

Chemerin-activated functions of CMKLR1 are regulated by G protein-coupled receptor kinase 6 (GRK6) and β -arrestin 2 in inflammatory macrophages

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ABSTRACT

Chemerin receptor (CMKLR1) is a G protein-coupled receptor (GPCR) implicated in macrophage-mediated inflammation and in several forms of human arthritis. Analogous to other GPCR, CMKLR1 is likely regulated by G protein-coupled receptor kinase (GRK) phosphorylation of intracellular domains in an activation-dependent manner, which leads to recruitment and termination of intracellular signaling via desensitization and internalization of the receptor. The ubiquitously expressed GRK family members include GRK2, GRK3, GRK5, and GRK6, but it is unknown which GRK regulates CMKLR1 cellular and signaling functions. Our data show that activation of CMKLR1 by chemerin in primary macrophages leads to signaling and functional outcomes that are regulated by GRK6 and β -arrestin 2. We show that arrestin recruitment to CMKLR1 following chemerin stimulation is enhanced with co-expression of GRK6. Further, internalization of endogenous CMKLR1, following the addition of chemerin, is decreased in inflammatory macrophages from GRK6- and β -arrestin 2-deficient mice. These GRK6- and β -arrestin 2-deficient macrophages display increased migration toward chemerin and altered AKT and Extracellular-signal Related Kinase (ERK) signaling. Our findings show that chemerin-activated CMKLR1 regulation in inflammatory macrophages is largely GRK6 and β -arrestin mediated, which may impact innate immunity and have therapeutic implications in rheumatic disease.

1. Introduction

The chemokine-like receptor 1 (CMKLR1), also known as ChemR23 or the chemerin receptor, was first cloned in 1996 and classified as a G protein-coupled receptor (GPCR) (Gantz et al., 1996; Samson et al., 1998). It is a 42 kDa, seven-transmembrane domain protein structurally similar to complement component 3a receptor (C3aR), C5a receptor (C5aR), and other chemoattractant receptors (Gantz et al., 1996; Samson et al., 1998). CMKLR1 is expressed on various leukocyte

subsets, including monocytes, macrophages, natural killer cells, immature plasmacytoid dendritic cells (pDCs), as well as adipocytes (Samson et al., 1998; Vermi et al., 2005; Parolini et al., 2007; Bozaoglu et al., 2007). In 2003, a CMKLR1 ligand, chemerin, was identified and was classified as both an adipokine and a chemoattractant peptide (Bozaoglu et al., 2007; Wittamer et al., 2003). Chemerin is secreted into the plasma as pro-chemerin and requires proteolytic processing for activation (Wittamer et al., 2003). Previous reports have demonstrated CMKLR1-mediated migration *ex vivo* of both human and mouse

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leukocytes, such as pro-inflammatory macrophages and pDCs (Wittamer et al., 2003; Zabel et al., 2005; Luangsay et al., 2009; Zabel et al., 2006).

Chemerin and CMKLR1 are implicated in various inflammatory diseases and metabolic syndromes, and in particular, rheumatic disease. Elevated levels of bioactive chemerin are found in rheumatoid arthritis (RA), osteoarthritis (OA), psoriatic arthritis (PA), and lupus nephritis (Bozaoglu et al., 2007; Wittamer et al., 2003; Huss et al., 2010; Huang et al., 2012; Berg et al., 2010; Kaneko et al., 2011; Zhao et al., 2011; Eisinger et al., 2012; Albanesi et al., 2010; Albanesi et al., 2009; De Palma et al., 2011). In contrast, Resolvin E1, another natural ligand of CMKLR1, has anti-inflammatory properties (Aoki et al., 2008; Seki et al., 2010).

RA is a chronic, inflammatory autoimmune disease that affects more than 1 million people in the United States (Helmick et al., 2008). It is characterized by inflammation coupled with leukocyte migration to the joint in a chemokine-dependent manner followed by articular destruction. Within the RA synovium, CMKLR1 is expressed by articular chondrocytes (Berg et al., 2010), macrophages, immature dendritic cells (DCs), and fibroblast-like synoviocytes (FLS) (Kaneko et al., 2011). In addition, chemerin was found *in vitro* to enhance secretion of pro-inflammatory proteins, including TNF- α , IL1- β , IL-6, IL-8, MMP-1 and MMP-8 by articular chondrocytes, IL-6 and MMP-3 by FLS, and to increase phosphorylation of AKT and ERK1/2 (p44/42 MAPK) in both chondrocytes and FLS (Berg et al., 2010; Kaneko et al., 2011). Circulating plasmacytoid dendritic cells have been shown to express CMKLR1 and migrate toward chemerin in transendothelial migration assays (Zabel et al., 2005), which further implicates chemerin and CMKLR1 in RA disease pathogenesis.

Analogous to the established activation paradigm of other GPCRs, it is likely that CMKLR1 undergoes a conformational change after binding chemerin. Activation of GPCR typically leads to dissociation of intracellular heterotrimeric G proteins (G α and G $\beta\gamma$ subunits) and subsequent downstream signaling via second messengers (Rajagopal et al., 2010). G protein-coupled receptor kinases (GRKs) regulate GPCRs via phosphorylation of their cytoplasmic domains in an activation-dependent manner, which leads to termination of receptor signaling via desensitization and subsequent internalization of the receptor (Lefkowitz, 1998). Previous reports have identified 12 serine/threonine residues within the intracellular domains of CMKLR1 that are putative sites for GRK phosphorylation (Samson et al., 1998). Site-directed mutagenesis of CMKLR1 Ser343, one of the predicted GRK phosphorylation sites, results in loss of receptor internalization after stimulation by chemerin (Zhou et al., 2014). Although not all GPCRs employ this mechanism after agonist activation, desensitization by GRKs is often followed by β -arrestin recruitment to the receptor, which targets the receptor for internalization (Lefkowitz and Shenoy, 2005). There are two ubiquitously expressed, non-visual arrestin isoforms in mammals, β -arrestin 1 (also called arrestin-2) and β -arrestin 2 (arrestin-3), as well as two “visual arrestins” (arrestin-1 and arrestin-4), which are restricted to the eye (Smith and Rajagopal, 2016). Previous work by De Henau, et al. has shown that chemerin binding to CMKLR1 can induce the recruitment of both β -arrestin-1 and β -arrestin-2, but that downstream signaling to ERK1/2 requires β -arrestin-2, but not β -arrestin-1 (De Henau et al., 2016). To our knowledge, specific GRK-mediated regulation of chemerin/CMKLR1 functions has not been previously defined.

