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Ras-Related Protein Rab5a Regulates Complement C5a Receptor Trafficking, Chemotaxis, and Chemokine Secretion in Human Macrophages

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Abstract
Complement activation and Rab GTPase trafficking are commonly observed in inflammatory responses. Recruitment of innate immune cells to sites of infection or injury and secretion of inflammatory chemokines are promoted by complement component 5a (C5a) that activates the cell surface protein C5a receptor1 (C5aR1). Persistent activation can lead to a myriad of inflammatory and autoimmune diseases. Here, we demonstrate that the mechanism of C5a induced chemotaxis of human monocyte-derived macrophages (HMDMs) and their secretion of inflammatory chemokines are controlled by Rab5a. We find that C5a activation of the G protein coupled receptor C5aR1 expressed on the surface of HMDMs, recruits β-arrestin2 via Rab5a trafficking, then activates downstream phosphatidylinositol 3-kinase (PI3K)/Akt signaling that culminates in chemotaxis and secretion of pro-inflammatory chemokines from HMDMs. High-resolution lattice light-sheet microscopy on live cells showed that C5a activates C5aR1-GFP internalization and colocalization with Rab5a-tdTomato but not with dominant negative mutant Rab5a-S34N-tdTomato in HEK293 cells. We found that Rab5a is significantly upregulated in differentiated HMDMs and internalization of C5aR1 is dependent on Rab5a. Interestingly, while knockdown of Rab5a inhibited C5aR1-mediated Akt phosphorylation, it did not affect C5aR1-mediated ERK1/2 phosphorylation or intracellular calcium mobilization in HMDMs. Functional analysis using transwell migration and μ-slide chemotaxis assays indicated that Rab5a regulates C5a-induced chemotaxis of HMDMs. Further, C5aR1 was found to mediate interaction of Rab5a with β-arrestin2 but not with G proteins in HMDMs. Furthermore, C5a-induced secretion of pro-inflammatory chemokines (CCL2, CCL3) from HMDMs was attenuated by Rab5a or β-arrestin2 knockdown or by pharmacological inhibition with a C5aR1 antagonist or a PI3K inhibitor. These findings reveal a C5a-C5aR1-β-arrestin2-Rab5a-PI3K signaling pathway that regulates chemotaxis and pro-inflammatory chemokine secretion in HMDMs and suggests new ways of selectively modulating C5a-induced inflammatory outputs.

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Introduction

The complement system is an important signaling network for immunity. Depending on the activation stimuli, the complement system can be activated through the classical, alternative, lectin, and/or extrinsic pathways [1, 2]. These pathways converge and activate key inflammatory proteins C3 and C5 through proteolytic cleavage catalyzed by C3 and C5 convertases, leading to small complement protein byproducts, C3a and C5a [3]. C5a is a potent anaphylatoxin that signals primarily through its cognate G protein-coupled receptor (GPCR) called C5a receptor 1 (C5aR1) that is highly expressed on innate immune cells, such as neutrophils, monocytes, and macrophages. C5a activation of C5aR1 is generally acknowledged to have pro-inflammatory roles in many human diseases [2, 4], including COVID-19-associated inflammation [5], cancers [6], rheumatoid arthritis [7], and Alzheimer's disease [8]. During inflammatory responses, monocytes are recruited into tissues and differentiated into different macrophage subsets with different roles [9]. These human monocyte-derived macrophages (HMDMs) have been identified as important immune cells in diseases such as rheumatoid arthritis [10], colitis [11], asthma [12], and COVID-19 [13]. The development and differentiation of macrophages into different functional subsets are driven by granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF). GM-CSF stimulation leads to a pro-inflammatory M1-like subset, whereas M-CSF leads to an anti-inflammatory M2-like subset in response to pathogenic stimuli [14, 15]. From a signaling transduction perspective, HMDMs expressed high levels of C5aR1 and C5a-mediated C5aR1 activation is implicated in ERK1/2 phosphorylation, calcium mobilization, Rho activation, and β-arrestin2 recruitment [16, 17] leading to downstream functions, such as secretion of cytokines, chemotaxis, and phagocytosis.

The oncogenic Ras-related Rab proteins belong to the family of small GTPases that regulate membrane receptors internalization, endosome maturation and intracellular vesicular trafficking, recycling and degradation through interactions with specific effectors [18]. Given their important role in regulating these cellular processes, Rab proteins also control trafficking of immune receptors, such as toll-like receptors [19], endosome formation [20], antigen presentation [21], and cytokine secretion [22] in macrophages. Dysfunction of Rab proteins or impairment of Rab-related signaling pathways has been associated with a variety of cancers, neurological disorders, and immunodeficiencies [23, 24]. Rab5a facilitates GDP and GTP binding, GTPase activity, and is a key regulator of early endosome formation and transport, exocytosis, and trafficking of some GPCRs between membrane compartments [25, 26]. Rab5a-S34N dominant negative mutant has been reported to inhibit internalization of GPCRs, such as β_2 adrenergic receptor, neurokinin 1 receptor, and dopamine receptor D_2 [27–29] but has no effect on internalization of angiotensin II type 1A receptor [30]. This raises an intriguing question as to whether Rab5a regulates C5aR1 internalization and signaling in pro-inflammatory M1-like HMDMs. Despite extensive reports describing C5a-induced C5aR1 signal transduction [31–33] and immune processes [16, 34], no clear role has been reported for Rab5a in regulating C5aR1 signaling. Here, we show for pro-inflammatory M1-like HMDMs that Rab5a is crucial in mediating C5aR1 trafficking, chemotaxis, and secretion of chemokines.

Materials and Methods

Plasmids and Materials

C5aR1-GFP plasmid, C5aR1 plasmid, and recombinant human C5a protein were purchased from Sino Biological. Rab5a-tdTomato was a gift from Michael Davidson (Addgene plasmid #58126), and Rab5a-S34N-tdTomato dominant negative mutant was generated using a Q5 site-directed mutagenesis kit (New England Biolabs) according to manufacturer's instructions. Fluorescein-labeled C5aR1 peptide agonist (FITC-FKP-dChaCha-dR-OH) [35], antagonist PMX53 (AcF([OPdChaWR]) – also called 3D53 [4, 36, 37] and fluorescein-labeled PMX53 (FITC-F([OPdChaWR]) were synthesized and characterized in-house by our reported methods.

Cell Culture and Transfection

Human embryonic kidney (HEK293) cells were grown in Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 50 U/mL penicillin, 50 μg/mL streptomycin, and 2 mM GlutaMAX and maintained in an incubator at 37°C with 5% CO_2. Cells were transfected with plasmids C5aR1-GFP, Rab5a-tdTomato, or Rab5a-S34N-tdTomato using Lipofectamine 3000 (ThermoFisher Scientific). Stably transfected cells were selected with 300 μg/mL hygromycin and 750 μg/mL geneticin then sorted by Queensland Brain Institute flow cytometry facility (BD Influx Cell Sorter). For siRNA knockdown experiments, Rab5a, β-arrestin2, or universal negative control siRNAs (50 nM) (Sigma-Aldrich) were transfected into M1-like HMDM using DharmaFECT2 Transfection Reagent (Horizon Discovery) according to manufacturer's instructions.

Isolation and Differentiation of M1-like HMDMs

M1-like HMDMs were isolated from buffy coats provided by the Australian Red Cross LifeBlood. Mononuclear cells were harvested as previously described [38]. $CD14^+$ monocytes were then positively selected using CD14 MicroBeads (Miltenyi Biotec)

and further differentiated to M1-like HMDMs using 20 ng/mL recombinant human GM-CSF (PeproTech) for 7 days in Iscove's modified Dulbecco's media (IMDM) supplemented with 10% FBS, 50 U/mL penicillin, 50 μg/mL streptomycin, and 2mM GlutaMAX. M1-like HMDMs were supplemented after 5 days with fresh medium containing 10 ng/mL GM-CSF and collected by gentle scraping on day 7.

Lattice Light-Sheet Microscopy

For 3D live cell imaging, cells were seeded onto 5 mm #1 coverslips (Warner Instruments, #64-0700) and allowed to adhere overnight. On the day of experiment, media were changed to phenol-free DMEM supplement with 10% FBS, 50 U/mL penicillin, and 50 μg/mL streptomycin. Cells were then treated with recombinant human C5a (50 nM). Image acquisitions were obtained using a 3i lattice light-sheet microscope (V2) using a 0.55/0.493 annular mask, with lattice patterns for 488 nm (0.96 μm spacing, 52 beams) and 561 nm (1.102 μm spacing, 45 beams). 3D volumes were captured with 0.495 μm axial spacing (0.268 μm post-Deskew) with individual Hammamatsu Orca Flash 4.0 sCMOS cameras, with samples incubated at 37°C. Post-image deskewing and deconvolution (20 cycles) were performed using Microvolution Deconvolution software and visualized with Imaris v9.2.1 software (Bitplane AG).

