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C5L2 is required for C5a-triggered receptor internalization and ERK signaling

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C5L2 is a receptor that binds to C5a and belongs to the family of G protein-coupled receptors, but its role in physiological C5a-mediated responses remains under debate. Here we show that, like the canonical C5a receptor C5aR, C5L2 plays a pro-inflammatory role in a murine model of acute experimental colitis. We demonstrate that C5L2 physically interacts with C5aR and is required for optimal C5a-mediated C5aR internalization and associated ERK activation. Abrogation of C5a-induced receptor internalization by treatment with the dynamin inhibitor dynasore™ impaired C5a-induced MEK and ERK signaling. Although the presence of C5aR alone was sufficient to recruit the scaffold protein β-arrestin1 to the cell membrane in response to C5a stimulation, it was inadequate to mediate AP2 recruitment and subsequent C5aR internalization. Expression of C5L2 allowed normal internalization of C5aR in response to C5a stimulation, followed by normal ERK signaling. Thus, our work reveals an essential role for C5L2 in C5a-triggered, AP2-dependent C5aR internalization and downstream ERK signaling.

1. Introduction

The complement system is composed of multiple soluble zymogens and regulatory factors, and is one of the major effectors of innate immunity [1]. Once activated by pathogen infection, all three pathways of complement activation converge on C3b, which contributes to the assembly of the C5 convertase. C5 convertase cleaves C5 into the C5a and C5b fragments. While C5b promotes the formation of the membrane attack complex (MAC) on the microbe surface and the eventual osmotic lysis of the target, C5a functions as an anaphylatoxin that triggers the host inflammatory response [2–4].

C5a is a 9 kDa peptide that activates both leukocytes and non-hematopoietic cells. In neutrophils, C5a stimulates NADPH oxidase assembly and triggers the production of reactive oxygen species (ROS) to destroy pathogens [5]. Local C5a is also a chemoattractant for neutrophil recruitment [6], and induces cytokine and chemokine secretion by endothelial cells that promotes local inflammation and additional leukocyte recruitment [7,8]. These effects are mediated mainly by the canonical C5a receptor (C5aR), which belongs to the seven transmembrane G protein-coupled receptor (GPCR) family [9,10].

Upon engagement by C5a, C5aR associates with the Gαi2 or Gα16 protein through its G protein-interacting DRY domain [11,12]. This complex transduces the downstream calcium efflux signal to activate mitogen-activated protein kinase (MAPK) [11,13]. In 2000, a second C5a receptor called C5L2 was discovered and found to be a GPCR-like protein that lacks the conserved Gα protein-coupling DRY motif [10,14]. C5L2 was initially thought to be a decoy receptor because C5L2 overexpression in L1.2 cells was unable to mediate C5a-induced calcium signaling or ERK activation. This deficit was erased when the mutated DRY motif in C5L2 was replaced with the C5aR DRY motif, suggesting that it is its DRY mutation that prevents C5L2 from transducing signaling initiated by C5a engagement [10].

Studies using a murine model of immune complex (IC)-induced lung injury have suggested that C5L2 has an anti-inflammatory function. Gene-targeted mice deficient for C5L2 (*c5l2*$^{-/-}$ mice) showed greater leukocyte lung infiltration and higher serum levels of TNFα and IL-6 than did IC-treated wild-type (WT) mice [15]. However, the results of two other studies have indicated that C5L2 can promote C5a-mediated pro-inflammatory responses. We previously showed that C5a-mediated ERK, p38 MAPK and Protein Kinase B (PKB)/Akt activation were all diminished in *c5l2*$^{-/-}$ neutrophils and macrophages [16,17]. In addition, methacholine-induced airway hyper-responsiveness and OVA-induced leukocyte infiltration in OVA-sensitized *c5l2*$^{-/-}$ mice were markedly reduced compared with sensitized WT littermates [16]. Consistent with these observations, in a cecum-ligated

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puncture (CLP)-induced mouse model of sepsis, both *c5ar*$^{-/-}$-CLP and *c5l2*$^{-/-}$-CLP mice survived longer than WT-CLP mice [17]. Furthermore, *c5l2*$^{-/-}$-CLP mice had lower serum levels of IL-1β, MIP1α and MIP2 compared to WT-CLP mice, and C5L2 was required for HMGB1 release by macrophages [17]. Thus, C5L2 appears to have both anti- and pro-inflammatory functions, but how these roles are regulated in response to C5a signaling is unclear.

