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, C5a desArg, 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 in vitro. 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

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 (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.

The Impact of C5aR2 on the C5a-/Fcy-receptor Crosstalk

In the past the critical bidirectional interaction between C5a and FcyR activation on cells expressing both receptor families, including neutrophils has been described. Accordingly, C5a generated as a result of immune complex-mediated cellular activation can lead to up-regulation of activating FcyRs and down-regulation of inhibitory FcyRlb 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 FcyRs 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 FcyRs. We found that C5a stimulation led to up-regulation of all FcyRs, including FcyRlb. Interestingly, although we did not detect a significant difference between FcyR 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 FcyRs.

Neutrophils Stimulated with C5a

An important factor not addressed in our recent publication is the higher affinity of C5aR2 for C5adesArg compared with C5aR1. C5adesArg 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, C5adesArg can induce several of the biological activities as C5a when administered at higher concentrations [5,16,4748]. The rapid degradation of C5a to C5adesArg 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 circulation and express high numbers of C5aR1 [5].

To evaluate the impact of C5adesArg on neutrophil activation, we repeated the in vitro neutrophil stimulation experiments presented in Seiler et al., 2022 [29] using C5adesArg as a stimulant. We found that although C5adesArg induced neutrophil chemotaxis and activation, the overall response to C5adesArg 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 C5adesArg. Moreover, C5adesArg triggered neutrophil activation and chemotaxis were completely absent when C5aR1 was missing (Figure 2b-d), confirming the important role of C5aR1 in C5a/C5adesArg-driven neutrophil function.
Is C5aR2 an Intracellular Modulator of C5aR1 Signaling in Neutrophils?

One of the several controversies about C5aR2 relates 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-driven 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 classical pathway (CP) [51,52], the alternative pathway (AP) C3 [53,54], or the terminal pathway C5aR1 [55] and the successful treatment
of paroxysmal nocturnal hemoglobinuria (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 (lectin pathway, 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, atypical hemolytic uremic syndrome (aHUS) or antineutrophil cytoplasmic antibodies (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/C5aΔdesArg-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 ANCA-associated vasculitis [55]. While the specific targeting of C5aR1 in this case was justified with findings from mouse studies of anti-myeloperoxidase (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 hC5aΔdesArg is a partial agonist for hC5aRs and, to a lesser extent, for mC5aRs. In contrast, mC5aΔdesArg 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 seven-transmembrane 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 (WT), C5ar1−/− and C5ar2−/− (both on C57BL/6J background) mice were isolated as described [29]. 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 FcγRIIV. Surface expression of FcγRs on C5a-stimulated and nonstimulated Ly6G+/CD11b+ cells were determined using a BD LSRII 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+), 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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