Immunoblots and Immunoprecipitations

For immunoblots, cell lysates were prepared and separated as described [39]. Membranes were blocked with 5% skim milk for 1 h at room temperature and incubated overnight at 4°C with appropriate primary antibody against Rab5a (1:1,000, Abcam), pAkt (1:3,000), Akt (1:2,000), pERK1/2 (1:5,000), ERK1/2 (1:2,000, Cell Signaling Technology), C5aR1 (1:1,000, Abcam), or GAPDH (1:30,000 Sigma-Aldrich). For immunoprecipitation using GFP-Trap, cell lysates were incubated with GFP-Trap magnetic beads (ChromoTek) for 1 h at 4°C, followed by 3× washes in lysis buffer. Cell lysates were separated and transferred as above. The membranes were incubated with C5aR1 (1:300, Santa Cruz Biotechnology) or Rab5a (1:300, Cell Signaling Technology) using iBind Western System (ThermoFisher Scientific). Chemiluminescence acquisition was performed using ChemiDoc MP (Bio-Rad Laboratories) and densitometric analysis of bands was quantified using ImageJ.

Flow Cytometry

Cells were lifted with Versene Solution (ThermoFisher Scientific) and blocked using human TruStain FcX (BioLegend). Surface C5aR1 was stained using primary mouse anti-human C5aR1 (1:500, BioLegend) for 20 min at 4°C followed by secondary goat anti-mouse APC (1:200, BioLegend). CytoFLEX flow cytometer (Beckman Coulter) and FlowJo software were used to quantify surface C5aR1.

Confocal Microscopy

For live cell imaging, M1-like HMDMs were seeded into 35 mm glass bottom coverslip dishes (MatTek) and allowed to adhere overnight. Cells were stimulated with FITC-C5aR1 peptide agonist (500 nM) for 30 min followed by Hoechst staining. For antagonist, cells were pretreated with PMX53 (500 nM) or FITC-PMX53 (500 nM) for 30 min prior to stimulation. Live cells were imaged using a confocal microscope (Zeiss LSM 880, 63x 1.4NA Plan Apochromat oil immersion objective, running Zen Black, with full environmental control). For immunofluorescence, M1-like HMDMs were seeded onto coverslips and treated with C5a (50 nM) for 30 min. M1-like HMDMs were fixed with 4% formaldehyde at 37°C for 15 min, then permeabilized with 0.1% saponin at room temperature for 10 min, followed by blocking with 5% goat serum at room temperature for 1 h. Primary antibodies against C5aR1 (1:500, BioLegend) and Rab5a (1:400, Cell Signaling Technology) were incubated overnight at 4°C and secondary antibodies (Alexa Fluor 488, 1:500 or Alexa Fluor 633, 1:400) were incubated at room temperature for 1 h. M1-like HMDMs were then mounted onto slides using Fluoroshield with DAPI and imaged with a confocal microscope (as above).

Transwell Chemotaxis

M1-like HMDMs were seeded into transwell inserts (polycarbonate 5 μm membrane) at a density of 3×10^5 cells in serum-free IMDM. Cells were pretreated for 30 min in the upper chamber with PMX53 (1 μM) or wortmannin (1 μM) before the addition of C5a (3 nM) to the bottom chamber to stimulate chemotaxis. After overnight incubation, cells on the upper side of the membrane were removed using a cotton swab, then fixed in 4% formaldehyde. Migrated cells on the underside of the membrane were stained with DAPI, acquired using a Nikon Ti-U inverted widefield microscope stand with Nikon DS-Fi1 10× 0.30NA plan Fluor objective, and Qi1 CCD camera running NIS Elements and quantified with FIJI software [40].

μ-Slide Chemotaxis

M1-like HMDMs were seeded into the μ-slide chemotaxis (ibidi) at a density of 3×10^6/mL in serum-free IMDM and allowed to incubate at 37°C overnight. After incubation, reservoirs were filled with serum-free IMDM and C5a (10 nM) was added as the chemoattractant on one side of the reservoir. Live cell imaging was immediately recorded at 10 min intervals for 16 h using a Nikon TI-E inverted microscope with a 20× 0.45NA objective with a Hamamatsu Orca Flash 4.0 sCMOS camera running NIS Elements. Movement tracks of 30 cells/donor were determined with ImageJ Manual Tracking plugin. Chemotaxis parameters for forward migration index, velocity, Euclidean distance, and total distance were calculated with ibidi plugin Chemotaxis and Migration Tool [41].

β-Arrestin2 TANGO Recruitment

HTLA cells were a gift from the laboratory of Bryan Roth and β-arrestin2 recruitment was measured as described [42]. Briefly, HTLA cells were transfected with C5aR1-TANGO plasmid (Addgene #66232) using calcium phosphate. The next day, varying concentrations of C5a were incubated overnight with transfected HTLA-C5aR1 TANGO cells. For the antagonist assay, HTLA-C5aR1 TANGO cells were pretreated with PMX53 for 30 min, followed by overnight incubation with C5a (30 nM). Luminescence was measured using a PHERAstar plate reader (BMG Labtech).

Proximity Ligation Assay

A proximity ligation assay was performed using Duolink in situ Detection Reagents Red (Sigma-Aldrich) according to the manufacturer's instructions. M1-like HMDMs were probed with primary antibodies (rabbit anti-Rab5a (1:500, Abcam), rabbit anti-C5aR1 (1:250, Abcam), mouse, anti-β-arrestin2 (1:750, Abcam),

mouse anti-$G\alpha_{q/11/14}$ (1:50), mouse anti-$G\alpha_{s/o}$ (1:100), mouse anti-$G\alpha_{12}$ (1:100), mouse anti-$G\alpha_{13}$ (1:50, Santa Cruz Biotechnology), then detected with the supplied secondary antibodies and reagents. M1-like HMDMs were imaged using a confocal microscope (as above) and serial z-sections were acquired at 0.31 μm intervals. The number of proximity ligation fluorescence spots in a single cell was quantified using the "Spots" function, with consistent settings across all images in Imaris v9.2.1 software (Bitplane AG).

CCL2 and CCL3 Measurement

M1-like HMDMs were seeded at a density of 5×10^5/mL in a 48-well plate and allowed to adhere overnight. For inhibitor studies, M1-like HMDMs were pretreated with PMX53 (1 μM) or wortmannin (1 μM) for 30 min prior to C5a (50 nM) stimulation. After overnight incubation, supernatants were collected for analysis. CCL2 was quantified using human CCL2 kit (Cisbio) and CCL3 was quantified using human CCL3 kit (PeproTech) according to manufacturers' instructions.

Statistical Analysis

Data were plotted and analyzed using Prism 8 (GraphPad). Data point represents mean ± SEM ($n \geq 3$). For HMDMs studies, experiments were performed for at least 3 independent donors. Statistical significance was analyzed using unpaired *t* test for two variables, or one-way ANOVA for three or more variables. *p* values of * 0.033, ** 0.002, *** <0.001 were considered statistically significant.

Results

Rab5a Regulates Internalization and Trafficking of C5a-C5aR1 in HEK293 Cells

Upon activation by an agonist, some GPCRs are known to internalize and traffic with Rab proteins, but the role for Rab5a in the activation and trafficking of the specific GPCR known as C5aR1 in live human cells is currently unknown. To study interactions between Rab5a and activated C5aR1, we used lattice light-sheet microscopy to visualize C5aR1-GFP and Rab5a-tdTomato in HEK293 cells. These transfected HEK293 cells showed that C5a stimulation induced time-dependent phosphorylation of ERK1/2 (online suppl. Fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000530012), suggesting that the HEK293 cell line is a relevant model system and that fusion of GFP to C5aR1 did not alter the receptor's biological activity. Lattice light-sheet microscopy enables high-speed high-resolution (both laterally and axially) imaging of live cells [43] and was recently used to monitor Rab13 in macrophages during membrane ruffling and formation of macropinosomes [44]. Prior to C5a stimulation, we found that C5aR1-GFP was predominantly expressed on the cell surface, whereas Rab5a-tdTomato was located within the cytoplasm (Fig. 1a–c). Following C5a stimulation, C5aR1-GFP was observed at about 30 min to have internalized and colocalized with Rab5a-tdTomato in endocytic vesicles within the cytoplasm (Fig. 1d–g). To establish whether Rab5a was responsible for the internalization of C5aR1, we used the Rab5a-S34N dominant negative mutant to inhibit the function of Rab5a [45]. Similar to wildtype Rab5a, Rab5a-S34N-tdTomato was located within the cytoplasm and C5aR1-GFP was predominantly expressed on the cell surface prior to C5a stimulation (Fig. 2a–c). However, upon stimulation with C5a, Rab5a-S34N-tdTomato expression significantly attenuated internalization of C5aR1-GFP (Fig. 2d–g), which consequently did not colocalize with Rab5a-S34N-tdTomato endosomes (Fig. 2d–g).

