D, Jackson J, Lim YS *et al.* The C5a receptor (C5aR) C5L2 is a modulator of C5aR-mediated signal transduction. *J Biol Chem* 2010; **285**(10)**:** 7633-44.

13. Scola AM, Johswich KO, Morgan BP, Klos A, Monk PN. The human complement fragment receptor, C5L2, is a recycling decoy receptor. *Mol Immunol* 2009; **46**(6)**:** 1149-62.

14. Chen N-J, Mirtsos C, Suh D, Lu Y-C, Lin W-J, McKerlie C *et al.* C5L2 is critical for the biological activities of the anaphylatoxins C5a and C3a. *Nature* 2007; **446**(7132)**:** 203-207.

15. Gao H, Neff TA, Guo RF, Speyer CL, Sarma JV, Tomlins S *et al.* Evidence for a functional role of the second C5a receptor C5L2. *FASEB J* 2005; **19**(8)**:** 1003-5.

16. Croker DE, Halai R, Kaeslin G, Wende E, Fehlhaber B, Klos A *et al.* C5a2 can modulate ERK1/2 signaling in macrophages via heteromer formation with C5a1 and beta-arrestin recruitment. *Immunol Cell Biol* 2014; **92:** 631-639.

17. Rittirsch D, Flierl MA, Nadeau BA, Day DE, Huber-Lang M, Mackay CR *et al.* Functional roles for C5a receptors in sepsis. *Nat Med* 2008; **14**(5)**:** 551-7.

18. Ward PA, Guo RF, Riedemann NC. Manipulation of the complement system for benefit in sepsis. *Crit Care Res Pract* 2012; **2012:** 1-8.

19. Riedemann NC, Neff TA, Guo RF, Bernacki KD, Laudes IJ, Sarma JV *et al.* Protective Effects of IL-6 Blockade in Sepsis Are Linked to Reduced C5a Receptor Expression. *J Immunol* 2003; **170**(1)**:** 503-507.

20. Li R, Coulthard LG, Wu MC, Taylor SM, Woodruff TM. C5L2: a controversial receptor of complement anaphylatoxin, C5a. *FASEB J* 2013; **27**(3)**:** 855-64.

21. Halai R, Bellows-Peterson ML, Branchett W, Smadbeck J, Kieslich CA, Croker DE *et al.* Derivation of ligands for the complement C3a receptor from the C-terminus of C5a. *Eur J Pharmacol* 2014; **745:** 176-81.

22. Johswich K, Klos A. C5L2--an anti-inflammatory molecule or a receptor for acylation stimulating protein (C3a-desArg)? *Adv Exp Med Biol* 2007; **598:** 159-80.

23. Johswich K, Martin M, Thalmann J, Rheinheimer C, Monk PN, Klos A. Ligand Specificity of the Anaphylatoxin C5L2 Receptor and Its Regulation on Myeloid and Epithelial Cell Lines. *J Biol Chem* 2006; **281**(51)**:** 39088-39095.

24. Reis ES, Chen H, Sfyroera G, Monk PN, Kohl J, Ricklin D *et al.* C5a receptor-dependent cell activation by physiological concentrations of desarginated C5a: insights from a novel label-free cellular assay. *J Immunol* 2012; **189**(10)**:** 4797-805.

25. Kenakin T. Collateral efficacy in drug discovery: taking advantage of the good (allosteric) nature of 7TM receptors. *Trends Pharmacol Sci* 2007; **28**(8)**:** 407-15.

26. Monk PN, Bellows-Peterson ML, Smadbeck J, Floudas CA, Kieslich CA, Morikis D *et al.* De novo protein design of agonists and antagonists of C5a receptors. *Immunobiology* 2012; **217**(11)**:** 1162-1163.

27. Croker DE, Halai R, Fairlie DP, Cooper MA. C5a, but not C5a-des Arg, induces upregulation of heteromer formation between complement C5a receptors C5aR and C5L2. *Immunol Cell Biol* 2013; **91:** 625-633.

28. Woodruff TM, Nandakumar KS, Tedesco F. Inhibiting the C5-C5a receptor axis. *Mol Immunol* 2011; **48**(14)**:** 1631-42.

29. Bosmann M, Haggadone MD, Zetoune FS, Sarma JV, Ward PA. The interaction between C5a and both C5aR and C5L2 receptors is required for production of G-CSF during acute inflammation. *Eur J Immunol* 2013; **43**(7)**:** 1907-13.

30. Seow V, Lim J, Iyer A, Suen JY, Ariffin JK, Hohenhaus DM *et al.* Inflammatory responses induced by lipopolysaccharide are amplified in primary human monocytes but suppressed in macrophages by complement protein C5a. *J Immunol* 2013; **191**(8)**:** 4308-16.

31. Jagels MA, Hugli TE. Neutrophil chemotactic factors promote leukocytosis. A common mechanism for cellular recruitment from bone marrow. *J Immunol* 1992; **148**(4)**:** 1119-28.

32. Pundir P, MacDonald CA, Kulka M. The Novel Receptor C5aR2 Is Required for C5a-Mediated Human Mast Cell Adhesion, Migration, and Proinflammatory Mediator Production. *J Immunol* 2015; **195**(6)**:** 2774-87.

