

The Importance of C5aR2 in Neutrophil Function and Its Impact on Neutrophil-mediated Diseases

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Abstract

The two receptors for the anaphylatoxin C5a are critically involved in the recruitment of immune cells and activate these cells at sites of inflammation. The pro-inflammatory function of C5aR1 in these processes is well established, whereas the functional properties of the second C5a receptor, C5aR2, in inflammation remain enigmatic. We recently reported a pro-inflammatory contribution of C5aR2 to the pathogenesis of the prototypical autoimmune skin blistering disease *epidermolysis bullosa acquisita* (EBA). Deficiency of C5aR2 ameliorated the disease phenotype in an antibody transfer model of EBA and reduced neutrophil migration and activation *in vitro*. Here, we discuss not only these data, but the crosstalk of C5aR2 with Fcγ receptors, and the effect of C5a^{desArg} stimulation on neutrophils. In addition, we highlight the cellular location of C5aR2, its functional dependence on concomitant C5aR1 expression, and its importance for therapeutic strategies targeting the C5a receptor pathways in neutrophil-mediated diseases.

Keywords: C5aR2, C5a, C5adesArg, Neutrophil, Heterodimerization, Autoimmunity

Introduction

C5aR2 serves as the second receptor for the anaphylatoxin C5a. It was identified about 10 years after identification of the first cognate receptor, C5aR1. Initially, C5aR2 was considered a mere decoy receptor for C5a. According to this view, its function was to scavenge excess C5a from C5aR1 and thereby exert anti-inflammatory effects. However, this initial view of C5aR2 had been oversimplified. Several reports were published pointing toward the functional relevance of this receptor in cell homeostasis as well as in inflammatory conditions driven by immune cells that express both C5a receptors. Importantly, the functions of C5aR2 in such diseases varied substantially from anti- to pro-inflammatory effects and appeared to depend on the cellular and spatial expression of this receptor. Using *C5ar2*^{-/-} mice, we recently

demonstrated a pro-inflammatory contribution of *C5aR2* to the pathogenesis of *epidermolysis bullosa acquisita* (EBA). EBA is a rare autoimmune disease of the skin in which autoantibodies to type VII collagen cause neutrophil-mediated destruction of the dermal-epidermal junction, leading to subepidermal blistering and inflammation. A critical role of C5aR1 in the development and progression of EBA has been reported previously. Mice with a targeted deletion of the *C5ar1* gene were almost completely protected from the development of skin lesions and inflammation [1,2]. This protection from disease development was associated with reduced neutrophil function and migration. Because neutrophils are the major effector cells in EBA and express both C5a receptors at high levels, we additionally examined the effects of deleting *C5ar2* on C5a-mediated neutrophil function and migration *in vitro*. We found significantly impaired function and migration of

neutrophils from C5aR2^{-/-} mice compared with corresponding cells from wild-type mice. Interestingly, these C5a-mediated functions were entirely dependent on the presence of C5aR1.

The Enigmatic C5aR2

In humans, C5aR2 is abundantly expressed in immune cells such as myeloid cells, natural killer cells, and some T cells [3-8]. In mice, the C5aR2 gene is located on chromosome 7 adjacent to its paralog C5aR1 and encodes for a 344-amino acid seven transmembrane receptor. Using the recently established floxed tandem dye (td) Tomato-C5aR2 knock-in reporter mouse, C5aR2 has been shown to have an expression pattern comparable to humans in mice, but without a positive signal for C5aR2 in T cells [6,9,10]. For a detailed overview

of the expression patterns of C5aR1 and C5aR2 in mice and humans, see the review by Laumonnier et al. [11]. Like C5aR1, C5aR2 contains a potential N-linked glycosylation site at Asn3 and several serine and threonine residues at the C-terminal domain that serve as phosphorylation sites for GPCR kinases [6,12]. However, despite these possible phosphorylation sites and the general seven-transmembrane structure of C5aR2 (Figure 1a), C5aR2 does not bind to G- proteins. This is in part due to (I) a change in the DRY motif, in which the arginine residue important for Gα-protein coupling has been replaced by a leucine residue, (II) missing serine/threonine residues in the third intracellular loop, which is important for G protein recognition in C5aR1, and (III) a change in the NPXXY motif of the seventh transmembrane helix, which functions as an important signal transduction sequence in