To establish direct interaction between C5aR1-GFP and Rab5a-tdTomato, we performed immunoprecipitation experiments at different timepoints following C5a stimulation. Using GFP-Trap magnetic beads targeting C5aR1-GFP, Rab5a-tdTomato was found to be co-precipitated with C5aR1-GFP (Fig. 3a). By contrast, Rab5a-S34N-tdTomato did not coprecipitate with C5aR1-GFP at any timepoints following C5a stimulation (Fig. 3b), suggesting that C5a-activated C5aR1 directly interacts with wildtype Rab5a but not with Rab5a-S34N dominant negative mutant. These co-immunoprecipitation findings are consistent with the colocalization microscopy imaging results for C5aR1-GFP, Rab5a-tdTomato, and Rab5a-S34N-tdTomato (Fig. 1, 2). Since these microscopy and co-immunoprecipitation experiments established internalization of C5aR1, colocalization with Rab5a, and direct interaction between C5aR1 with Rab5a, we next sought to quantify the internalization of unlabeled C5aR1 using flow cytometry. As expected, C5a stimulation induced significant C5aR1 internalization (~50%) in Rab5a-expressing HEK293 cells in a time-dependent manner (online suppl. Fig. 2), but C5aR1 internalization was significantly attenuated in the Rab5a-S34N dominant negative mutant (online suppl. Fig. 2). Taken together, the data from lattice light-sheet microscopy, co-immunoprecipitation, and flow cytometry indicate that C5a-mediated C5aR1 internalization is dependent on Rab5a and requires direct interaction between C5aR1 and Rab5a in HEK293 cells.

Rab5a Is Upregulated in M1-Like HMDMs and Required for Internalization of C5aR1

Macrophages are one of the most important innate immune cell types responsible for host defense, inflammation, and homeostasis, while HMDMs are known to be involved in various inflammatory pathologies [10, 11, 13]. To determine the significance of Rab5a in M1-like HMDMs, we measured Rab5a protein levels during

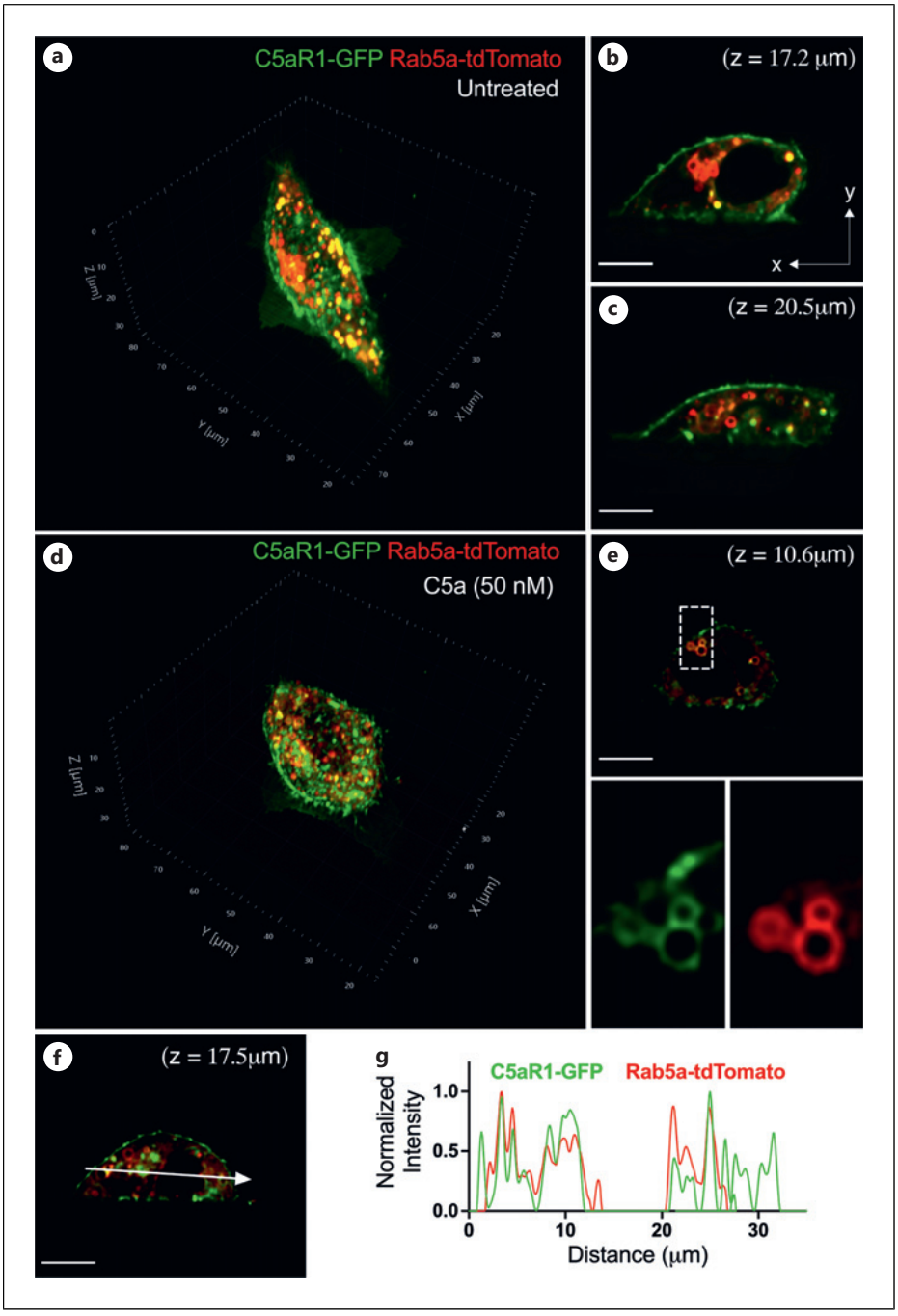

Fig. 1. C5a induced C5aR1-GFP internalization and colocalization with Rab5a-tdTomato in HEK293 cells. Representative images of lattice light-sheet microscopy of HEK293 transfected with C5aR1-GFP and Rab5a-tdTomato. **a–c** In untreated cells, C5aR1-GFP is predominantly expressed on the cell surface, while Rab5a-tdTomato is within the cytoplasm. Different representative re-slice xy planes (**b**, **c**) were shown to illustrate the distribution of C5aR1-GFP and Rab5a-tdTomato. Scale bar represents 10 μm. **d–g** Stimulation by C5a (50 nM) for 30 min induced internalization of C5aR1-GFP from the cell surface into endocytic vesicles where it colocalized with Rab5a-tdTomato endosomes. **e** Representative re-slice of xy plane showed visual colocalization of C5aR1-GFP and Rab5a-tdTomato endosomes. Scale bar represents 10 μm. **f**, **g** Line scan analysis of a representative re-slice xy plane showing colocalization of C5aR1-GFP and Rab5a-tdTomato in endosomes. Representative re-slice of xy planes were shown relative to corresponding z depth.

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differentiation of M1-like HMDMs over 7 days (Fig. 4a). In the undifferentiated monocytes (day 0), Rab5a was observed to be expressed at minimal levels but became upregulated during the course of GM-CSF differentiation, with substantial expression by day 5 and maximal levels in fully differentiated M1-like HMDMs (day 7) (Fig. 4b). This suggests that Rab5a is important in differentiated M1-like HMDMs, consistent with previous studies showing upregulated Rab5a expression in pro-inflammatory or infected human macrophages [46, 47].

To determine whether Rab5a has an important role in C5aR1 trafficking, we took advantage of prior knowledge of the endogenous expression of C5aR1 [15] and upregulated Rab5a expression (Fig. 4a, b) in M1-like HMDMs. We first used live cell microscopy and fluorescent ligands (FITC-C5aR1 peptide agonist or FITC-PMX53) to visualize the internalization and formation of endocytic vesicles containing endogenous C5aR1 in M1-like HMDMs. No background fluorescence signal was detected in the untreated control HMDMs (Fig. 4c), but stimulation with FITC-C5aR1 peptide agonist for 30 min resulted in the formation of endocytic vesicles within HMDMs (Fig. 4d). The specificity of the FITC-C5a peptide agonist to C5aR1 was further confirmed using a selective insurmountable C5aR1 antagonist (PMX53) [37], pretreatment with PMX53 for 30 min ablating the binding of FITC-C5aR1 peptide agonist to M1-like HMDMs (Fig. 4e). In a similar fashion, FITC-PMX53 pretreatment resulted only in plasma membrane staining and no intracellular staining (Fig. 4f), indicating that C5aR1 activation leads to its internalization into endocytic vesicles, whereas C5aR1 antagonist PMX53 inhibits C5aR1 activation and internalization.