There are seven known GRK isoforms (GRK1 - 7) further sub-divided into three families, all of which have a high degree of sequence homology, particularly within sub-families (Benovic et al., 1991; Haribabu and Snyderman, 1993). GRK2, GRK3, GRK5, and GRK6 are expressed ubiquitously (Benovic et al., 1991; Kunapuli and Benovic, 1993; Benovic et al., 1989), whereas the expression patterns of GRK1, GRK4 and GRK7 are more tissue restricted (Hisatomi et al., 1998; Weller et al., 1975; Andresen, 2010; Premont et al., 1996). GRK2 and GRK3 are members of the same subfamily (share 85% sequence homology) (Benovic et al., 1991) and GRK5 and GRK6 comprise

another subfamily (share 80% sequence homology) (Haribabu and Snyderman, 1993). The GRK2/GRK3 subfamily are cytosolic proteins that require a C-terminal pleckstrin homology (PH) domain for receptor ligand-induced translocation to the membrane (Pitcher et al., 1998). In contrast, the GRK5/GRK6 subfamily are predominately localized to the membrane via C-terminal post-translational modifications and N-terminal Phosphatidylinositol 4,5-bisphosphate (PIP₂) binding regions and lack the PH domain (Pitcher et al., 1998).

While similar expression patterns and shared sequence homology suggest overlapping GRK function, there is growing evidence that specific GRKs can 1) selectively regulate different GPCRs through differential phosphorylation patterns and/or recruitment, and 2) can be selectively activated by specific ligands (Busillo et al., 2010; Chen et al., 2004; Raghuvanshi et al., 2012; Tarrant et al., 2013, 2008). In addition, recent studies have suggested that GPCRs can initiate G protein independent signaling (e.g., GRK/ β -arrestin-dependent signaling), leading to biased physiologic outcomes (Lefkowitz and Shenoy, 2005; Ibrahim and Kurose, 2012; DeWire et al., 2007; Luttrell and Gesty-Palmer, 2010; Watari et al., 2014), suggesting that not only do GRKs regulate GPCRs directly, but also influence and control downstream signals.

Given the relevance of chemerin/CMKLR1 in inflammatory macrophages and in the pathogenesis of rheumatic diseases, such as RA (Kaneko et al., 2011; Eisinger et al., 2012), we sought to better understand the regulatory role of GRK isoforms 2, 3, 5 and 6 after receptor activation by pro-inflammatory chemerin. These investigations have identified specific regulation by GRK6 and β -arrestin 2 that is important to chemerin/CMKLR1-mediated cellular functions, which could, in turn, better inform the development and utilization of future therapeutics for rheumatic diseases.

2. Materials and methods

2.1. Mice

All mouse primary cells used in *ex vivo* experiments were harvested from C57BL/6 J mice (WT) or mice deficient in *Grk6* (GRK6^{-/-}) or *β -arrestin 2* (Barr2^{-/-}) backcrossed (> 12 generations) onto the C57BL/6 J background. All mice were cared for under the Institutional Animal Care and Use Committee (IACUC) approved protocols at the University of North Carolina at Chapel Hill.

2.2. Cells

Human fibroblast-like synoviocytes (FLS) were generated from identified, IRB-exempt samples isolated from surgical explanted tissue of patients with RA, OA, or normal synovium. Murine white blood cells were harvested 5–7 days post-treatment with injection of 3% Brewer thioglycollate (Sigma-Aldrich, St. Louis, MO) into the peritoneal cavity. Contaminating red blood cells were lysed. This procedure resulted in a heterogeneous white blood cell population where the only population of CMKLR1 expressing cells were F4/80+ pro-inflammatory murine peritoneal elicited monocyte/macrophages (referred to as “pro-inflammatory macrophages” in text).

2.3. Quantitative PCR (qRT-PCR)

RNA was isolated from human FLS using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA synthesized using BioRad iScript™ cDNA synthesis kit (BioRad, Hercules, California, USA) and the Eppendorf Mastercycler pro S (Eppendorf, Hamburg, Germany). qRT-PCR was performed using BioRad SsoAdvanced™ Universal SYBR Green Supermix on the BioRad CFX96™ Real-Time System. Total volume for each reaction was 20 μ L (10.0 μ L Supermix 2x, 1.0 μ L primers at 20 μ M, 8.0 μ L RNase-free H₂O, and 1 μ L template cDNA at 25 ng/ μ L). The qRT-PCR was carried out using the following thermocycling conditions:

95 °C for 30 s, 95 °C for 5 s, 55 °C for 30 s, plate-read and repeat for 39 cycles, followed by melt-curve analysis. The following published human primers were used: IDUA forward (5'-CTC GGG CCA CTT CAC TGA C-3'), IDUA reverse (5'-CAG TCC GTA CCT ACC GAT GTA T-3'), GRK2 forward (5'-ACT TCA GCG TGC ATC GCA T-3'), GRK2 reverse (5'-GCT TTT TGT CCA GGC ACT TCA T-3'), GRK3 forward (5'-AGC TGT AGA ACA CGT ACA AAG TC-3'), GRK3 reverse (5'-ATG TCA CCT CGA AGG CTT TCA-3'), GRK6 forward (5'-TAG CGA ACA CGG TGC TAC TC-3'), GRK6 reverse (5'-GCT GAT GTG AGG GAA CTG GA-3') (Balabanian et al., 2008), and CMKLR1 forward (5'-ACC TGC ATG GGA AAA TAT CCT-3'), CMKLR1 reverse (5'-GAG GTT GAG TGT GTG GTA GGG-3') (Eisinger et al., 2012). IDUA served as our reference gene for delta Ct (Δ Ct) analysis, as per our previously published protocol (Tarrant et al. (2012)).