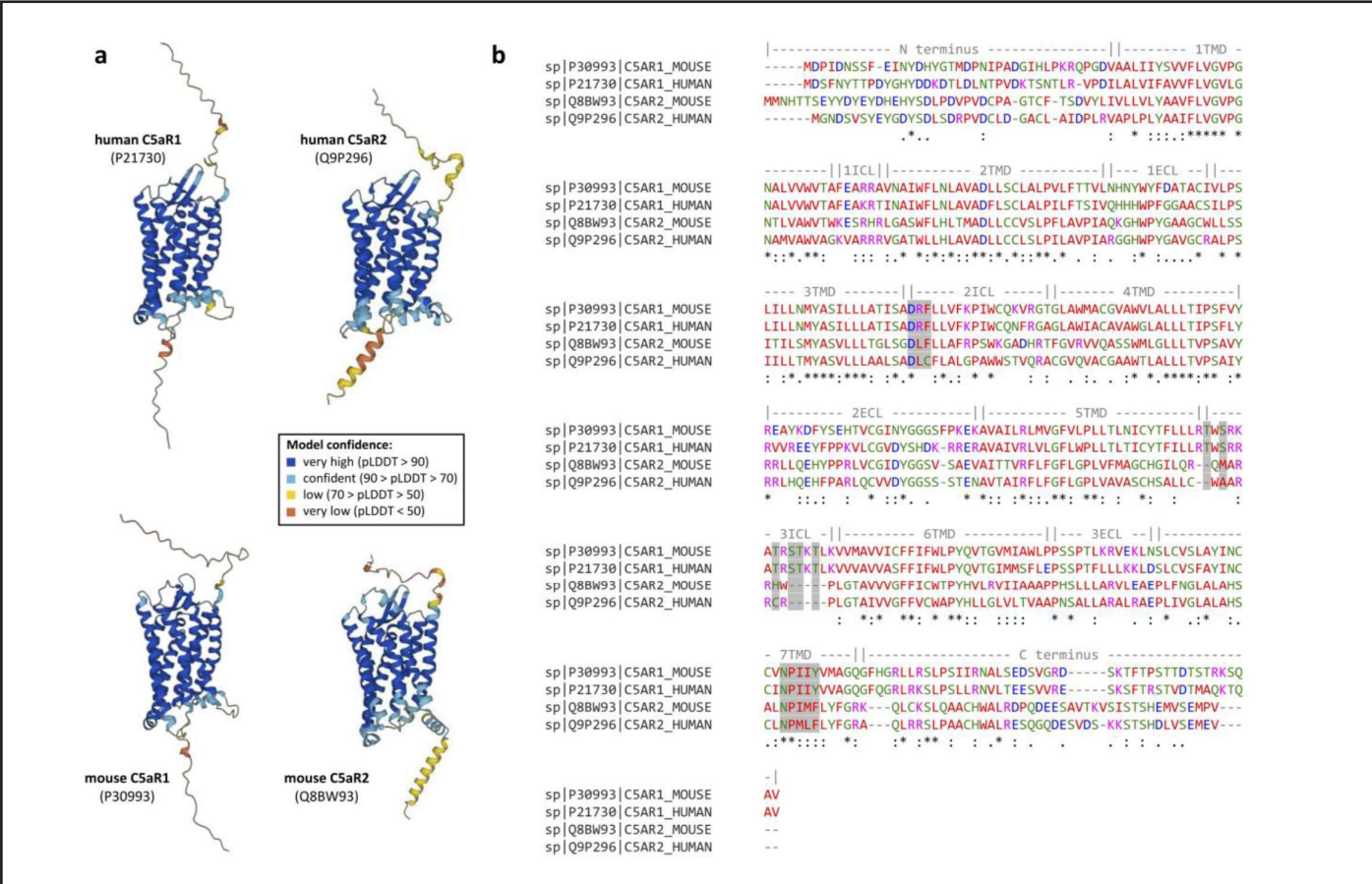


Figure 1. Predicted protein structures and amino acid sequence alignment of human and mouse C5a receptors. (a) AlphaFold protein structure prediction [68,69] of human C5aR1 (UniProt: P21730) and C5aR2 104 (UniProt: Q9P296) as well as mouse C5aR1 (UniProt: P30993) and C5aR2 (UniProt: Q8BW93). AlphaFold generates a per-residue confidence score (pLDDT) between 0 and 100. Some regions below 50 pLDDT may be unstructured in isolation. (b) Multiple sequence alignment of mouse C5aR1 (first row), human C5aR1 (second row), mouse C5aR2 (third row), and human C5aR2 (fourth row) generated using Clustal Omega v1.2.4 with default settings (<https://www.ebi.ac.uk/Tools/msa/clustalo/>; [70]). The seven transmembrane domains (TMD) as well as the intracellular loops (ICL) and extracellular loops (ECL) are indicated at the top of the alignment. Regions important for G-protein coupling in C5aR1 (DRY motif in the 2ICL, serine and threonine phosphorylation sites in the 3ICL, NPXXY motif in the 7TMD) but absent in C5aR2 are highlighted by light gray background. Amino acid colors indicate common amino acid properties: red = small, hydrophobic; blue = acidic; magenta = basic (without H); green = hydroxyl, sulfhydryl, or amine groups (including G). Multiple sequence alignment consensus is indicated by symbols at the bottom of the alignment: * single, fully conserved residue; : conservation between groups of strongly similar properties; . conservation between groups of weakly similar properties.

GPCRs (**Figure 1b**) [6,7,13-16]. The fact that C5aR2 can bind C5a and its degradation product C5a^{desArg} without triggering G protein-dependent signaling had led to the view of C5aR2 as a decoy receptor acting as a regulator of C5a and C5a^{desArg} availability for C5aR1. However, C5aR2 has been shown to recruit β -arrestins and modulate the recruitment of β -arrestins to C5aR1 [17-19]. Further, several studies have shown that C5aR2 has several functional properties. Interestingly, both pro-inflammatory and anti-inflammatory properties have been reported for C5aR2 – for a detailed overview of the conflicting roles of C5aR2 in pathophysiology, see the review by Li et al. [6]. Anti-inflammatory functions of C5aR2 have been demonstrated in studies of LPS- [20] and IC-mediated lung injury [21], allergic contact dermatitis [22], and recently also in a model of intestinal ischemia-reperfusion injury [23]. On the contrary, C5aR2 exerted pro-inflammatory function in experimental sepsis [24], experimental allergic asthma [25], thioglycolate-induced peritonitis and air-pouch inflammation [26], and surprisingly renal ischemia-reperfusion injury [27,28], and passive EBA [29]. In most cases, C5aR2 acted in concert with C5aR1, suggesting that C5a receptor crosstalk is required for the full development of certain pathological conditions.

Our previously published *in vitro* results are consistent with this perception of C5aR2 being dependent on the presence of C5aR1, as neutrophil activation, e.g., measured by the increase in intracellular calcium concentration and the up-regulation of CD11b, was completely absent when neutrophils lacked C5aR1. In support we found that pharmacological targeting by the C5aR1 antagonist PMX53 [30-32] or the dual C5aR1/C5aR2 inhibitor A8 Δ^{71-73} , abolished C5a-mediate activation of mouse neutrophils (unpublished observation). Such dependence of C5aR2 function on the presence of C5aR1 might be partially explained by the heterodimerization between the two C5a receptors as described previously [4,5,33,34]. It has been reported that heterodimer formation is required for C5a-induced internalization of C5aR1 and downstream PI3K/ERK signaling in a clathrin adaptor protein complex 2 (AP-2)-dependent manner [33].