To assess the regulatory role of Rab5a on C5aR1 trafficking, we performed siRNA knockdown of Rab5a and immunofluorescence staining on M1-like HMDMs. In both negative control siRNA and Rab5a-knockdown groups, C5aR1 was primarily expressed on the plasma membrane of unstimulated HMDMs and distinct from Rab5a as visualized using immunofluorescence confocal microscopy (Fig. 5a, b). After C5a stimulation, significant differences in C5aR1 and Rab5a distribution were observed in the negative control siRNA HMDMs (Fig. 5a). C5a promoted internalization of C5aR1 from the plasma membrane into endocytic vesicles, formation of Rab5a positive endosomes, and colocalization with C5aR1-Rab5a. In contrast, stimulation of C5a in the Rab5a-knockdown M1-like HMDMs showed predominant localization of C5aR1 on the plasma membrane, with no endocytic vesicle formation albeit slight internalization of C5aR1 into the cytoplasm (Fig. 5b). This immunofluorescence colocalization data for C5aR1 and Rab5a in M1-like HMDMs (Fig. 5a, b) is in agreement with the live cell lattice light-sheet microscopy data for transfected C5aR1-GFP and Rab5a-tdTomato HEK293 cells (Fig. 1, 2).

To examine the possible role of Rab5a in relation to C5aR1-mediated signaling, we knocked down Rab5a and measured C5a-induced signaling responses (Fig. 5c). Notably, C5a-induced Akt phosphorylation at 5 min was significantly reduced by Rab5a knockdown in M1-like HMDMs, as compared to control M1-like HMDMs stimulated with C5a (Fig. 5d). In contrast, C5a-induced ERK1/2 phosphorylation and intracellular calcium mobilization were unaffected by Rab5a knockdown in M1-like HMDMs (Fig. 5e, f). These data suggested that Rab5a modulates Akt signaling following C5a stimulation but is not involved in ERK1/2 phosphorylation or intracellular calcium mobilization. Previous studies on other Rab proteins have shown that Rab35 [48], Rab11 [49], and Rab8 [22] each regulate Akt signaling pathways to control endosomal trafficking in cancers, inflammatory, and metabolic diseases.

Rab5a Regulates C5a-Mediated Chemotaxis of HMDMs

C5a is known to be a potent chemoattractant involved in recruiting inflammatory cells. We therefore assessed the role of Rab5a in C5a-mediated chemotaxis of M1-like HMDMs using both transwell migration and μ-slide chemotaxis assays. In the transwell migration assay, C5a induced the migration of M1-like HMDMs through the pores to the underside of the transwell membrane (Fig. 6a). In contrast, the Rab5a knockdown M1-like HMDMs failed to migrate in response to C5a stimulation (Fig. 6b), suggesting that Rab5a is involved in C5a-mediated migration. As the transwell migration is an endpoint assay and lacks chemotaxis features, we further investigated the role of Rab5a using the μ-slide chemotaxis assay. Time-lapse imaging enabled tracking of individual cells and analysis of chemotaxis parameters [41]. Over a period of 16 h, the trajectory plots of C5a stimulated M1-like HMDMs showed significant chemotaxis in a C5a concentration-dependent manner as compared to unstimulated M1-like HMDMs (Fig. 6c). In contrast, significant inhibition of chemotaxis toward an increasing C5a gradient was observed in the Rab5a knockdown M1-like HMDMs compared to the C5a stimulated M1-like HMDMs control (Fig. 6c). Likewise,

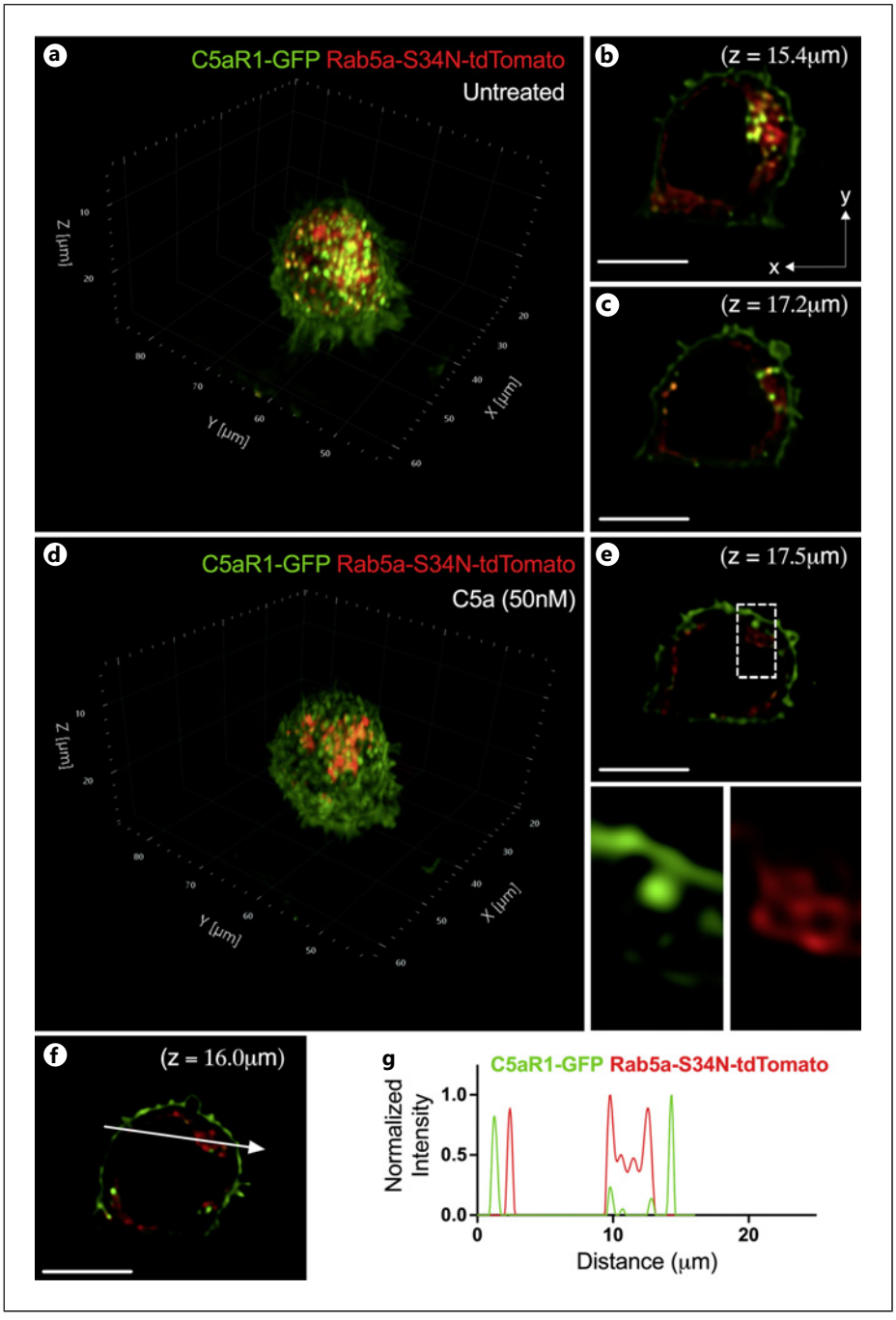

Fig. 2. C5a stimulation did not induced C5aR1-GFP internalization and colocalization with Rab5a-S34N-tdTomato in HEK293 cells. Representative images of lattice light-sheet microscopy of HEK293 transfected with C5aR1-GFP and Rab5a-S34N-tdTomato. **a–c** In untreated cells, C5aR1-GFP are predominantly expressed on the cell surface while Rab5a-S34N-tdTomato is within the cytoplasm. Different representative re-slice xy planes (**b**, **c**) were shown to illustrate the distribution of C5aR1-GFP and Rab5a-S34N-tdTomato. Scale bar represents 10 μm. **d–g** After stimulation with C5a (50 nM) for 30 min, C5aR1-GFP did not internalize into endocytic vesicles and not colocalized with Rab5a-S34N-tdTomato endosomes. **e** Representative re-slice xy plane showed C5aR1-GFP remained on the cell surface and distinct from Rab5a-S34N-tdTomato endosomes. Scale bar represents 10 μm. **f**, **g** Line scan analysis of a representative re-slice xy plane showed no colocalization of C5aR1-GFP and Rab5a-S34N-tdTomato endosomes. Representative re-slice of xy planes was shown relative to corresponding z depth.

other chemotaxis parameters, such as forward migration index, velocity, Euclidean distance, and total distance, were significantly inhibited in the C5a stimulated Rab5a knockdown M1-like HMDMs compared to the control M1-like HMDMs (Fig. 6d). However, these inhibitory effects were not completely suppressed in the C5a stimulated Rab5a knockdown M1-like HMDMs as compared to the untreated Rab5a knockdown M1-like HMDMs (Fig. 6d).