33. Diehl KH, Hull R, Morton D, Pfister R, Rabemampianina Y, Smith D *et al.* A good practice guide to the administration of substances and removal of blood, including routes and volumes. *J Appl Toxicol : JAT* 2001; **21**(1)**:** 15-23.

34. Hsu WC, Yang FC, Lin CH, Hsieh SL, Chen NJ. C5L2 is required for C5a-triggered receptor internalization and ERK signaling. *Cell Signal* 2014; **26**(7)**:** 1409-19.

35. Defea K. Beta-arrestins and heterotrimeric G-proteins: collaborators and competitors in signal transduction. *Br J Pharmacol* 2008; **153 Suppl 1:** S298-309.

36. Fan H, Bitto A, Zingarelli B, Luttrell LM, Borg K, Halushka PV *et al.* Beta-arrestin 2 negatively regulates sepsis-induced inflammation. *Immunology* 2010; **130**(3)**:** 344-51.

37. Schorr CA, Dellinger RP. The Surviving Sepsis Campaign: past, present and future. *Trends Mol Med* 2014; **20**(4)**:** 192-4.

38. Scola AM, Higginbottom A, Partridge LJ, Reid RC, Woodruff T, Taylor SM *et al.* The role of the N-terminal domain of the complement fragment receptor C5L2 in ligand binding. *J Biol Chem* 2007; **282**(6)**:** 3664-71.

39. Reid RC, Yau MK, Singh R, Hamidon JK, Reed AN, Chu P *et al.* Downsizing a human inflammatory protein to a small molecule with equal potency and functionality. *Nature Commun* 2013; **4:** 2802.

40. Bellows-Peterson ML, Fung HK, Floudas CA, Kieslich CA, Zhang L, Morikis D *et al.* De novo peptide design with C3a receptor agonist and antagonist activities: theoretical predictions and experimental validation. *J Med Chem* 2012; **55**(9)**:** 4159-68.

41. Klepeis JL, Floudas CA, Morikis D, Tsokos CG, Argyropoulos E, Spruce L *et al.* Integrated Computational and Experimental Approach for Lead Optimization and Design of Compstatin Variants with Improved Activity. *J Am Chem Soc* 2003; **125:** 8422-8423.

42. Verreck FAW, Boer Td, Langenberg DML, Hoeve MA, Kramer M, Vaisberg E *et al.* Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco)bacteria. *Proc Natl Acad Sci U S A* 2004; **101**(13)**:** 4560-4565.

43. Halili MA, Andrews MR, Labzin LI, Schroder K, Matthias G, Cao C *et al.* Differential effects of selective HDAC inhibitors on macrophage inflammatory responses to the Toll-like receptor 4 agonist LPS. *J Leukoc Biol* 2010; **87**(6)**:** 1103-14.

44. Halai R, Croker DE, Suen JY, Fairlie DP, Cooper MA. A Comparative Study of Impedance versus Optical Label-Free Systems Relative to Labelled Assays in a Predominantly Gi Coupled GPCR (C5aR) Signalling. *Biosensors* 2012; **2**(3)**:** 273-290.

45. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72:** 248-54.

46. Dutertre S, Croker D, Daly NL, Andersson A, Muttenthaler M, Lumsden NG *et al.* Conopressin-T from Conus tulipa reveals an antagonist switch in vasopressin-like peptides. *J Biol Chem* 2008; **283**(11)**:** 7100-8.

47. Kocan M, Dalrymple MB, Seeber RM, Feldman BJ, Pfleger KD. Enhanced BRET Technology for the Monitoring of Agonist-Induced and Agonist-Independent Interactions between GPCRs and β-Arrestins. *Front Endocrinol* 2011; **1:** 12.

FIGURE LEGENDS

Figure 1: C5aR2 binding affinity for the complement proteins C3a, C3a-des Arg, C5a and C5a-des Arg. Binding affinities for complement proteins: C5a K_i = 1.3 ±0.3 nM, C5a-des Arg K_i = 1.5 ±0.4 nM, C3a and C3a-des Arg K_i > 1 μM. Data is representative of at least three independent experiments performed in triplicate (n = 3-5). Data is normalised to C5a response for each experiment. Error bars represent SEM.

Figure 2: Identification and verification of hits at C5aR2 from 61-peptide library. (A) Testing of 61-peptide library at 100 μM for inhibition of ^{125}I-C5a binding to C5aR2 membranes. Asterisks indicate hits that show significant ($P < 0.05$) inhibition of ^{125}I-C5a binding compared to buffer control. **(B)** Sigmoidal dose-response curves for the hits P32, P59 and negative control peptide P48 for inhibition of ^{125}I-C5a binding to C5aR2 membranes**. (C)** Sigmoidal dose-response curves for the significant hits P32, P59 and negative control peptide P48 for inhibition of ^{125}I-C5a binding to C5aR1 membranes. Data is normalised to C5a response for each experiment. Data (mean ± SEM) are representative of at least three independent experiments performed in triplicate (n = 3-4). Error bars represent SEM; *$P < 0.05$ by one-way ANOVA followed by Dunnett's *post hoc* test.