2.4. Modified Tango assay of β -arrestin mobilization

A modified Tango assay was used to measure β -arrestin 2 recruitment to chemerin-stimulated receptors, based on the previously reported technique (Barnea et al., 2008). HTLA cells were transfected with a CMKLR1-TCS-tTA receptor construct (Barnea et al., 2008). This construct has the C-terminal tail of the V2 vasopressin receptor removed to ensure GRK effects were CMKLR1-specific (Fig. 2A). A previous report indicated that removal of the V2 tail from the CMKLR1 construct increased the ligand-induced responses of the receptor in the Tango assay (Kroeze et al., 2015). The following plasmids were used for GRK over-expression as obtained from Origene (Rockville, MD, USA): pCMV6-XL5 GRK2, pCMV6-XL5 GRK3, pCMV6-XL5 GRK5, pCMV6-XL5 GRK6 transcript 1, and pCMV6-XL5 vector as a negative control. Equivalent overexpression of the GRK proteins was confirmed by Western blot (Supplemental Fig. 2). A separate expression vector encoding yellow-fluorescent protein (YFP) was simultaneously transfected for use as a transfection control and epifluorescence detection was consistently > 70%. HTLA cells were transiently transfected with 6.5 μ g of total plasmid DNA per 10-cm plate (3 μ g of CMKLR1-Tango, 0.5 μ g of YFP, and either 3 μ g of empty-vector control, GRK2, GRK3, GRK5 or GRK6) via calcium-phosphate precipitation and arrestin-recruitment and data normalization was performed as previously described (Billard et al., 2016). Normalized data was combined and graphed using a log (agonist) vs response (three-parameter) dose-response curve. An extra sum-of-squares F test was performed comparing the Emax (Top) and the LogEC₅₀ for each GRK over-expression curve compared to control curve. Our p-value was set at 0.001.

2.5. Receptor internalization assay

Internalization of CMKLR1 upon activation by chemerin was measured by flow cytometry using pro-inflammatory macrophages from WT (control), *Grk6*^{-/-}, and *Barr2*^{-/-} mice. Cells were aliquoted (2.5 × 10⁵ cells/ tube), starved for 1 h in serum-free RPMI 1640, and then stimulated with either 6.25 nM or 200 nM chemerin (R&D systems) for 30 s, 1 min, 5 min, and 10 min at 37 °C + 5% CO₂. Cells were not stimulated at the 0 min time point in order to determine maximum receptor expression before addition of ligand. At each time point, receptor internalization was arrested with 4 °C buffer (HBSS + 5% FBS + 2 mM EDTA) and by placing cells immediately on ice. Cells were then stained for CMKLR1 (anti-mouse CMKLR1 PE, eBioscience, 12-7582, clone BZ194, discontinued, or anti-mouse CMKLR1, Miltenyi, 130-106-902, clone REA461) and F4/80 (rat anti-mouse F4/80:APC, AbC serotec, MCA497APCT, clone CI:A3-1) as previously described (Rama et al., 2011). CMKLR1 surface expression was measured via flow cytometry by gating on F4/80 positive cells to confirm the myeloid lineage and measuring mean fluorescence intensity (MFI) of CMKLR1. Data was normalized as a percent relative to the MFI at 0 min for each cell type.

2.6. Real-time fluorophore-based detection of cell migration

Cell migration to chemerin was examined using the Falcon™ HTS FluoroBlok 96-Multiwell Insert System (3 μ m pore-size) (BD Biosciences, Bedford, MA) and pro-inflammatory macrophages from WT (control), *Grk6*^{-/-}, and *Barr2*^{-/-} mice obtained as previously described. Cells were starved in RPMI 1640 + 1% BSA at 37 °C for one hour and labeled with calcein (485 nm/527 nm). Cells were washed and suspended in RPMI 1640 + 1% BSA + 10 mM HEPES, and 1 × 10⁵ cells were loaded into Falcon™ HTS FluoroBlok 96-Multiwell Insert System chemotaxis chambers (Corning, NY). Cells were allowed to migrate to RPMI/BSA/HEPES medium alone or to medium with addition of 6.25 nM chemerin (R&D Systems, Minneapolis, MN). Fluorescence was measured at 2 min intervals over the course of 100 min at 37 °C using a Fluoroskan Ascent Microplate Fluorometer (Thermo Scientific, Waltham, MA). Data was normalized by subtracting time 0 from each subsequent time point for each condition and graphed as fluorescence vs time for the first 100 min interval. Analysis was done using linear mixed models and tested for a significant group effect using likelihood ratio test.

2.7. Western immunoblotting

Serum starved pro-inflammatory macrophages from WT, *Grk6*^{-/-}, and *Barr2*^{-/-} mice either were not stimulated (0 min) or were stimulated with 6.25 nM chemerin (R&D Systems) for 1, 5, 10, 15, and 20 min, and then lysed in ice cold RIPA buffer (plus protease and phosphatase inhibitors) to arrest signaling. Protein lysates were normalized using a BCA assay (Thermo Scientific, Rockford, IL, USA) and run on a Mini-PROTEAN TGX™ gel (Any kD™, 15-well comb, 15 μ l well volume) (Bio-Rad, Hercules, CA, USA). Between 2–4 μ g total cellular protein was run per independent experiment. Lysates from *Barr2*^{-/-} and *Grk6*^{-/-} samples were always run on the same gel as the WT control lysates for direct comparison by densitometry of signaling strength and duration. Antibodies used were p44/42 MAPK (ERK1/2) (137F5) Rabbit mAb (1:2000), phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (D13.14.4E) XP™ Rabbit mAb (1:2000), phospho-AKT (Ser473) (D9E) XP™ Rabbit mAb (1:2000), pan-AKT (C67E7) Rabbit mAb (1:2000), and anti-rabbit IgG, HRP-linked Antibody (1:5000) (Cell Signaling Technology, Danvers, MA, USA). Images were scanned at 600 DPI resolution and imported into ImageJ where images were converted into an 8-bit black and white image. Quantification of band density was performed and graphs represent the ratio of phospho-protein to total-protein (relative density).

2.8. Statistics

Linear mixed models were used to determine differences between control and *Barr 2*^{-/-}, or *Grk6*^{-/-} migration curves of primary inflammatory macrophage/monocytes to chemokine ligand stimulation over time according to our previously published methodology (Tarrant et al., 2013; Giguere et al., 2013). Briefly, quadratic terms were included to account for the curve of the data and likelihood ratio tests (LRT) with three degrees of freedom (df) were used to test for significant group differences over time. Statistical significance was established at 0.05. All analyses were carried out using SAS, version 9.3 (Cary, NC).