Of note, to elicit optimal immune responses it also has been shown that complement receptors interact with pattern recognition receptors, particularly TLRs [6,35,36]. Such TLR activation has been shown to enhance C5a-induced pro-inflammatory responses such as the release of IL-6 and IL-8 in the absence of C5a-induced Ca²⁺ mobilization through negative modulation of C5aR2 [37]. On the other hand, this down-regulation of C5aR2 also negatively affected LPS-induced production of the nuclear protein high mobility group box 1 (HMGB1), an important mediator of inflammation in sepsis. Importantly, the release of HMGB1 is associated with PI3K/Akt activation and depends on C5aR2 but not C5aR1 [6,24,38,39]. In addition, C5aR2 can signal independently of C5aR1 through β -arrestin-scaffolded kinases [6,40], which is why it also has been proposed as an arrestin coupling receptor (ACR) [40].

The Impact of C5aR2 on the C5a-/Fc γ -receptor Crosstalk

In the past the critical bidirectional interaction between C5a and Fc γ R activation on cells expressing and downregulation of inhibitory Fc γ RIIb on myeloid cells [1,41-43]. The resulting change in the A/I ratio toward the activating phenotype primes the cells for an inflammatory response through a self-reinforcing feedback loop [29,42,44-46]. With our recently published data, we provide evidence that C5aR2 is involved in regulating the expression of Fc γ Rs on neutrophils and thereby modulates the A/I ratio. In further experiments, we stimulated bone marrow-derived neutrophils with C5a and analyzed the effects on the expression of Fc γ Rs. We found that C5a stimulation led to up-regulation of all Fc γ Rs, including Fc γ RIIb. Interestingly, although we did not detect a significant difference between Fc γ R expression in neutrophils from WT and C5aR2^{-/-} mice in response to C5a stimulation, similar to other C5a-mediated neutrophil responses analyzed, we noted that this up-regulation appeared to be dependent on the presence of C5aR1 (**Figure 2a**). This finding may suggest that the regulation of the A/I ratio by C5aR2 is an indirect effect due to modulation of C5aR1 signaling and does not act directly on Fc γ Rs.

Neutrophils Stimulated with C5a^{desArg} Show Similarly Diminished Responses when C5aR2 is Lacking

An important factor not addressed in our recent publication is the reported higher affinity of C5aR2 for C5a^{desArg} compared with C5aR1. C5a^{desArg} is the desarginated form of C5a, which is the primary degradation product of C5a in response to serum carboxypeptidase N (SCPN) with often reduced biological activity at C5aR1. However, C5a^{desArg} can induce several of the biological activities as C5a when administered at higher concentrations [5,16,47,48]. The rapid degradation of C5a to C5a^{desArg} in serum by SCPN is one mechanism to protect cells from uncontrolled C5aR1 activation, in particular on neutrophils, which are the most abundant leukocyte population in the circulation and express high numbers of C5aR1 [5].

To evaluate the impact of C5a^{desArg} on neutrophil activation, we repeated the *in vitro* neutrophil stimulation experiments presented in Seiler et al., 2022 [29] using C5a^{desArg} as a stimulant. We found that although C5a^{desArg} induced neutrophil chemotaxis and activation, the overall response to C5a^{desArg} was reduced compared to C5a, as expected. However, the effects of reduced neutrophil responsiveness in the absence of C5aR2 that we recently reported after stimulation with C5a were also observed after stimulation with C5a^{desArg}. Moreover, C5a^{desArg}-triggered neutrophil activation and chemotaxis were completely absent when C5aR1 was missing (**Figure 2b-d**), confirming the important role of C5aR1 in C5a/C5a^{desArg}-driven neutrophil function.