Figure 3: BRET assay testing hits for β-arrestin 2 recruitment by C5aR1 or C5aR2 and ligand induced heteromer formation between C5a receptors. (A) Ligand-induced BRET ratio for β-arrestin 2 recruitment by C5aR2 for C5a (500 nM), P32 or P59 (100 μM). **(B)** Sigmoidal dose response curves for C5a, P32 or P59 for β-arrestin 2 recruitment by C5aR2 calculated at ~40 min after ligand addition. **(C)** Ligand-induced BRET ratio for β-arrestin 2 recruitment by C5aR1 for C5a (500 nM), P32 or P59 (100 μM). **(D)** Ligand-induced BRET ratio for up-regulation of heteromer formation between C5aR1-C5aR2 for C5a (500 nM), P32

or P59 (100 μM). Data (mean ± SEM) are representative of at least three independent experiments performed in triplicate (n = 3-4).

Figure 4: P32 and P59 inhibit C5a-induced ERK1/2 activation in HMDM but have no effect on CHO-C5aR1 ERK1/2 activation. (A) Measurement of pERK1/2 activation in HMDM: HMDM were pre-incubated with media, C5aR1 antagonist PMX53 (10 μM), P32, P48 or P59 (100 μM) for 30 min prior to stimulation with media control (buffer), C5a (5 nM), PMX53 (10 μM), P32, P48 or P59 (100 μM) for 10 min. Data is normalised to C5a 5 nM (100%) for each donor. (**B**) Measurement of pERK1/2 activation in CHO-C5aR1: cells were pre-incubated with media, PMX53 (10 μM), P32, P48 or P59 (100 μM) for 30 min prior to stimulation with media control (buffer), C5a (5 nM), PMX53 (10 μM), P32, P48 or P59 (100 μM) for 10 min. Data is normalised to C5a 5 nM (100%) for each experiment. Data (mean ± SEM) are representative of at least four independent HMDM donors or CHO-C5aR1 experiments performed in triplicate (n = 4-8); **** $P < 0.0001$ by one-way ANOVA followed by Dunnett's *post hoc* test.

Figure 5: Testing P32 and P59 for effects on cytokine release from LPS stimulated HMDM. Measurement of human inflammatory cytokines IL-6 **(A, B)**, TNFα **(C, D)** and IL-10 **(E, F)** release from HMDM, 24 h after co-stimulation with LPS (10 $ngml^{-1}$) and C5a (0.1-100 nM), P32, P48 or P59 (0.1-100 μM) with or without C5aR1 antagonist PMX53 (10 μM) present. Cytokine release quantified by ELISA. CTRL represents non-stimulated HMDM. Data is normalised to LPS response (100%) for each donor. Data (mean ± SEM) are representative of at least five independent HMDM donors performed in triplicate (n = 5-8); *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ by one-way ANOVA followed by Dunnett's *post hoc* test or Sidak's *post hoc* test for comparisons +/- PMX53.

Figure 6: P32 inhibits C5a-induced neutrophil mobilization *in vivo* in a C5aR2 dependent manner. Blood neutrophil counts quantified 30 min after i.v. injection of PBS, C5a (50 μg/kg),

P32 (3 mg/kg), or P32 (3 mg/kg) with and without C5a (50 µg/kg) in either wild-type (**A**) or C5aR2$^{-/-}$ (**B**) mice. Data (mean ± SEM) represent 4-6 mice per treatment group; * $P < 0.05$, *** $P < 0.001$ by one-way ANOVA followed by Sidak's *post hoc* test.