3. Results

3.1. CMKLR1, GRK2, GRK5, and GRK6 are expressed in human fibroblast-like synoviocytes

Differential expression of GRK isoforms have been reported in various diseases and disease models, including RA (Billard et al., 2016; Nakaya et al., 2013; Bychkov et al., 2011; Lombardi et al., 1999). In

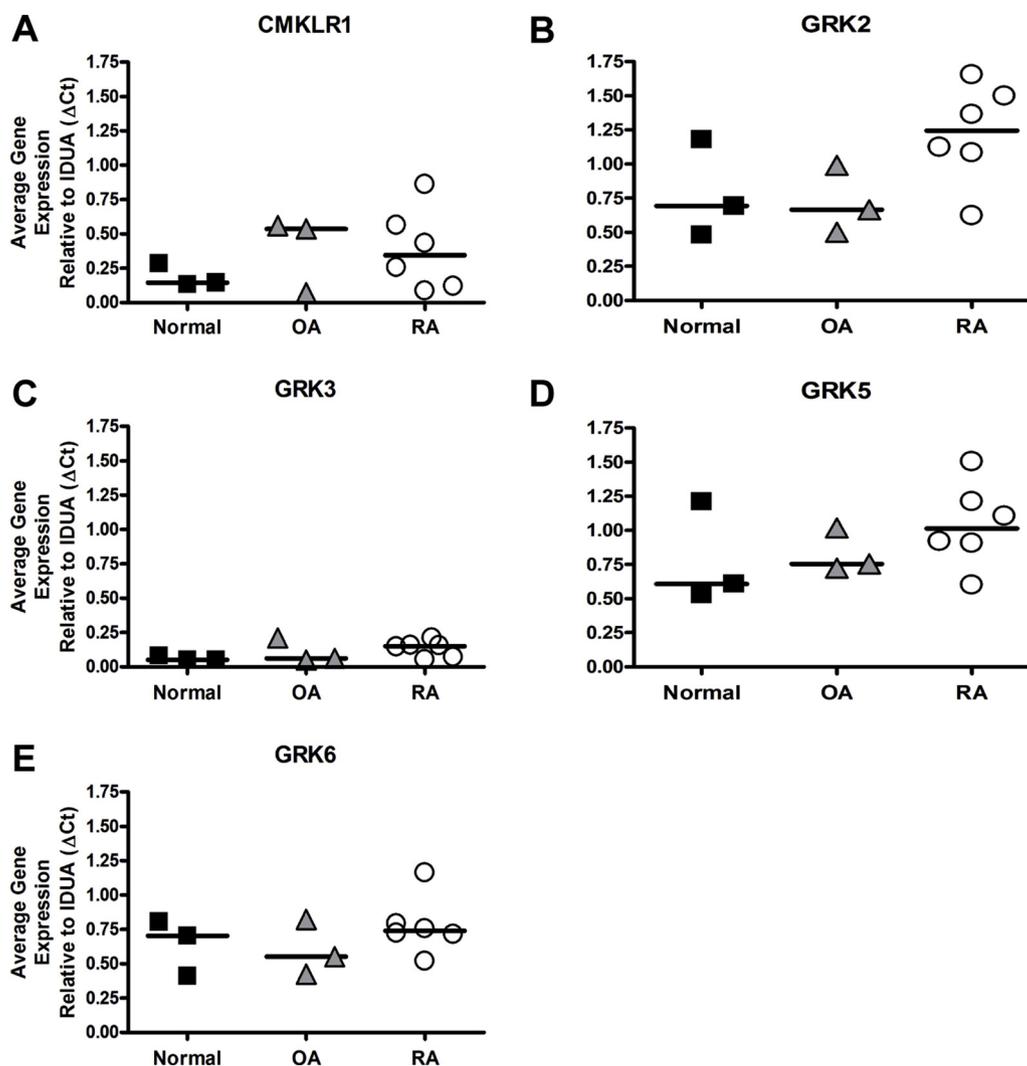


Fig. 1. Normal, Osteoarthritis (OA), and Rheumatoid arthritis (RA)-derived fibroblast-like synoviocytes (FLS) express *CMKLR1*, *GRK2*, *GRK5*, and *GRK6*. Human FLS from normal, OA, and RA samples were cultured, and gene expression of (A) *CMKLR1*, (B) *GRK2*, (C) *GRK3*, (D) *GRK5*, and (E) *GRK6* was determined via qRT-PCR. Data was analyzed relative to the housekeeping gene *IDUA* (Δ Ct). Horizontal bar represents the median expression for each group. All sample types tested expressed *CMKLR1*, *GRK2*, *GRK5*, and *GRK6*. Minimal expression of *GRK3* was observed in this cell type.

addition, previous reports have detected variable *CMKLR1* within RA and OA synovial tissue (Kaneko et al., 2011). Therefore, we chose to look at mRNA expression levels of *CMKLR1* and *GRKs* that could potentially regulate chemerin-specific GPCR responses in human fibroblast-like synoviocytes (FLS). Our results confirm that FLS from normal, OA, and RA synovial tissue express *CMKLR1*. The data further indicate slight increases in *CMKLR1* transcript expression observed in FLS cells from diseased tissue compared to normal FLS (Fig. 1A). In addition, we show that *GRK2*, -5, and -6 isoform transcripts are expressed in all FLS, independent of arthritis, but that *GRK3* is minimally expressed compared to the other isoforms (Fig. 1B-E).

3.2. *GRK6* recruits β -arrestin 2 to *CMKLR1* after chemerin stimulation

RA FLS express *CMKLR1*, *GRK2*, *GRK5*, and *GRK6*, suggesting that these kinases may play a role in *CMKLR1* regulation. We used a modified Tango assay (described in Fig. 2A-B and *Experimental Procedures*) to compare the effects of *GRKs* on β -arrestin 2 recruitment to chemerin-stimulated *CMKLR1* (Barnea et al., 2008). Our *CMKLR1* modified Tango assay system behaves similar to other previous reports (Barnea et al., 2008) with an EC_{50} to chemerin of 3.5 nM in control cells. HTLA cells transiently co-transfected with the cDNA of *CMKLR1* and *GRK2*, *GRK3*, *GRK5* or *GRK6* did not exhibit significantly altered EC_{50} values for

chemerin response as compared to control cells (Fig. 2C). However, HTLA cells transfected with *GRK6* resulted in up to 3-fold increase in reporter gene activity compared to control (100%) in response to chemerin (Fig. 2C). This increase in *GRK6*-mediated β -arrestin 2 recruitment was significant ($p < 0.001$), whereas cells transfected with *GRK2*, *GRK3*, or *GRK5* did not show a significant increase in reporter gene activity compared to control at any concentration tested (Fig. 2C). These data show that *GRK6* specifically increases β -arrestin 2 recruitment to *CMKLR1* following stimulation with chemerin.