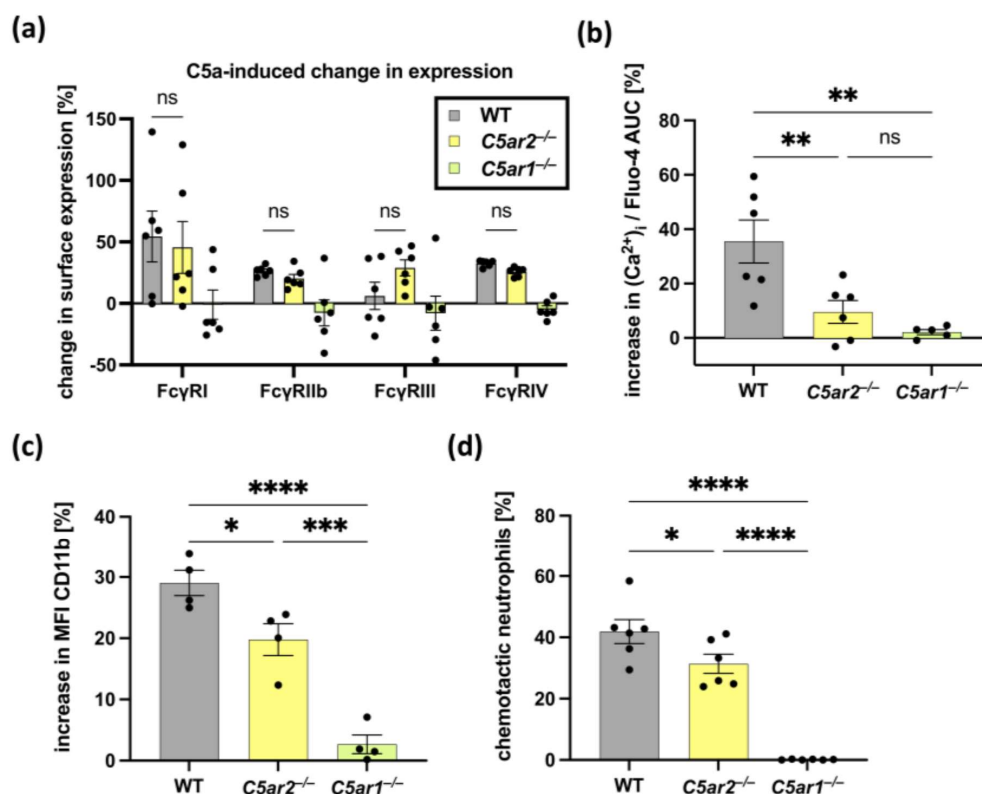


Figure 2. C5a-induced changes in the expression pattern of FcγRs on neutrophils and C5a^{desArg}-triggered neutrophil responses. (a) Change in surface expression of FcγRs on BM-derived neutrophils following stimulation with C5a (n = 6/group). **(b)** Changes in (Ca²⁺)_i concentration of neutrophils following stimulation with 20 nM C5a^{desArg} (n ≥ 5/group). **(c)** C5a^{desArg}-mediated activation of neutrophils quantified by up-regulation of surface expression of CD11b (n = 4/group). **(d)** Percentage of chemotactic bone marrow neutrophils towards C5a^{desArg} (n = 6/group). Shown are combined data from at least two independent experiments, each performed with 2-3 mice per genotype. Differences between groups were analyzed by ordinary one-way ANOVA with Holm-Šidák multiple comparison test.

Is C5aR2 an Intracellular Modulator of C5aR1 Signaling in Neutrophils?