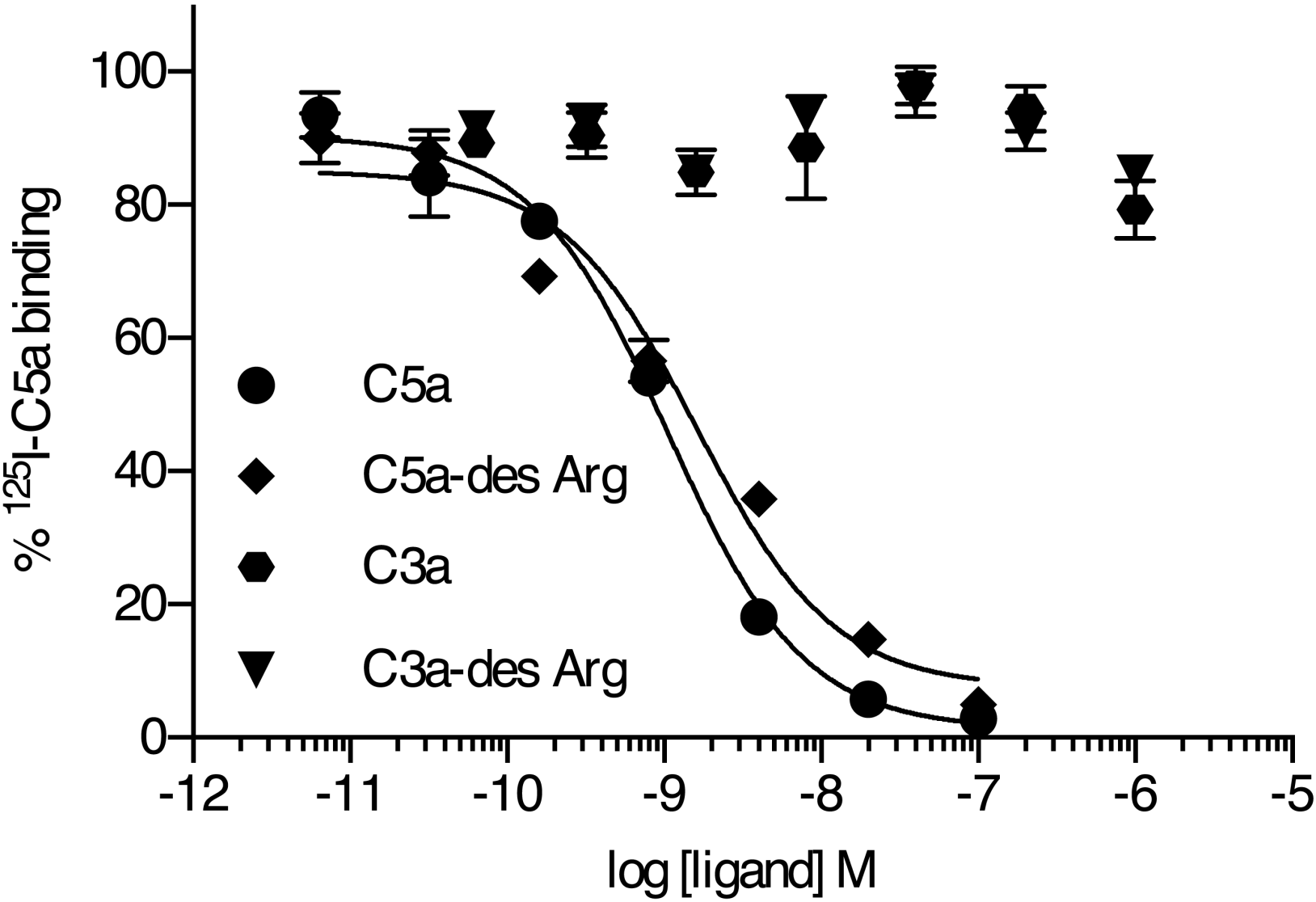
100
80
60
40
20
0
% 125I-C5a binding
C5a
C5a-des Arg
C3a
C3a-des Arg
-12
-11
-10
-9
-8
-7
-6
-5
log [ligand] M

Figure 1

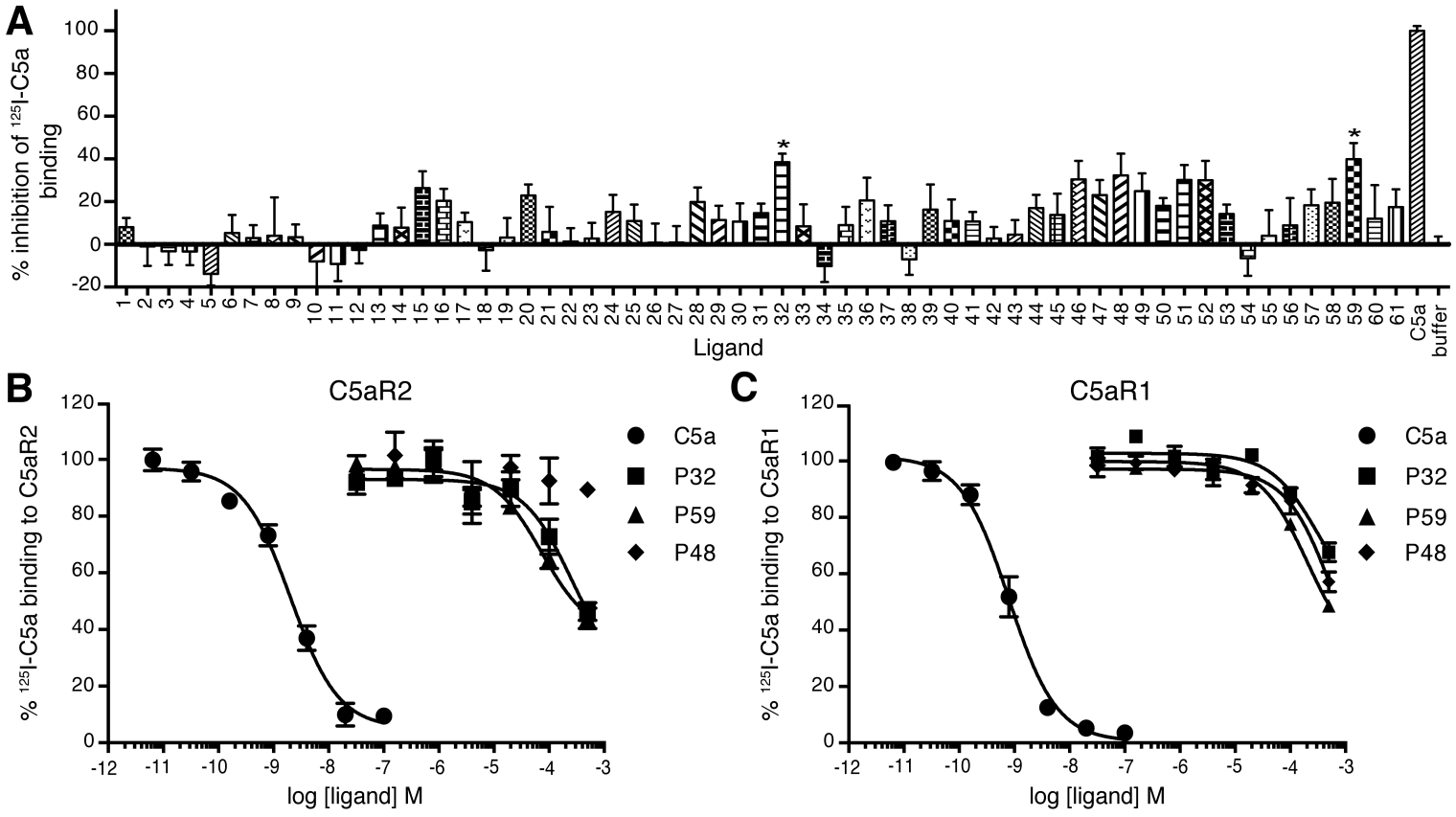
A
% inhibition of 125I-C5a binding
100
80
60
40
20
0
-20
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61
C5a
buffer
Ligand
*
*
B
C5aR2
% 125I-C5a binding to C5aR2
120
100
80
60
40
20
0
-12 -11 -10 -9 -8 -7 -6 -5 -4 -3
log [ligand] M
C5a
P32
P59
P48
C
C5aR1
% 125I-C5a binding to C5aR1
120
100
80
60
40
20
0
-12 -11 -10 -9 -8 -7 -6 -5 -4 -3
log [ligand] M
C5a
P32
P59
P48