3.3. *CMKLR1* internalization in pro-inflammatory myeloid cells following chemerin stimulation is mediated by β -arrestin 2

Phosphorylation of the GPCR C-terminus and intracellular loops by *GRKs* after ligand stimulation desensitizes the receptor and leads to β -arrestin recruitment and subsequent internalization of the GPCR (Zhou et al., 2014; Kohout and Lefkowitz, 2003). Pro-inflammatory macrophages are invasive hematopoietic cells that invade synovial tissue in RA (Kinne et al., 2007; Kim et al., 2009) and expresses high levels of *CMKLR1* (Wittamer et al., 2003; Zabel et al., 2006) and *GRK6* (Lombardi et al., 1999). To examine the role of β -arrestin and *GRK6* in the regulation of *CMKLR1* surface expression, we examined endogenous *CMKLR1* internalization using pro-inflammatory macrophages.

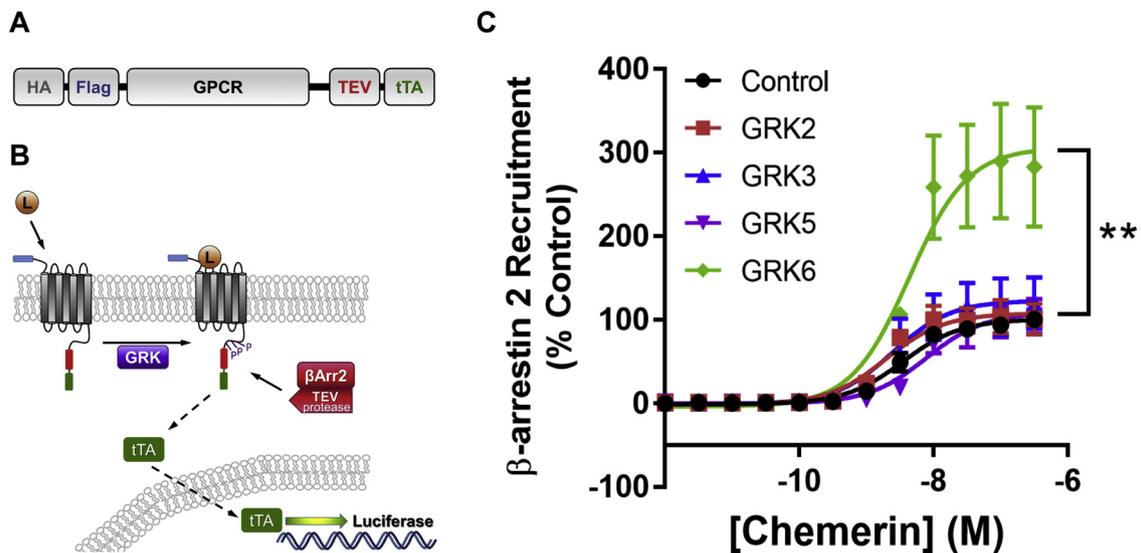


Fig. 2. GRK6 overexpressing HTLA cells show enhanced recruitment of β -arrestin 2 after stimulation with chemerin. The CMKLR1 Tango construct (A) consists of hemagglutinin (HA), flag epitope tag, CMKLR1 (or other GPCR), TEV protease, and tetracycline transactivator (tTA). To ensure GRK effects of β -arrestin recruitment were CMKLR1-specific, this construct removed the C-terminal tail of the V2 vasopressin receptor (that promotes β -arrestin recruitment) that was present in the original construct design (Kroeze et al., 2015). (B) The CMKLR1 Tango assay is initiated by the chemerin ligand (L) binding to the CMKLR1 GPCR (construct shown in A), followed by G protein-coupled receptor kinase (GRK) phosphorylation of the native GPCR cytoplasmic domains and recruitment of a modified β -arrestin (with attached TEV protease). TEV protease cleavage releases the tTA transcription factor which translocates into the nucleus to activate transcription of the luciferase reporter gene, providing a luminescence assay readout. (C) HTLA cells were transfected with 3 μ g of CMKLR1-Tango plasmid, 0.5 μ g YFP, and 3 μ g of either pCMV6-XL5 empty vector (control), or *GRK2*, *GRK3*, *GRK5*, and *GRK6* DNA for a Tango arrestin recruitment assay and stimulated with up to 300 nM of chemerin. Shown are dose-response curves of chemerin stimulation. Error bars represent SEM. An extra sum-of-squares F test was done on the log(agonist) vs response (three-parameter) dose-response curves with significance set at $p = 0.001$. HTLA cells overexpressing GRK6 showed a significantly higher Emax (Top) compared to control cells ($p < 0.0001$). There was no statistical difference in Emax between GRK2, 3, or 5 overexpressing cells and Control cells. In addition, there was no statistical difference in EC_{50} in any condition tested compared to Control cells. $n = 3-6$.

CMKLR1 receptor internalization tested after stimulation by 6.25 nM chemerin (near EC_{50}) resulted in $> 50\%$ internalization after 1 min and $> 70\%$ after 10 min in wild type control cells. However, β -arrestin 2 deficient (Barr2^{-/-}) pro-inflammatory macrophages had decreased internalization compared to controls at all time points (Fig. 3A). The difference between CMKLR1 surface expression on Barr2^{-/-} deficient cells compared to WT cells was also statistically significant when cells were treated with 200 nM (supersaturating range). Stimulation with this high concentration of chemerin resulted in $> 70\%$ CMKLR1 internalization by 1 min and $> 80\%$ in wild type control cells. Again, Barr2^{-/-} cells had significantly less internalization for each time point (Fig. 3B).