C5aR1 Induces β-Arrestin2-Rab5a-PI3K Interaction to Regulate Secretion of CCL2 and CCL3

Since β-arrestin2 is considered an important regulator for GPCR internalization, signaling, and degradation [50,

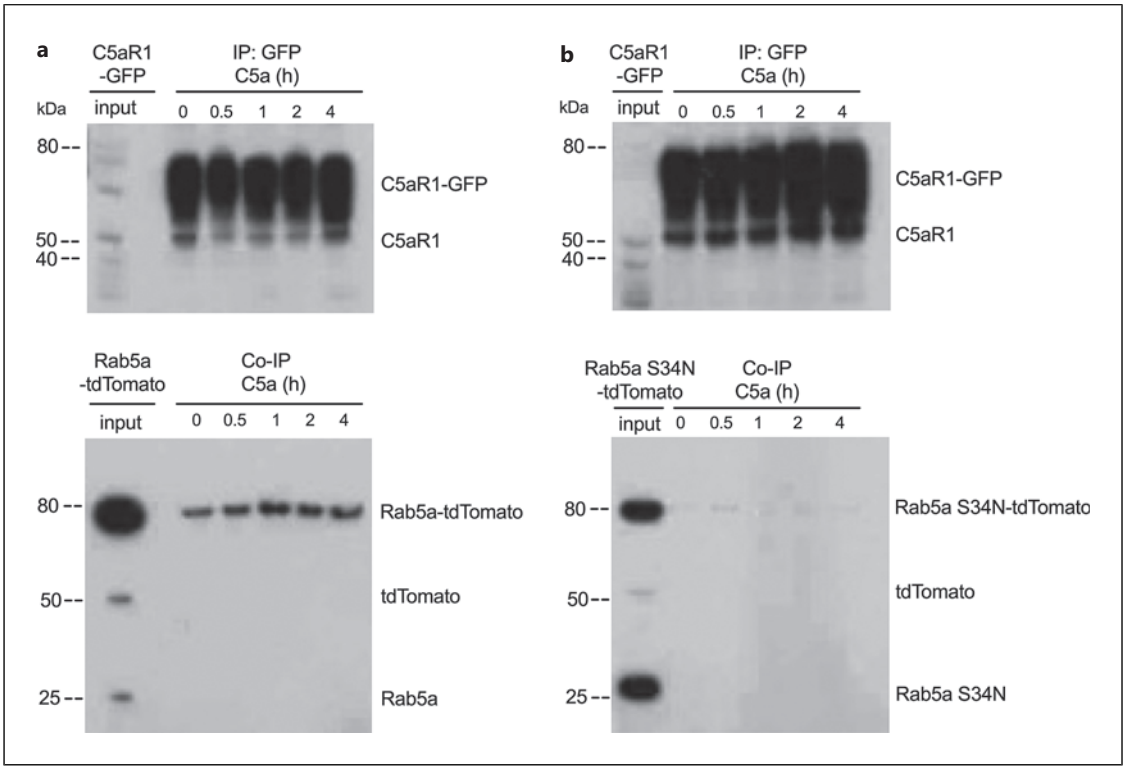

Fig. 3. C5a induced C5aR1 interaction with Rab5a but not with Rab5a-S34N in HEK293 cells. HEK293 cells expressing (**a**) C5aR1-GFP and Rab5a-tdTomato or (**b**) C5aR1-GFP and Rab5a S34N-tdTomato were treated with C5a (50 nM) for 0.5, 1, 2, or 4 h. Co-immunoprecipitations were performed using GFP-Trap magnetic beads and cell lysates were probed with anti-Rab5a.

51], we next examined C5a-mediated β-arrestin2 recruitment using the C5aR1 TANGO assay [42]. C5a induced the recruitment of C5aR1-mediated β-arrestin2 (EC_{50} 2.3 nM) (Fig. 7a), but pretreatment with C5aR1 antagonist (PMX53) inhibited (IC_{50} 44 nM) C5a-induced β-arrestin2 recruitment in the C5aR1-TANGO assay (Fig. 7b). We next performed proximity ligation assays to confirm the possible interaction between C5aR1 and β-arrestin2 in C5a stimulated M1-like HMDMs. The proximity ligation assay is based on specific labeling of two proteins in close proximity (<40 nm) with primary antibodies and oligonucleotide-linked fluorescent probes [52]. As expected C5a stimulation significantly increased the proximity ligation fluorescence signal for C5aR1 and β-arrestin2 in M1-like HMDMs, unlike untreated control (Fig. 7c). We then performed similar proximity ligation assays to examine possible interaction between β-arrestin2 and Rab5a. We observed a significant increase in proximity ligation fluorescence signal for β-arrestin2 and Rab5a in C5a-stimulated M1-like HMDMs compared to untreated M1-like HMDMs (Fig. 7d). By contrast, other different G proteins ($G\alpha_{q/11/14}$, $G\alpha_{s/o}$, $G\alpha_{12}$, and $G\alpha_{13}$) did not show any increase in proximity ligation fluorescence signal with Rab5a in C5a stimulated M1-like HMDMs (online suppl. Fig. 3). These findings suggested that activated C5aR1 mediates interaction of Rab5a with β-arrestin2 but not with G proteins ($G\alpha_{q/11/14}$, $G\alpha_{s/o}$, $G\alpha_{12}$, and $G\alpha_{13}$) in M1-like HMDMs.

We next measured the secretion of chemokines CCL2 and CCL3 after C5aR1 activation, these chemokines being important macrophage-derived mediators in diseases such as asthma, arthritis, and cancer [53, 54]. C5a was able to stimulate significant increases in secreted CCL2 and CCL3 chemokines from M1-like HMDMs compared to unstimulated control (Fig. 8). These responses were inhibited by siRNA knockdown of either β-arrestin2 or Rab5a (Fig. 8a). Alternative pharmacological inhibition of C5aR1 with antagonist PMX53 inhibited C5a-induced CCL2 and CCL3 secretion in M1-like HMDMs (Fig. 8b). Since Akt phosphorylation is known to be regulated by phosphatidylinositol 3-kinases (PI3K), and as Rab5a endosomal trafficking is also