Figure 2

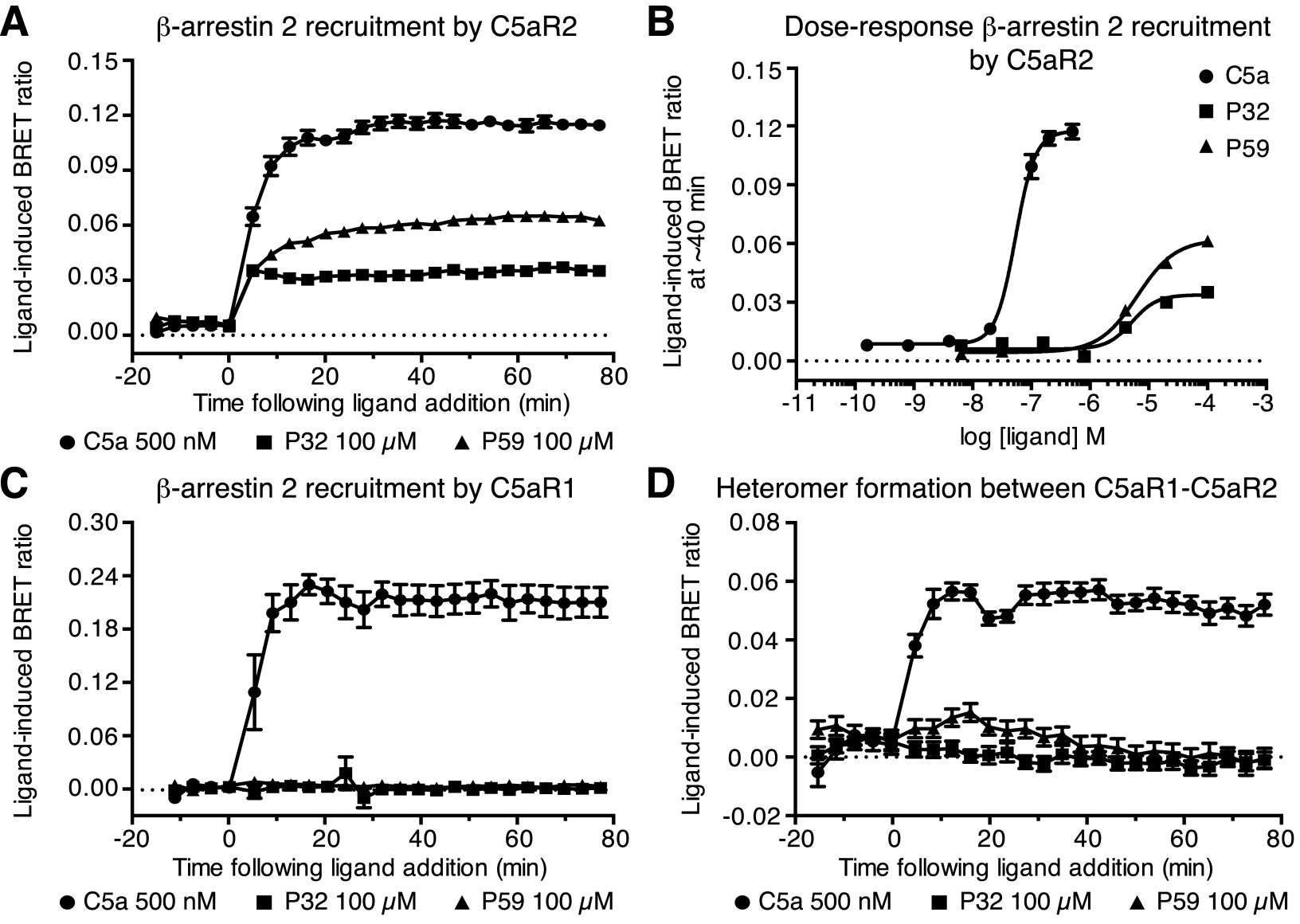
A
β-arrestin 2 recruitment by C5aR2
Ligand-induced BRET ratio
0.15
0.12
0.09
0.06
0.03
0.00
-20
0
20
40
60
80
Time following ligand addition (min)
C5a 500 nM
P32 100 μM
P59 100 μM
B
Dose-response β-arrestin 2 recruitment by C5aR2
Ligand-induced BRET ratio at ~40 min
C5a
P32
P59
-11
-10
-9
-8
-7
-6
-5
-4
-3
log [ligand] M
C
β-arrestin 2 recruitment by C5aR1
0.30
0.24
0.18
0.12
0.06
0.00
D
Heteromer formation between C5aR1-C5aR2
0.08
0.06
0.04
0.02
0.00
-0.02