GRK6 is known to have strong regulatory roles in arthritis inflammation (Tarrant et al., 2008; Lombardi et al., 1999, 2001). Because of this and our Tango data suggesting a prominent role for GRK6 in β -arrestin 2 recruitment, we focused on the mechanistic effects of GRK6 on CMKLR1 internalization. GRK6-deficient (GRK6^{-/-}) pro-inflammatory macrophages exhibited decreased internalization of CMKLR1 over time compared to control cells after 6.25 nM chemerin stimulation (Fig. 3A), though this difference was not significant. The impact of GRK6 deficiency was lost when stimulating macrophages with high dose chemerin (200 nM) (Fig. 3B). Taken together, these data show that internalization of CMKLR1 by pro-inflammatory macrophages is strongly dependent upon β -arrestin 2, and while GRK6 appears to affect internalization at physiologically functional chemerin concentrations, it may be unnecessary or overwhelmed at high concentrations of chemerin.

3.4. β -arrestin 2 and GRK6 negatively regulate migration of pro-inflammatory macrophages toward chemerin

Evidence suggests that chemerin is involved in recruiting pro-inflammatory macrophage/monocytes to synovial tissue in arthritis

(Wittamer et al., 2003; Zabel et al., 2005; Kaneko et al., 2011); thus, we examined how deletion of GRK6 and β -arrestin 2 affected the migration of CMKLR1-expressing pro-inflammatory macrophages to chemerin. Our hypothesis was that increased migration would be observed in cells deficient in either GRK6 or β -arrestin 2, which we have established as components of the CMKLR1 desensitization/internalization machinery. Barr2^{-/-}, GRK6^{-/-}, and wild type controls all showed significant increases in macrophage migration toward chemerin over media alone (Fig. 4). In addition, Barr2^{-/-} macrophage chemotaxis to chemerin was significantly enhanced above that seen by control macrophages to chemerin. GRK6^{-/-} macrophage migration toward chemerin was significantly increased over both control and Barr2^{-/-} macrophages (Fig. 4 and Supplemental Fig. 1). These results support GRK6 and β -arrestin 2 involvement in chemerin/CMKLR1 regulation of cell migration.

3.5. β -arrestin 2 and GRK6 function as negative regulators of CMKLR1 signaling

Barr2^{-/-} and GRK6^{-/-} pro-inflammatory macrophages show decreased CMKLR1 internalization and enhanced migration to chemerin; therefore, to determine whether CMKLR1 signaling is also altered, we first examined the Ras-Raf-MEK-ERK signaling cascade, which is known to be a downstream effector of G protein and β -arrestin 2 activation. We hypothesized that loss of either Barr2 or GRK6 would result in prolonged and/or enhanced ERK1/2 signaling (Roskoski, 2012; Luo et al., 2008). Upon stimulation with 6.25 nM chemerin, Barr2^{-/-} pro-inflammatory macrophages showed significantly enhanced signaling over time compared to control (Fig. 5A and 5B). GRK6^{-/-} macrophages appear to have a similar trend of enhanced signaling to ERK1/2 that persists throughout the time course shown (Fig. 5C). However, densitometric analysis of the Western blot data show that this difference is not significant (Fig. 5D).

Because GPCR signaling through the PI3K/AKT pathway can

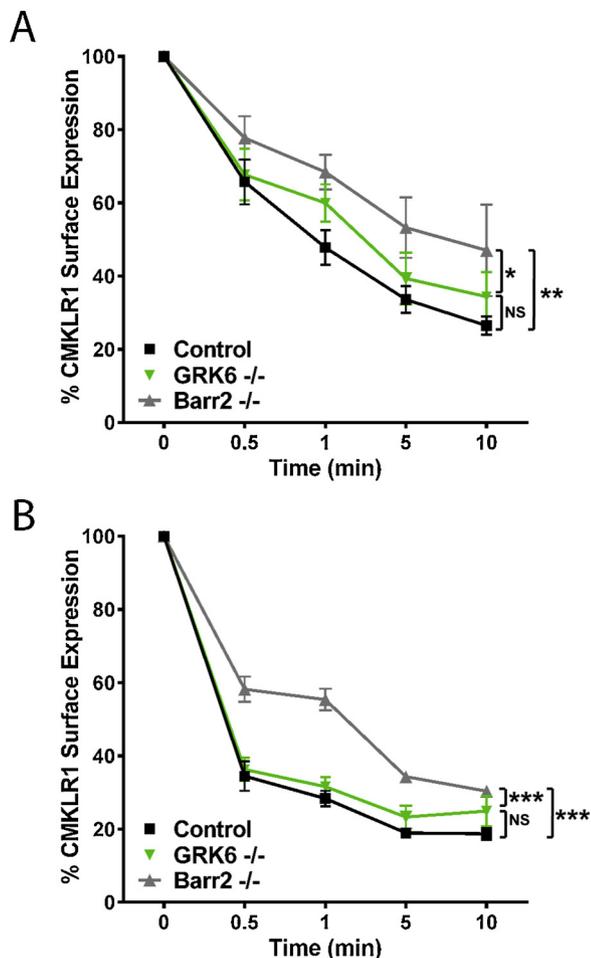


Fig. 3. β -arrestin-2 deficient (Barr2^{-/-}) and GRK6 deficient (GRK6^{-/-}) pro-inflammatory macrophages show delayed CMKLR1 internalization. (A) Wild type control, GRK6^{-/-}, and Barr2^{-/-} pro-inflammatory macrophages were stimulated with 6.25 nM chemerin *ex vivo*. Cell surface expression of CMKLR1 protein on F4/80 positive myeloid cells was determined using flow cytometry. (B) Control, GRK6^{-/-}, and Barr2^{-/-} pro-inflammatory macrophages were stimulated with 200 nM chemerin *ex vivo*. Data are graphed by using the ratio mean fluorescence intensity (MFI) of surface CMKLR1 compared to unstimulated cells (0 min) as a percent of maximum. Error bars represent SEM. A 2-way ANOVA was performed for statistical analysis. n = 4-6. N.S. = not significant; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

potentially influence cellular functions that contribute to inflammation (e.g. migration, survival), the activation of this pathway was also examined in Barr2^{-/-} and GRK6^{-/-} macrophages stimulated with 6.25 nM chemerin by Western blot for phospho-AKT (Fig. 6). We again hypothesized that loss of either Barr2 or GRK6 would result in prolonged and/or enhanced AKT signaling. Chemerin stimulation (6.25 nM) of Barr2^{-/-} pro-inflammatory macrophages did not appear to initially enhance phosphorylation of AKT and the observed prolonged activation of this pathway over time was not statistically significant (Figs. 6A and 6B). In contrast, GRK6^{-/-} macrophages stimulated with 6.25 nM chemerin showed a significant increase in AKT phosphorylation that appears to degrade at a similar rate to that seen in wild type controls (Figs. 6C and 6D). Taken together, these data suggest non-redundant roles for Barr2 and GRK6 in regulating chemerin/CMKLR1 signals to the ERK1/2 and AKT pathways.