One of the several controversies about C5aR2 relate to its cellular expression. Some studies found that only a small fraction (< 20%) of C5aR2 is located on the cell surface while most C5aR2 is predominantly localized within the cell [4,14]. In addition, BRET assays proposed that C5aR1 and C5aR2 form C5a-induced heterodimers, thereby regulating C5a signaling responses [5]. This heterodimerization required high C5a concentrations that are not present in the circulation under physiologic conditions but could be present locally under inflammatory conditions associated with strong complement activation such as in immune complex diseases like EBA.

Together with our previously published data, which demonstrated the dependence of C5aR2 function on the presence of C5aR1 in neutrophils, we propose that C5a is made available to the large pool of intracellular C5aR2 after binding of C5a to C5aR1 and subsequent internalization, which may be dependent on surface expressed C5aR2 [33].

In turn, intracellular C5aR2 may influence the function of C5aR1, either by triggering a signaling cascade itself or by transporting C5a or C5aR1 back to the cell surface to initiate repeated C5a/C5aR1-signaling [6]. This view is consistent with the observation that C5a, but not C5a^{desArg}, induces the formation of C5aR1/2 heterodimers [5]. Therefore, together with the lower affinity of C5a^{desArg} for C5aR1, this could also contribute to the lower overall neutrophil response to C5a^{desArg} compared to C5a.

Of note, based on its intracellular localization, C5aR2 may also contribute to immunometabolism, i.e., the impact of immune functions on cell metabolism [16,34,49,50].

C5a Receptor Targeting in Neutrophil-driven Diseases

The recent FDA approval of several compounds that target the complement system either at the level of the CP [51,52], the AP C3 [53,54], or the terminal pathway C5aR1 [55] and the successful treatment of PNH patients with the terminal

pathway inhibiting anti-C5 mAb eculizumab and derivatives [56-58] paved the way for an extensive pipeline of new therapeutic inhibitors targeting the complement system at several levels [59-61].

While specific blockade of one activation pathway might be an appropriate strategy to inhibit adverse complement activation in some complement-related diseases such as cold agglutinin disease (CP), IgA nephropathy and certain thrombotic microangiopathies (LP) as well as primary membranous nephropathy (AP), C5a and the membrane-attack complex formation, as effectors of the terminal pathway, critically contribute to the clinical manifestation of several complement-mediated diseases such as PNH, aHUS or ANCA-associated vasculitis [62]. Clearly, blocking C5a or C5a receptors might serve as an important immune target in neutrophil-mediated diseases, in particular in response to immune complex-driven diseases in the skin such as bullous pemphigoid diseases including EBA.

Due to the overwhelming evidence of C5aR1 as the main effector molecule of C5a/C5a^{desArg}-induced activation of immune cells and the still controversial and enigmatic role of C5aR2, C5aR1 has been considered the main C5aR target. Recently, avacopan (CCX168, Tavneos, ChemoCentryx), a small-molecule that effectively blocks C5aR1 but not C5aR2, has been approved for the treatment of antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis [55]. While the specific targeting of C5aR1 in this case was justified with findings from mouse studies of anti-MPO-induced necrotizing and crescentic glomerulonephritis that suggested a pro-inflammatory contribution of C5aR1 but a suppressive or anti-inflammatory mode of action for C5aR2 [63], our recent study clearly demonstrated that C5aR2 can also contribute to neutrophil-driven diseases following complement activation. Accordingly, therapeutic targeting of C5aR2 in addition to C5aR1 might be a strategy to consider in defined clinical settings such as autoimmune blistering of the skin or in severe sepsis [64,65].