Figure 3

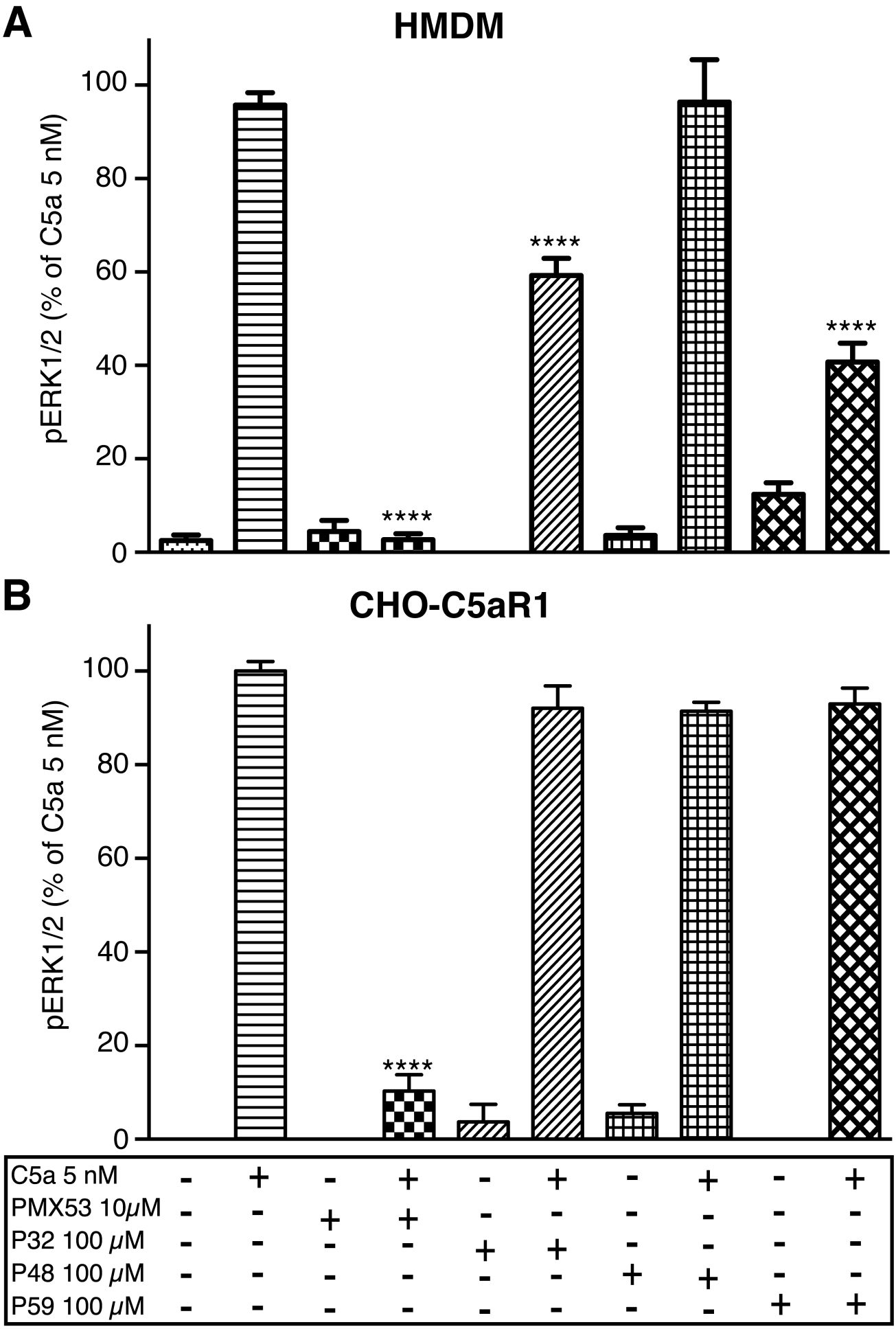
A
HMDM
pERK1/2 (% of C5a 5 nM)
100
80
60
40
20
0
****
****
****
B
CHO-C5aR1
pERK1/2 (% of C5a 5 nM)
100
80
60
40
20
0
****
C5a 5 nM - + - + - + - + - +
PMX53 10μM - - + + - - - - - -
P32 100 μM - - - - + + - - - -
P48 100 μM - - - - - - + + - -
P59 100 μM - - - - - - - - + +