4. Discussion

Chemerin is a known chemotactic agent for CMKLR1-expressing antigen-presenting cells (APCs), such as immature DCs, plasmacytoid

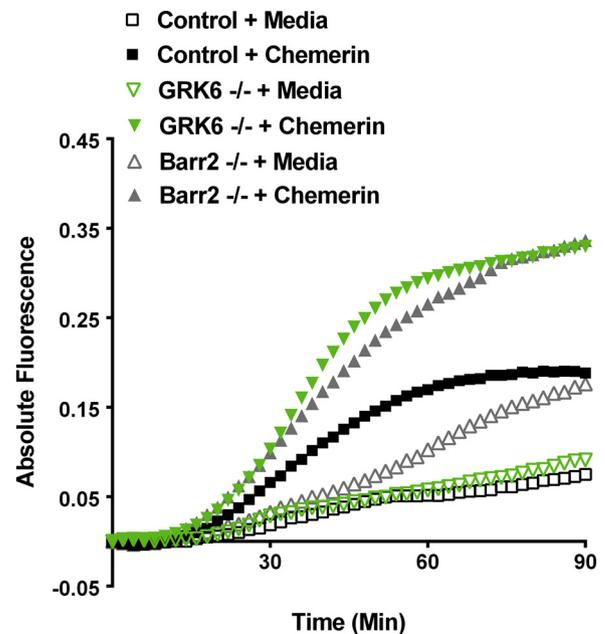


Fig. 4. β -arrestin-2 deficient (Barr2^{-/-}) and GRK6 deficient (GRK6^{-/-}) pro-inflammatory macrophages show enhanced migration to chemerin. Control, Barr2^{-/-}, and GRK6^{-/-} pro-inflammatory monocytes/macrophages were fluorescently labeled with calcein and loaded into the upper chamber wells of a Falcon[™] HTS FluoroBlok 96-Multiwell Insert System with either 6.25 nM chemerin in the lower chamber to stimulate migration or medium alone as a control. Shown is mean fluorescence intensity (MFI) (measured at 2 min intervals) of 3–5 independent experiments. Error bars are omitted for clarity. Data was normalized by setting 0 absolute fluorescence as the start point for each curve. The effect of chemerin was examined using an ANCOVA for statistical analysis (Supplemental Fig. 1). GRK6^{-/-} macrophage migration was significantly enhanced compared to Barr2^{-/-} and Control ($p < 0.0001$). Barr2^{-/-} macrophage migration was also significant compared to Control ($p < 0.01$).

DCs, and macrophages (Wittamer et al., 2003; Zabel et al., 2005), which are important to the pathogenesis of RA (Kinne et al., 2007; Lande et al., 2004; Santiago-Schwarz et al., 2001). In addition, high levels of chemerin are found in arthritic synovial fluid (Wittamer et al., 2003) and chemerin protein has been detected in RA and OA synovial tissue (Kaneko et al., 2011). Taken together, these findings suggest that chemerin/CMKLR1 signaling promotes inflammation in RA by recruiting inflammatory cells, such as macrophages, to the joint. Therefore, elucidation of the specific cellular machinery underpinning the regulation of chemerin/CMKLR1 signaling should provide a better understanding of RA pathogenesis, as well as highlight potential therapeutic targets. A previous study has shown that CMKLR1 undergoes internalization after chemerin stimulation via a non-clathrin-mediated pathway, and that this pathway is likely via caveolae (Zhou et al., 2014), which is generally considered to be GRK/arrestin independent (Barki-Harrington and Rockman, 2008; Pierce and Lefkowitz, 2001). However, a previous study by Rourke, et al. states that β -arrestin could be involved in CMKLR1 internalization, which our results confirm (Rourke et al., 2015). Our data show that using the modified Tango assay to monitor arrestin mobilization, expression of GRK6 enhances β -arrestin 2 recruitment to chemerin-activated CMKLR1 (Fig. 2). In addition, endogenously-expressed CMKLR1 internalization in inflammatory macrophages was β -arrestin 2-dependent. CMKLR1 internalization was not completely abolished, suggesting a possible role for β -arrestin 1 or contributions of a non-clathrin mediated mechanism of internalization (e.g. caveolae) (Zhou et al., 2014; Gong et al., 2008). Further, internalization, cellular migration, and downstream signaling to ERK1/2 and AKT was altered in macrophages deficient in β -arrestin 2 and GRK6, suggesting that these are likely the predominant receptor-