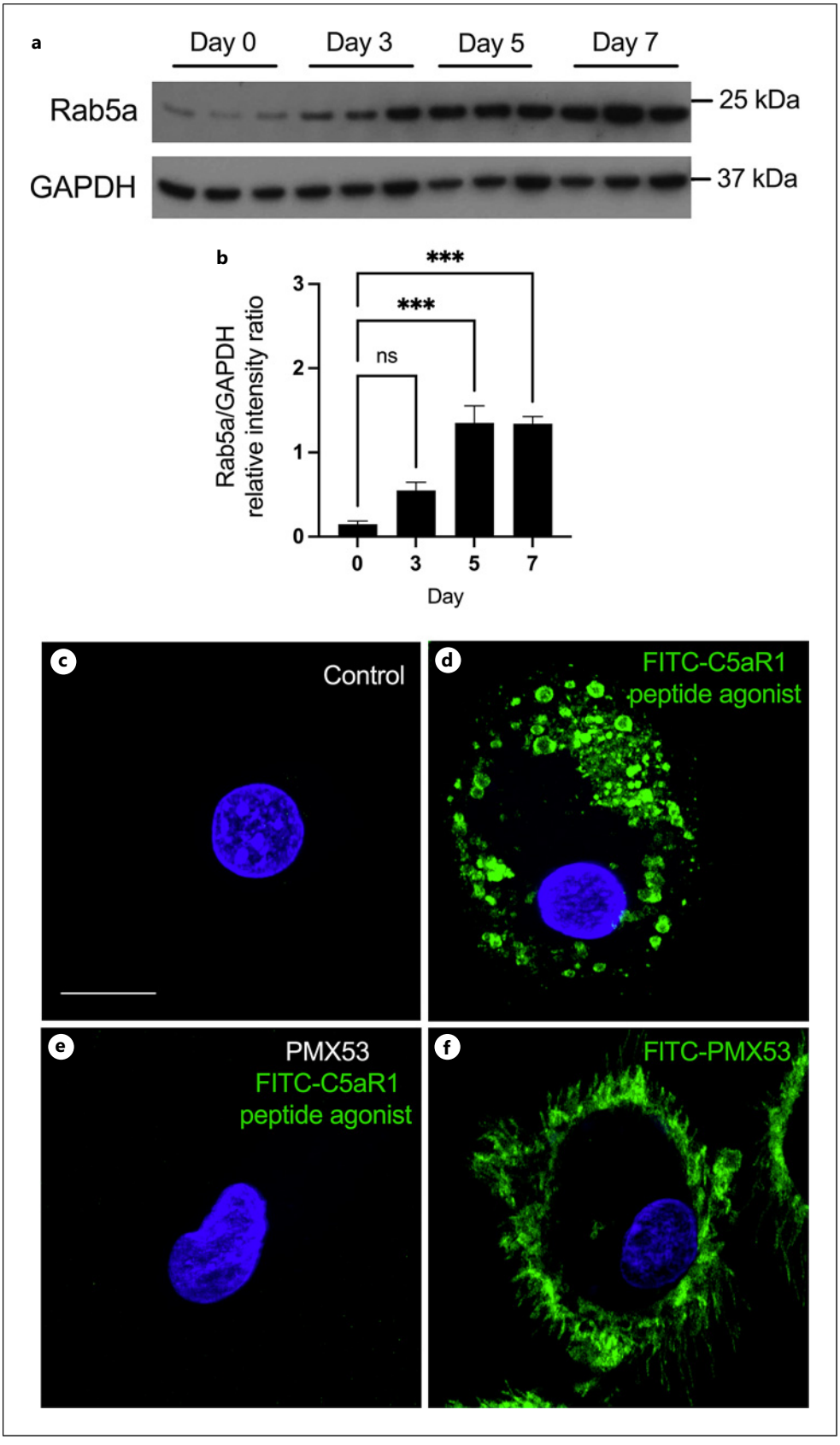

Fig. 4. Rab5a expression is upregulated in HMDMs and activation of C5aR1 on M1-like HMDMs leads to internalization. **a** Immunoblot analysis of Rab5a expression in monocytes (day 0) during differentiation to M1-like HMDMs (day 7). Each lane represents an individual donor (n = 3). **b** Densitometric analysis of Rab5a relative to GAPDH. **c**–**f** Representative images of live cell confocal microscopy of M1-like HMDMs. **d** Internalization and formation of endocytic vesicles were observed when M1-like HMDMs were stimulated with FITC-C5aR1 peptide agonist (500 nM) for 30 min. **e** Pre-treatment with C5aR1 antagonist (PMX53) (500 nM) for 30 min inhibited both the binding of FITC-C5aR1 peptide agonist to C5aR1 and formation of endocytic vesicles. **f** No internalization or endocytic vesicles were observed when M1-like HDMDs were treated with FITC-PMX53 (500 nM) for 30 min. Error bars represent mean ± SEM. Statistical significance was determined using one-way ANOVA. ns, not significant; p value of *** <0.001. Scale bar represents 10 μm.

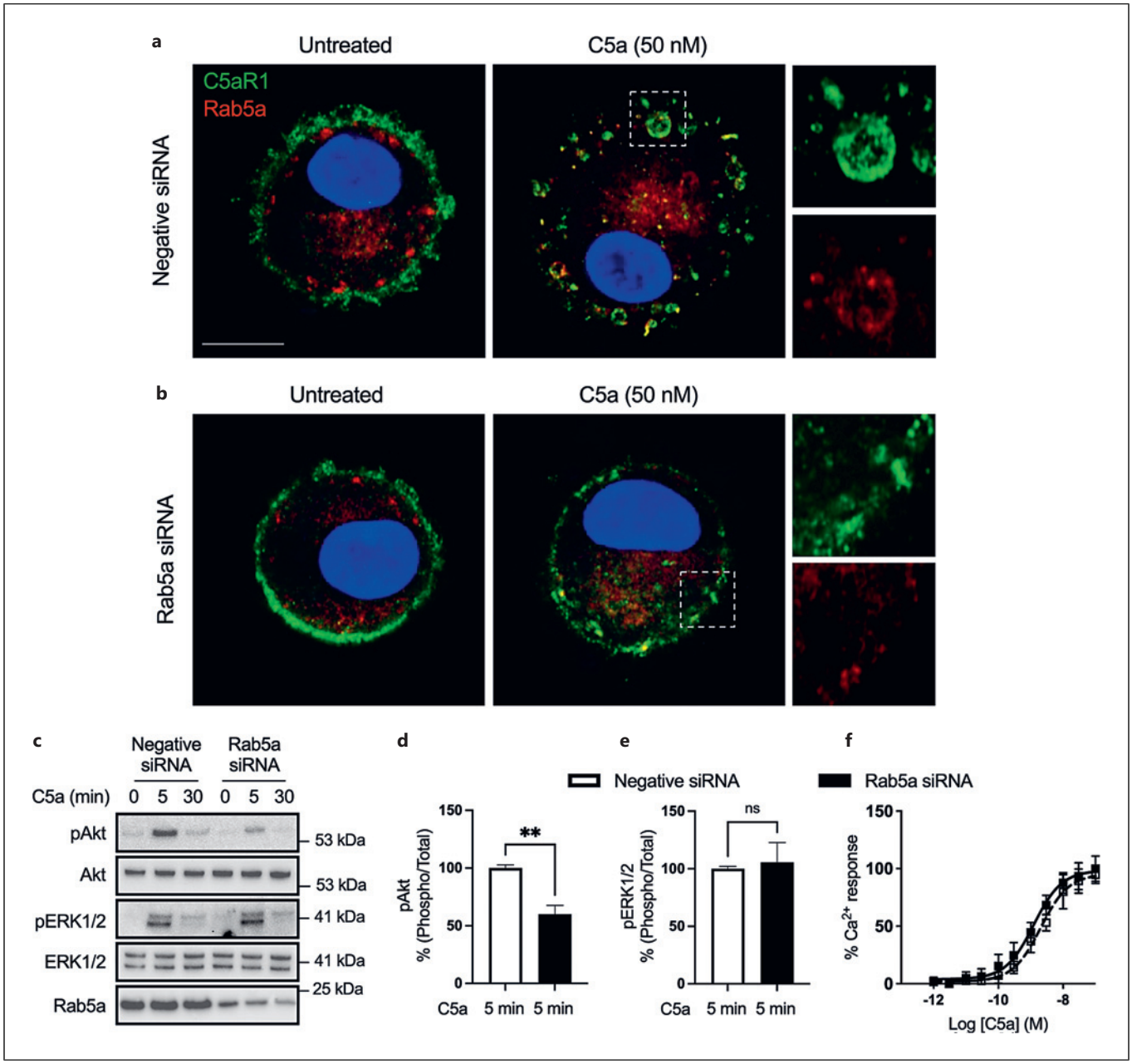

Fig. 5. Rab5a is required for C5aR1 internalization and Akt phosphorylation in M1-like HMDMs. **a**, **b** Immunofluorescence images of M1-like HMDMs using anti-C5aR1 (green), anti-Rab5a (red), and DAPI (blue). **a** In untreated control (negative siRNA) M1-like HMDMs, C5aR1 is primarily expressed on the plasma membrane while Rab5a is within the cytoplasm. C5a (50 nM) induces internalization of C5aR1 and formation of endocytic vesicles with Rab5a. **b** In untreated Rab5a knockdown M1-like HMDMs, expression of C5aR1 and Rab5a is similar to untreated control. However, C5a stimulation (50 nM) failed to induce internalization of C5aR1 and formation of endocytic vesicles with Rab5a. Images are representative of 3 donors, scale bar represents 10 μm. **c** Representative immunoblot analysis of C5a stimulation (50 nM) for negative control and Rab5a knockdown M1-like HMDMs. Densitometric analysis of (**d**) pAkt and (**e**) pERK1/2 at 5 min post-C5a stimulation. **f** Concentration-dependent of C5a-mediated intracellular calcium mobilization of negative control and Rab5a knockdown M1-like HMDMs. Error bars represent mean ± SEM and statistical significance was determined using unpaired *t* test. ns, not significant; *p* value of ** 0.002.