Figure 4

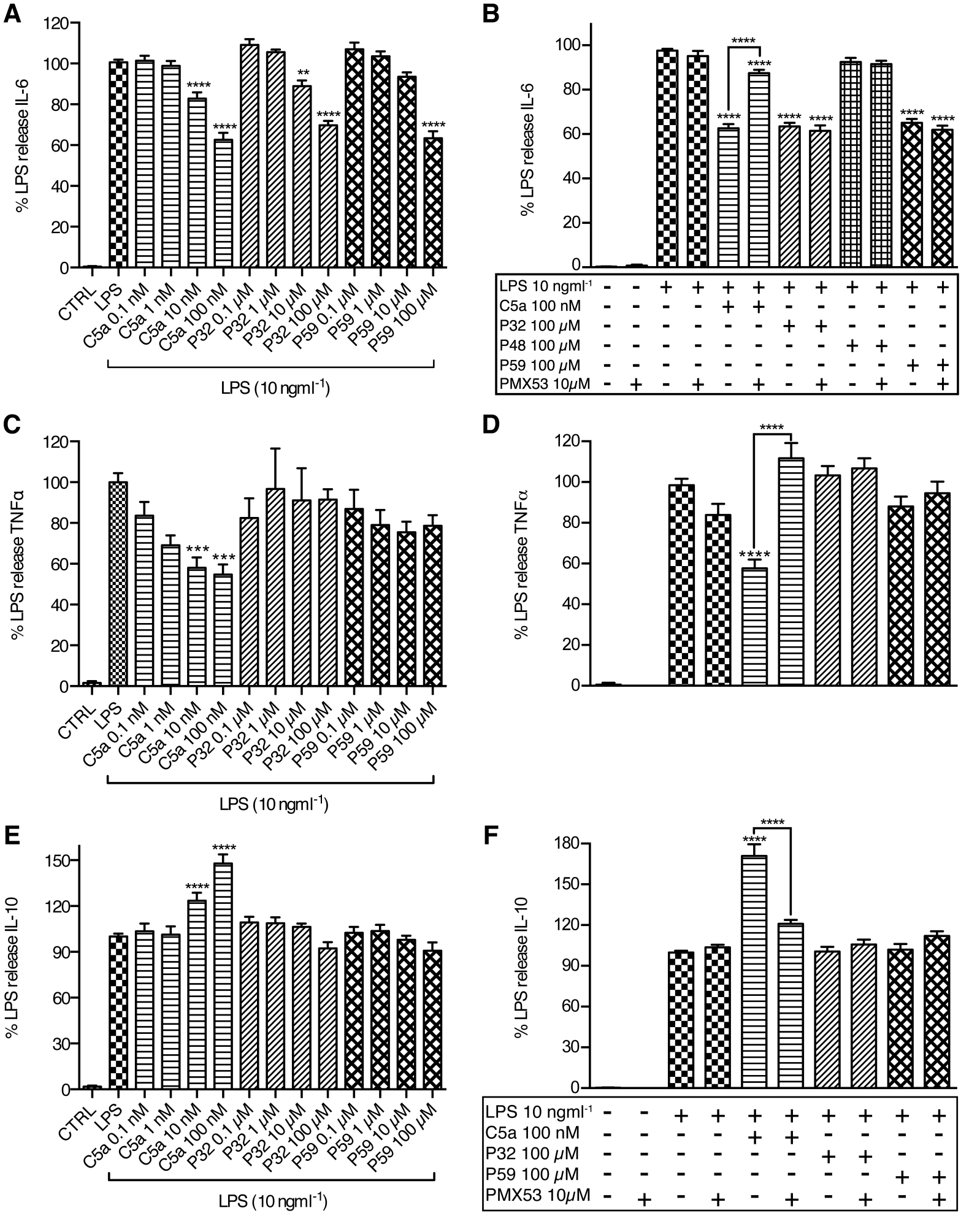
A
% LPS release IL-6
CTRL
LPS
C5a 0.1 nM
C5a 1 nM
C5a 10 nM
C5a 100 nM
P32 0.1 μM
P32 1 μM
P32 10 μM
P32 100 μM
P59 0.1 μM
P59 1 μM
P59 10 μM
P59 100 μM
LPS (10 ngml-1)
B
% LPS release IL-6
LPS 10 ngml-1
C5a 100 nM
P32 100 μM
P48 100 μM
P59 100 μM
PMX53 10μM
C
% LPS release TNFα
LPS (10 ngml-1)
D
% LPS release TNFα
E
% LPS release IL-10
LPS (10 ngml-1)
F
% LPS release IL-10
LPS 10 ngml-1
C5a 100 nM
P32 100 μM
P59 100 μM
PMX53 10μM