Given the widespread expression of C5aR2 in various immune (e.g., granulocytes) and non-immune (e.g., cardiomyocytes) cells, it is reasonable to speculate that C5aR2 may have different functions depending on the tissue, cell, and/or subcellular location, which should be considered for therapeutic modulation of this receptor. Moreover, therapeutic targeting of C5aR2, in contrast to C5aR1, may pose particular challenges to be addressed due to its predominantly intracellular location. Although there are compounds that have been reported to have both binding and functional activity at C5aR2, to the best of our knowledge only the linear peptides P32 and P59 [66] are specific (agonists) for C5aR2, and a specific receptor antagonist has yet to be discovered. Clearly, such discoveries then need to be tested experimentally in appropriate animal models to get a better understanding of C5aR2 function in the specific disease setting.

Of note, although C5a and C5aRs are well conserved across species, there are reports of defined differences between mouse and human C5a and C5aRs (e.g., [67]). According to this, both human C5a (hC5a) and murine C5a (mC5a) act as C5aRs agonists regardless of the receptor species, with mC5a exhibiting higher potency. The degradation product hC5a^{desArg} is a partial agonist for hC5aRs and, to a lesser extent, for mC5aRs. In contrast, mC5a^{desArg} is a partial agonist of hC5aRs but has full agonist potency for mC5aRs, albeit reduced compared to mC5a. Therefore, it will be crucial to verify the functions of C5aR2 found in animal models – such as the net pro-inflammatory contribution of C5aR2 in antibody-transfer experimental EBA, which we have previously reported – in humans to draw accurate conclusions for its clinical use.

Conclusion

Our recent publication highlighted the critical function of C5aR2 in EBA disease development and showed a clear pro-inflammatory contribution. In neutrophils, which are critical drivers of inflammation in EBA, these effects were associated with and depending on C5aR1. Our study also showed that C5aR2 can affect the expression of other receptors, such as FcγRs, whose activation is involved in innate effector functions, i.e., ROS release. At this point, the underlying molecular signals downstream of C5aR2 activation are still unknown as is the potential of C5aR2 to dimerize with other 7TM receptors. Clearly, further research is required to uncover the signaling pathways of C5aR2 in different immune cells and in different inflammatory conditions and its crosstalk with other receptors at the molecular level. Such knowledge will fuel our understanding of when and where C5aR2 targeting might prove useful. This could be of particular relevance in neutrophils, which are not only involved in innate host defense but play an important role as drivers of autoimmune diseases such as ANCA-associated vasculitis or skin blistering diseases.

Material & Methods

Mice

C57BL/6J (WT) as well as C5ar1^{-/-} and C5ar2^{-/-} mice on the C57BL/6J genetic background were bred and housed in a 12-hour light-dark cycle at the University of Lübeck animal facility (Lübeck, Germany).

Bone marrow cell preparation

Isolation of bone marrow cells from femurs and tibiae is described in Seiler et al., 2022 [29].

C5a-induced changes in surface expression of FcγRs on neutrophils

To investigate the influence of C5a stimulation on the surface expression of FcγRs on neutrophils, bone marrow cells from

C57BL/6J (wild-type), *C5ar1^{-/-}* and *C5ar2^{-/-}* (both on C57BL/6J background) mice were isolated as described. Isolated bone marrow cells were incubated in complete RPMI-1640 medium containing 12.5 nM C5a or no C5a as a control for 30 minutes at 37°C, 5% carbon dioxide. Cells were washed and stained for Ly6G, CD11b, FcγRI, FcγRIIb, FcγRIII, and FcγRIV. Surface expression of FcγRs on C5a-stimulated and nonstimulated Ly6G⁺/CD11b⁺ cells were determined using a Cytex Aurora spectral flow cytometer. Changes in FcγRs expression were quantified by calculating the relative increase in mean fluorescence intensity of the FcγRI, FcγRIIb, FcγRIII, or FcγRIV signal in response to C5a stimulation.

Assessment of (Ca²⁺)_i changes in bone marrow neutrophils

Flow cytometry-based analysis of intracellular calcium flux is described in Seiler et al., 2022 [29].

CD11b up-regulation

The assessment of C5a-induced CD11b up-regulation is described in Seiler et al., 2022 [29].

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