Upon ligand stimulation, the C-terminus of a GPCR is phosphorylated and associates with the important scaffold protein β-arrestin [18,19]. β-Arrestin subsequently recruits endocytosis-related proteins such as adaptin-2 (AP2), clathrin and dynamin, which mediate the internalization of the GPCR. When such GPCR endocytosis is blocked, as by knockdown of β-arrestin, the late stage of ERK signaling, called endocytosis-dependent signaling, is abolished [20–22]. GPCRs can also regulate their downstream signaling through the formation of homodimers, heterodimers or oligomers [23–25]. In line with these observations, C5aR can form a heterodimer with the chemokine receptor CCR5 such that CCR5 phosphorylation is increased after C5a engagement of C5aR. Interestingly, C5a can trigger the internalization of a CCR5:C5aR heterodimer [26]. The formation of a heterodimer between C5L2 and C5aR has been recently reported [27], but whether this interaction is crucial for C5a-mediated signaling is unknown.

It has been demonstrated using experimental dextran sulfate sodium (DSS)-induced mouse model of acute ulcerative colitis (UC) that C5aR contributes to the pathology of inflammatory bowel disease (IBD) [28]. Mice deficient in C5aR (*c5ar*$^{-/-}$ mice) showed a reduction in weight loss compared to DSS-treated WT mice. Histological analyses revealed that, compared to WT-DSS mice, *c5ar*$^{-/-}$-DSS mice displayed decreased damage to the colonic epithelium, reduced intestinal crypt loss, and less leukocyte infiltration. We wondered if C5L2 might play a similar role in UC, and report here that C5L2 does indeed contribute to the local inflammation observed in a DSS-induced acute colitis model. Our results demonstrate that C5L2 plays a novel and important role in mediating C5a-induced AP2 recruitment, C5aR internalization, and ERK signaling downstream of C5aR engagement.

2. Materials and methods

2.1. Reagents and antibodies

The dynamin inhibitor dynasore^{TM} and phosphatase inhibitor cocktail were purchased from Sigma. The PI3K inhibitor Ly294002 was purchased from Calbiochem. Recombinant mouse C5a and mouse M-CSF were purchased from R&D Systems. The protease inhibitor cocktail was purchased from Roche.

Antibodies recognizing phosphorylated ERK1/2 (pERK1/2) or pMEK1/2 were purchased from Cell Signaling; antibodies recognizing ERK, HSP60, β-tubulin, Myc or GFP were obtained from Santa Cruz; FITC-conjugated anti-C5aR Ab was obtained from Cedarlane; anti-β-arrestin was purchased from Epitomics; anti-AP2 was obtained from BD Transduction Laboratories; anti-FLAG was obtained from Sigma; Cy3-labeled anti-mouse Ig was purchased from Jackson Immunoresearch; and HRP-labeled anti-mouse Ig and anti-rabbit Ig were obtained from GE Healthcare.

2.2. Mice and induction of ulcerative colitis

Male WT Balb/c mice (8 to 12 weeks old) and age-matched *c5l2*$^{-/-}$ mice (Balb/c) were maintained in the animal center of NYMU according to the protocol of the Institutional Animal Care and Use Committee (IACUC). For induction of acute UC, animals were given ad libitum access for 8 days to drinking water containing 4% DSS (MP Biomedicals, Illkirch, France). Body weight, stool viscosity and occult blood in stools were monitored and recorded every day. Disease activity index scores were evaluated as follows: decrease in body weight during acute disease relative to weight on day 0 (0%–3%, scores of 0; 3%–10%, 1; 10%–20%, 2; 20%–30%, 3; 30% ~ 40%, and 4); stool viscosity (0–3); and occult blood (0–1). Mice were sacrificed for colon sample collection on day 8.