Figure 5

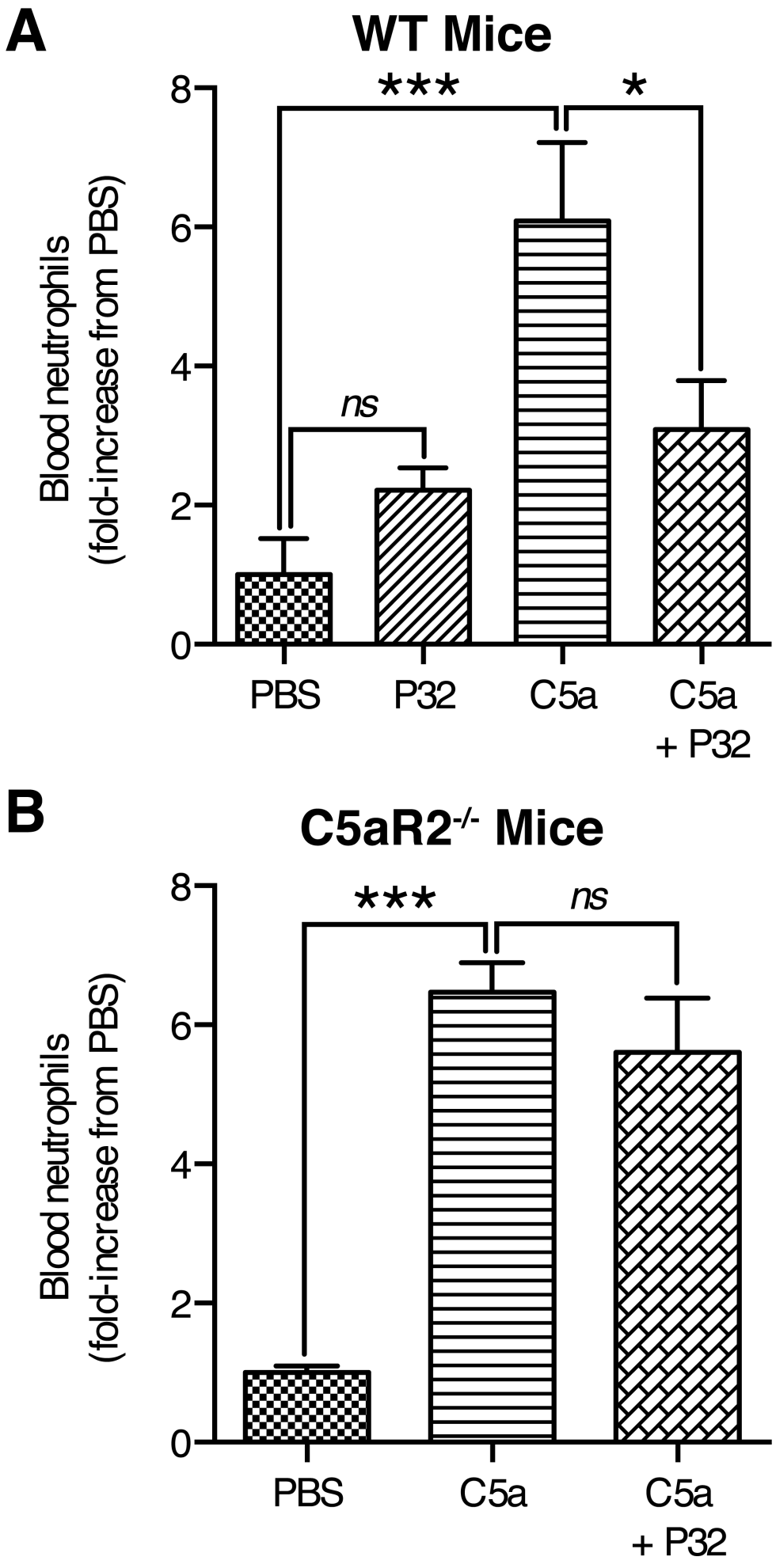
A
WT Mice
***
*
ns
Blood neutrophils
(fold-increase from PBS)
8
6
4
2
0
PBS
P32
C5a
C5a
+ P32
B
C5aR2-/- Mice
***
ns
Blood neutrophils
(fold-increase from PBS)
8
6
4
2
0
PBS
C5a
C5a
+ P32

Figure 6

TABLES

Table I: Binding affinity and efficacy for ligands at C5aR2 and C5aR1

	Sequence	C5aR2 binding	C5aR2-β-arrestin 2 BRET		C5aR1 binding	C5aR1 pERK1/2
		K_i ± SEM	EC_{50} ± SEM	Recruitment (% of C5a)	K_i ± SEM	% Inhibition of C5a 5 nM
C5a		1.8 ± 0.3 nM	53.7 ± 5.2 nM	100	0.57 ± 0.08 nM	**PMX53** 90
P32	RHYPYWR	223 ± 140 μM	5.2 ± 0.9 μM	28.8	234 ± 230 μM	8
P59	LIRLWR	64 ± 23 μM	6.4 ± 0.5 μM	52.9	466 ± 59 μM	7