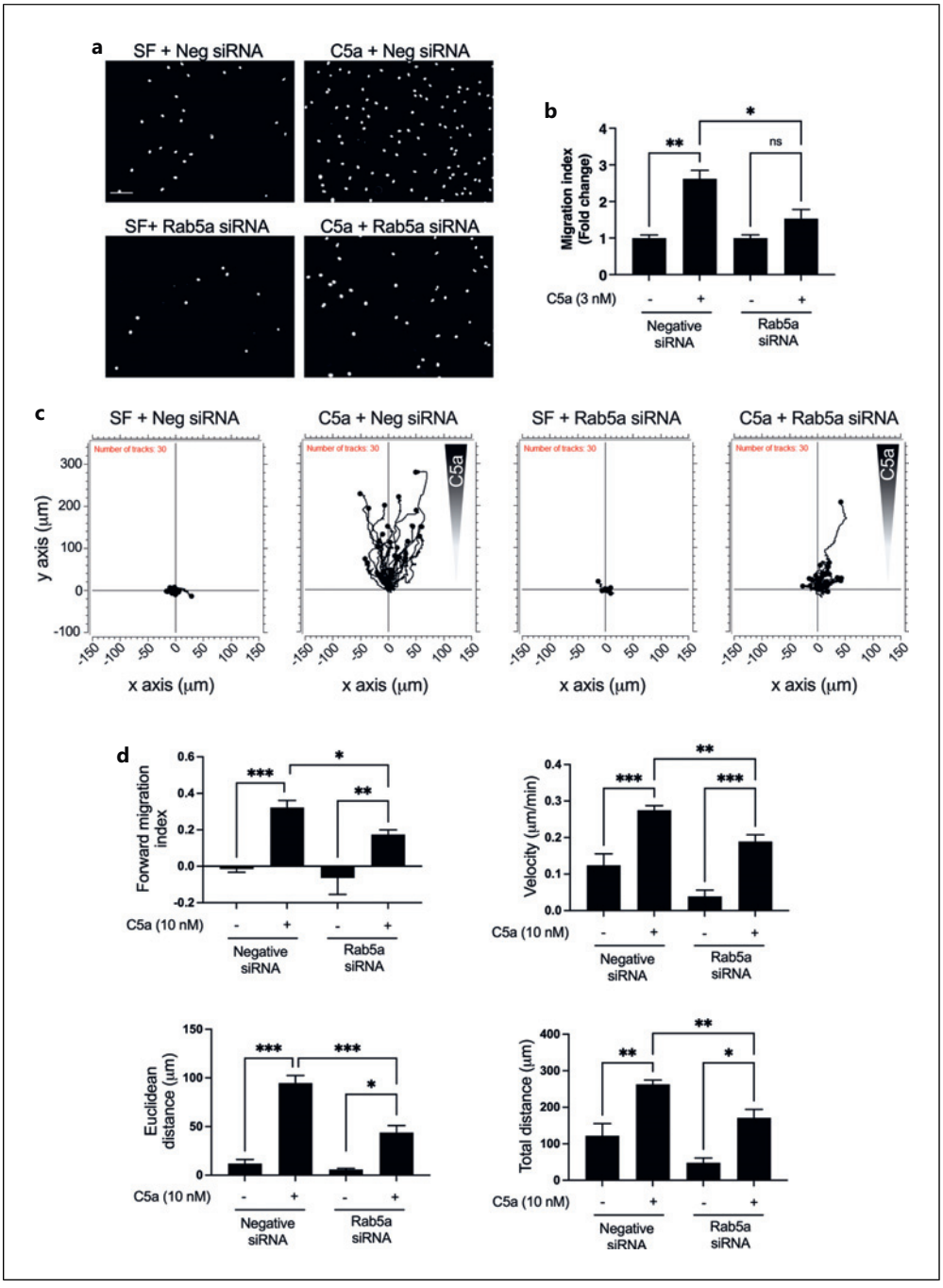

Fig. 6. Rab5a regulated C5a-mediated migration and chemotaxis of M1-like HMDMs. **a** Representative greyscale images of DAPI staining of migrated M1-like HMDMs in the transwell migration assay, scale bar represents 50 μm. **b** C5a induced migration of M1-like HMDMs, while Rab5a knockdown M1-like HMDMs failed to migrate in response to C5a. Migration index is the ratio of C5a-induced migration versus serum-free (SF) control. **c** Representative C5a-mediated trajectory plots for control (negative siRNA) and Rab5a knockdown M1-like HMDMs in μ-Slide chemotaxis slides. Trajectories of 30 cells per donor (n = 3) were analyzed by time-lapse microscopy for 16 h at 10 min intervals. Chemotaxis parameters from each trajectory plot were calculated for (**d**) forward migration index, velocity, Euclidean distance, and total distance. Error bars represent mean ± SEM n = 3 donors. Statistical significance was determined using one-way ANOVA. p value of * 0.033, ** 0.002, and *** <0.001.

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regulated by PI3K [55], we hypothesized that PI3K is involved in C5aR1-mediated secretion of CCL2 and CCL3. Indeed, pretreatment with a PI3K inhibitor, wortmannin, significantly reduced C5a-induced secretion of CCL2 and CCL3 in M1-like HMDMs (Fig. 8b). Taken together, these data showed that C5aR1-mediated secretion of chemokines CCL2 and CCL3 involves the C5aR1-β-arrestin2-Rab5a-PI3K signaling pathway in M1-like HMDMs.

Discussion and Conclusion

The complement system is a tightly regulated enzymatic cascade involving multiple regulatory protein-protein interactions and represents a key signaling network in host innate immunity. However, aberrant or persistent complement activation potentiates inflammatory signaling that can lead to a diverse range of inflammatory diseases [56, 57]. One common feature of many inflammatory diseases is a significant increase in concentration of C5a, which is one of the most potent anaphylatoxins known and is important in recruitment of immune cells to sites of infection or tissue damage and in production of other pro-inflammatory mediators. C5a activates two distinct GPCRs, C5aR1, and C5aR2, which are endogenously expressed on immune cells [58]. C5a-C5aR1 activation is associated with pro-inflammatory roles in human diseases, while C5a-C5aR2 activation does not exhibit G-protein coupling and has been proposed to serve as a negative regulator by removing excess C5a [32]. The potent C5aR1 antagonist PMX53 is highly selective for C5aR1 [37], not binding to C5aR2, and so it can be constructively used to implicate C5aR1 in mediating the actions of C5a as in this study.

Previously, it has been shown that activated C5aR1 accumulates in early endosomes, and then in lysosomes of C5aR1-transfected Chinese hamster ovary cells, however, in those cells the mechanisms of receptor internalization and recycling are different from human cells [59]. This suggested that the use of non-human cells may affect C5aR1 trafficking, signaling and function. In our study, we used a well-characterized model cell line, HEK293 cells, for expressing and visualizing C5aR1-GFP and Rab5a-tdTomato. This expression system for Rab proteins is known to strongly recapitulate canonical trafficking, internalization, recycling, and degradation mechanisms typical of other GPCRs including β2-adrenergic receptor, C5aR2, CXCR2, and neurokinin 1 receptor [27, 28, 60, 61]. Interestingly, yellow punctate vesicles were observed in the untreated state, indicating that the colocalization of C5aR1-GFP and Rab5a-tdTomato was observed prior to C5a stimulation (Fig. 1a). Rab5a endosomes are heterogenous in terms of morphology, localization, composition, and size. Although endosomes are typically represented in vesicles, they are also tubular extensions of the trans-Golgi network [62–64]. For GPCRs, such as C5aR1, these cell surface receptors are synthesized and assembled in the endoplasmic reticulum then transported to the cell surface through the trans-Golgi network [65, 66]. These observed colocalized punctate vesicles suggested that there are basal intracellular interactions of C5aR1 and Rab5a or that nascent anterograde transport of C5aR1 occurs through the trans-Golgi network with Rab5a as a trafficking chaperone protein. We demonstrated that C5aR1 internalization is dependent on Rab5a and that activated C5aR1 directly interacts with Rab5a in HEK293 cells. To gain further insight into the physiological role of Rab5a in C5a-mediated C5aR1 trafficking and function, we used pro-inflammatory M1-like HMDMs, which are relevant for human diseases. Furthermore, we used a combination of genetic and pharmacological targeting to elucidate the role of Rab5a in C5aR1-mediated chemotaxis and chemokine secretion (CCL2 and CCL3) in M1-like HMDMs.

The internalization of activated GPCRs is known to be dependent on the phosphorylation of intracellular loops or/and the carboxyl-terminal region, followed by the recruitment of β-arrestins and interaction with downstream endocytic machinery [67]. For C5aR1, previous reports have shown that phosphorylation-deficient mutants of serine and threonine residues on the carboxyl-terminal region are critical for C5a-mediated C5aR1 internalization [68, 69]. It is generally believed that recruitment of β-arrestins impedes further G protein-mediated signaling, leading to desensitization and degradation of GPCRs. However, β-arrestins have been shown to interact with Rab5a in endosomes [70, 71] and promote sustained endosomal signaling following activation of some GPCRs [51]. Our data showed that Rab5a regulates C5aR1-mediated Akt phosphorylation but not ERK1/2 phosphorylation and calcium mobilization in M1-like HMDMs. These findings suggest that Rab5a effector proteins and compartmental signaling in endosomes are both likely involved in Akt phosphorylation. Previous reports have shown that Akt signaling pathways depend on Rab5a effector proteins, such as APPL1, PI3K, mTORC2, or ZFYVE21 and the formation of endosomes [72–75]. Consistent with our findings, siRNA knockdown of early endosomes regulated angiotensin II receptor-mediated Akt phosphorylation but not ERK1/2 phosphorylation [76]. Based on results from this study and previous reports on