2.3. Histology

Colons were collected, rolled, fixed in 4% paraformaldehyde, and embedded in paraffin according to standard protocols. Sections (7 μm) of paraffinized tissue were cut, mounted on slides, and stained with hematoxylin and eosin (H&E). Images were acquired using a Nikon Eclipse 80i microscope.

2.4. Preparation of bone marrow neutrophils, bone marrow-derived macrophages and peritoneal macrophages

For neutrophil isolation, total bone marrow cells were collected and separated using a Histopaque-1083 (Sigma) gradient to remove the upper layer of mononuclear cells. The cell pellet was resuspended in 10 ml ACK lysis buffer (8.29 g/l NH_4Cl, 1 g/l $KHCO_3$, 37 mg/l Na_2EDTA, pH 7.2) to lyse red blood cells (RBCs). After repeated washing, the remaining neutrophils were counted before use in experiments.

For bone marrow-derived macrophages, whole bone marrow cells were collected, incubated with ACK buffer to remove RBCs, and cultured in 9 cm Petri dishes in 6 ml RPMI 1640 medium (Gibco) containing 10% heat-inactivated FBS (Hyclone, Thermo) and recombinant mouse M-CSF (20 ng/ml, R&D). Fresh medium (3 ml) containing 10% FBS and M-CSF was added every other day. After 5 days, macrophages that had adhered to the plate were collected and counted prior to use in experiments.

For peritoneal macrophages, mice were intraperitoneally injected with 1 ml thioglycollate broth (2.4% in H_2O; Difco). Infiltrating cells in the peritoneal cavity were harvested 3 days later and incubated in ACK to remove RBCs. The remaining cells were washed, counted and cultured at 37 °C for 1 h in Petri dishes containing RPMI 1640 medium with 10% FBS. Adherent macrophages were collected, washed and counted prior to use in experiments.

2.5. Plasmid preparation

The pLP-ECFP-C1 (Clontech), pLP-EYFP (modified from pLP-ECFP-C1), and pDsRed-monomer-C1 (Clontech) plasmids were the kind gifts of the CHL laboratory. The ECFP and EYFP genes were first subcloned into the pCMV-MycJ3 vector (Clontech). Mouse C5aR or C5L2 cDNA was then subcloned into the N-terminus of the CFP or YFP vector, respectively. β-Arrestin1 cDNA was PCR-amplified and fused at its N-terminus to DsRed in a pPGK-based vector. For FLAG tag experiments, mouse C5L2 cDNA was subcloned into the pBabePuro-FLAG vector, and YFP was then subcloned into the C-terminus of C5L2.

2.6. Transfections and C5a stimulation

HEK293T cells were obtained from the American Type Culture Collection and routinely cultured in DMEM containing 5% FBS plus 1% penicillin and streptomycin (Sigma) at 37 °C in 5% CO_2. For transfections, optimized amounts of DNA were first mixed with PEI (final concentration 40 ng/μl) (Polysciences) in Opti-MEM (Gibco) and incubated at room temperature (RT) for 15 min before addition to cultures containing 3×10^5 HEK293T cells. Cells were cultured for 16 h and washed in DPBS prior to C5a stimulation.

For C5a stimulation, cells were rested in serum-free medium for 3 h prior to incubation with C5a for the time periods indicated in the figures. C5a at 30 ng/ml was used for signaling detection by immunoblotting, while 450 ng/ml C5a was used for co-localization detection by confocal microscopy analysis, AP2-C5aR immunoprecipitation and C5aR internalization by flow cytometry (see below).