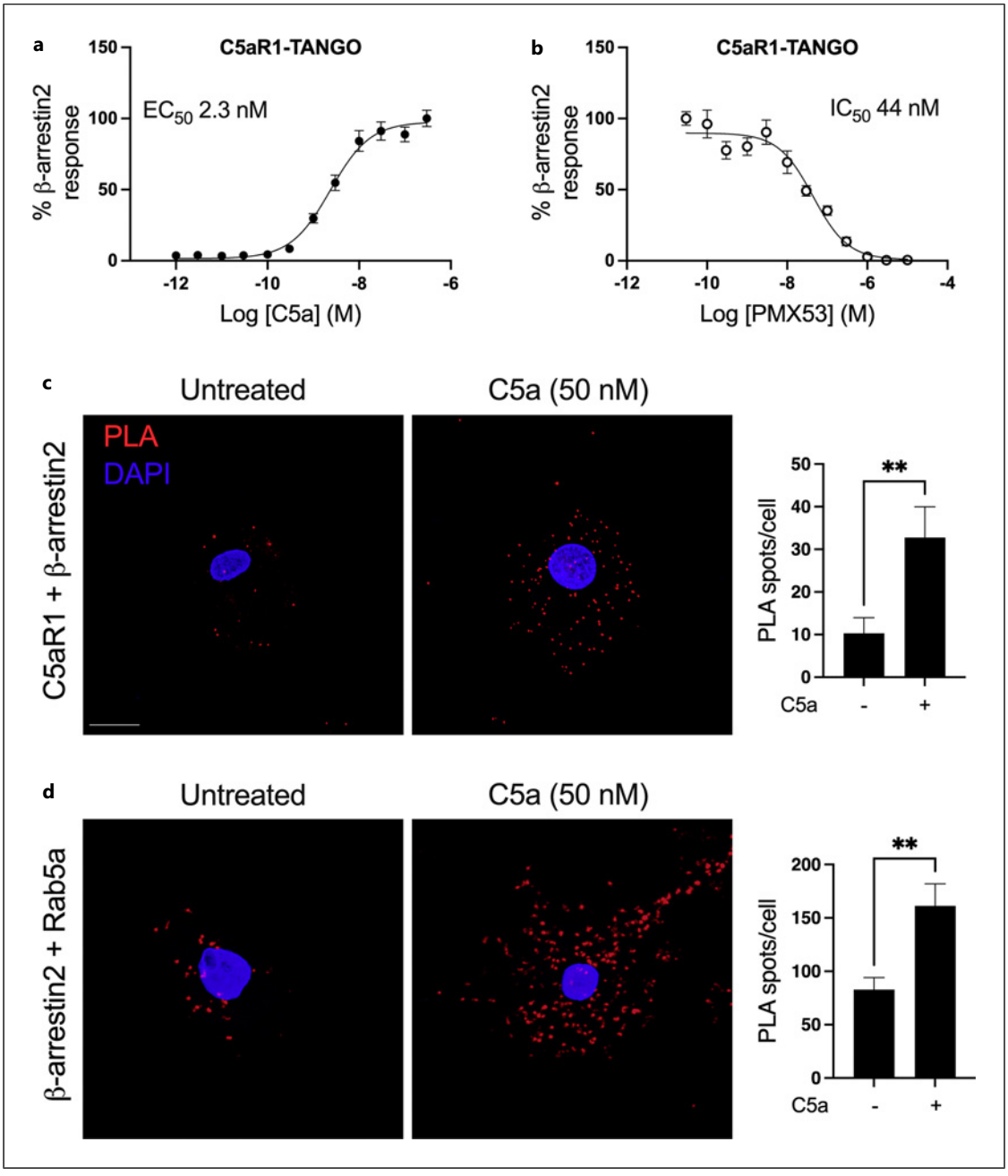

Fig. 7. C5a induced β-arrestin2 recruitment and interaction between β-arrestin2 and Rab5a. β-arrestin2 recruitment was measured using HTLA cells transfected with C5aR1-TANGO and (**a**) stimulated with varying concentrations of C5a. **b** For antagonist assay, cells were pretreated with PMX53 at indicated concentrations for 30 min prior to C5a (30 nM) stimulation. Proximity ligation assay (PLA) was performed on untreated or C5a stimulated (50 nM) M1-like HMDMs using (**c**) anti-C5aR1 and anti-β-arrestin2 or (**d**) anti-β-arrestin2 and anti-Rab5a. Each spot represents a molecular interaction and was quantified using Imaris software. Images are representative of 3 donors and scale bar represents 10 μm. Error bars represent mean ± SEM and statistical significance was determined using an unpaired *t* test. *p* value of ** 0.002.

C5aR1 activation, we propose that C5a-mediated C5aR1 activation leads to phosphorylation of the C5aR1 carboxyl-terminal region for β-arrestin2 recruitment, followed by initiation of C5aR1 internalization. This internalization process subsequently initiates formation of the C5aR1-β-arrestin2-Rab5a-PI3K complex in endosomes to control

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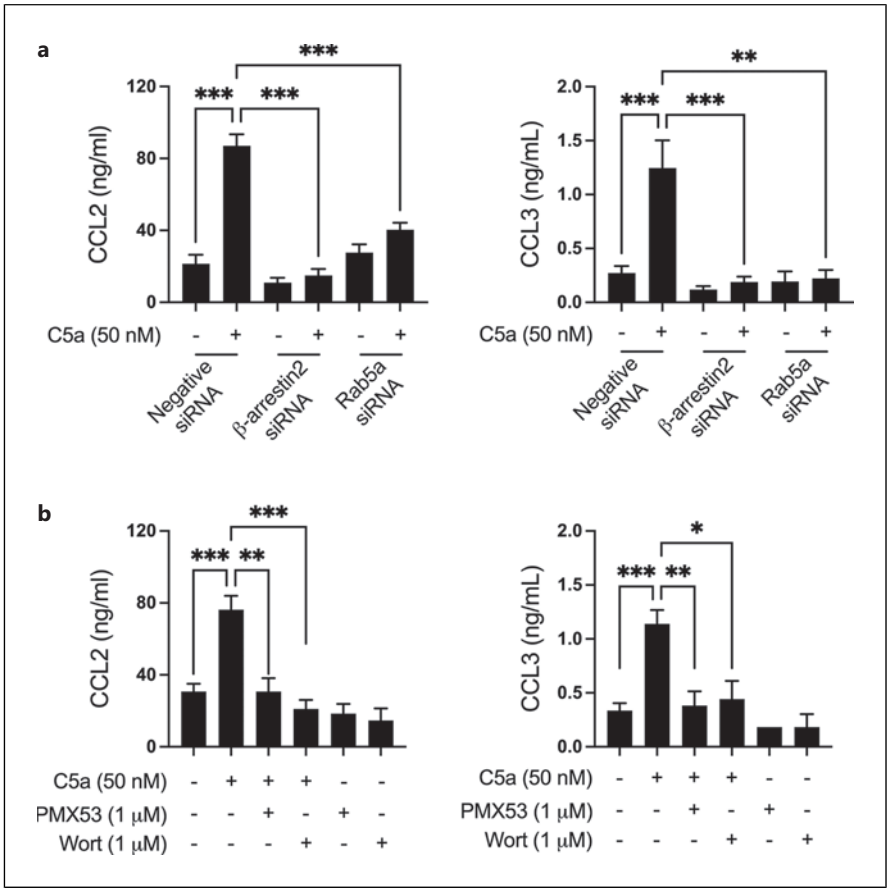

Fig. 8. C5aR1-mediated secretion of CCL2 and CCL3 is dependent on β-arrestin2-Rab5a-PI3K pathway. **a** C5a upregulated CCL2 and CCL3 secretion in M1-like HMDMs, while β-arrestin2 or Rab5a knockdown inhibited C5a-mediated CCL2 and CCL3 secretion. **b** Pretreatment with C5aR antagonist (PMX53) or PI3K inhibitor (wortmannin) inhibited C5a-mediated CCL2 and CCL3 secretion in M1-like HMDMs. Error bars represent mean ± SEM $n = 3$ donors. Statistical significance was determined using one-way ANOVA. p value of * 0.033, ** 0.002, and *** <0.001.

M1-like HMDMs inflammatory responses. It is important to note that $G\alpha_o$ [77] and $G\alpha_s$ [78] have been shown to interact with Rab5 to mediate trafficking and endocytosis, and thus it may well be possible for Gα proteins or β-arrestin1 to interact with other endosomal-related Rab proteins. Some limitations of the current study need to be acknowledged and require future investigation. First, whether a larger protein complex is formed between Rab5a, C5aR1, and β-arrestin2 within endosomes to modulate intracellular compartmentalized signaling remains unknown. Second, although we speculate that C5aR1 activation leads to β-arrestin2 recruitment and then Rab5a binding in a stepwise manner, we cannot exclude the possibility that Rab5a binds to β-arrestin2 then subsequently binds to C5aR1.

In summary, the results of this study provide critical insights to the mechanism of C5a-induced C5aR1-mediated intracellular signaling, which involves a C5a-C5aR1-β-arrestin2-Rab5a-PI3K axis that regulates chemotaxis and pro-inflammatory cytokine secretion in M1-like HMDMs.

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Statement of Ethics

The experiments on human cell lines and HMDMs were approved by the University of Queensland Research Ethics and Integrity (2021/HE002355). Written informed consent from donors was obtained by the Australian Red Cross Lifeblood to participate for research use.

Conflict of Interest Statement

The authors have no present conflicts of interest to declare.

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Author Contributions

Kai-Chen Wu, Junxian Lim, and David P. Fairlie designed, analyzed, and wrote the manuscript with input from Nicholas D. Condon, Timothy A. Hill, and Robert C. Reid. Kai-Chen Wu and Junxian Lim performed cellular experiments. Kai-Chen Wu and Nicholas D. Condon performed microscopy experiments and analysis. Timothy A. Hill and Robert C. Reid provided specialized reagents.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its online supplementary material. Further inquiries can be directed to the corresponding authors.

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