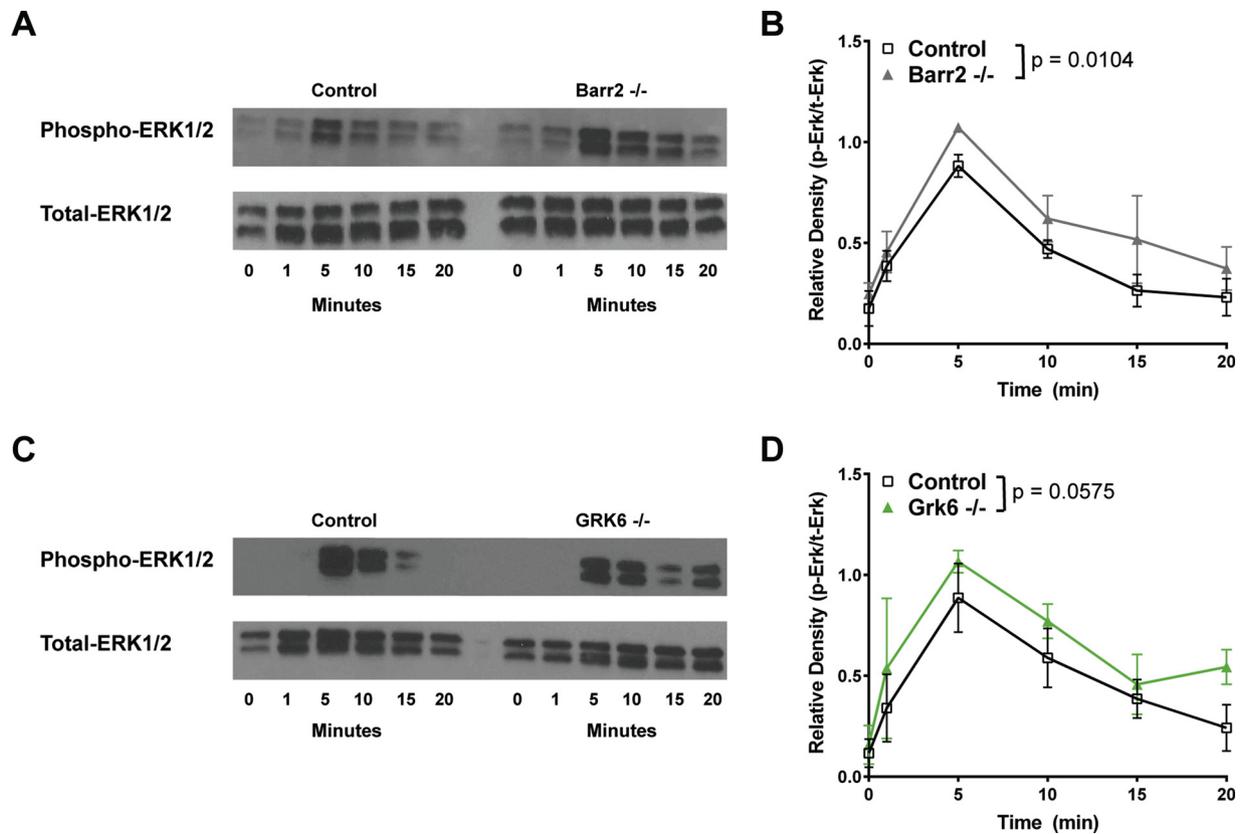


Fig. 5. Barr2^{-/-} pro-inflammatory macrophages show increased ERK1/2 phosphorylation after activation by chemerin. Control versus Barr2^{-/-} (A, B) or GRK6^{-/-} (C, D), C57BL/6 J peritoneal macrophages were either left unstimulated (0 min) or stimulated with 6.25 nM chemerin *ex vivo* for 1, 5, 10, 15, or 20 min. Total protein was normalized using a BCA assay. Western blot analysis was performed on cell lysates probed using phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (Phospho-ERK1/2) and p44/42 MAPK (ERK1/2) (Total-ERK1/2). Representative blots (A, C) of 3 (Barr2^{-/-}) or 4 (GRK6^{-/-}) independent experiments are shown. Quantification shown in (B, D) was done by densitometry using ImageJ software and is shown as a mean ratio of relative density \pm SEM (Barr2^{-/-} n = 3; GRK6^{-/-} n = 4).

proximal regulators of CMKLR1 in response to chemerin stimulation (Figs. 3–6), though to differing degrees.

To better define the functional effects of GRKs on chemerin stimulated CMKLR1, we interrogated GRK-mediated, β -arrestin 2 recruitment using a modified Tango assay to measure GRK specificity. As noted in the *Experimental Procedures*, this assay requires an 18–24-hour incubation *after* stimulation for reporter expression and irreversible cleavage of the transcription factor from the receptor. These caveats of the TANGO assay (and our modified version) do not strictly imitate the rapid time scale or catalytic activity of arrestins (Violin et al., 2006). However, the modified TANGO assay used does recapitulate concentration-dependent responses and broad-level regulatory functions by GRKs. Our results show that GRK6 was the only GRK isoform, of those ubiquitously expressed, that increased β -arrestin 2 recruitment to chemerin-stimulated CMKLR1 receptor (Fig. 2). As expected from these data, GRK6-deficient macrophages also exhibited enhanced migration (Fig. 4), and increased phosphorylation of Akt (Figs. 6) with chemerin stimulation. Additionally, the more pronounced defects in internalization observed by β -arrestin 2 deficient cells, as compared to GRK6, suggest that there may be GRK redundancy or GRK-independent mechanisms of CMKLR1 regulation and provide evidence of underlying signaling bias that is known to exist with many GPCR (Rajagopal et al., 2010; Pierce and Lefkowitz, 2001; Zidar et al., 2009).

Our results show that FLS cells express CMKLR1, GRK2, GRK5, and GRK6, but very low levels of GRK3 by comparison (Fig. 1). Although some trends are observed in the differential expression of CMKLR1, GRK2 and GRK5 in FLS from arthritic patients, the small sample size limited statistical comparisons. Previous studies have observed decreased expression of GRK2 and GRK6 in hematopoietic cells of patients with active RA, suggesting these two kinases could be relevant in GPCR

function specifically in inflammatory arthritis (Lombardi et al., 1999, 2001), but further investigation is needed to determine the roles of GRK regulation of CMKLR1 in these resident fibroblast-like synoviocytes. While data in arthritis models suggest a pro-inflammatory role for chemerin in joint synovium (Wittamer et al., 2003; Huang et al., 2012; Berg et al., 2010; Kaneko et al., 2011), a lung disease model has shown an anti-inflammatory role for chemerin using CMKLR1 knockout mice (Luangsay et al., 2009; Bondue et al., 2011). Therefore, understanding the regulatory mechanisms of CMKLR1 signaling could help characterize the role of the chemerin/CMKLR1 axis in inflammatory disease states.

GRK6^{-/-} mice in a K/BxN model of acute RA showed an increase in disease severity compared to wild type mice, further implicating the role of GRK6 in migration and inflammation (Tarrant et al., 2008). Our observations using real-time fluorophore-labeled cell migration paralleled these findings in that we found absence of β -arrestin 2 or GRK6 caused enhanced migration of pro-inflammatory macrophages to chemerin (Fig. 4), thereby supporting a regulatory role of these two proteins in normal CMKLR1 desensitization and chemotaxis. In addition to enhanced chemotaxis of Barr2^{-/-} cells to chemerin, we observed significant migration to medium alone in these cells ($p < 0.0001$), which is possibly the result of chemokinesis that has been described previously in migration assays utilizing β -arrestin 2 deficient cells (Fong et al., 2002; Sharma et al., 2013).

To explore if the functional phenotypes observed were the result of changes in downstream signaling, we examined the MAPK and AKT signaling cascades. Previous results by the Lefkowitz group have shown differential effects of GRK knockdown on receptor internalization and that ERK1/2 phosphorylation are GRK- and receptor-dependent (Zidar et al., 2009; Kim et al., 2005; Ren et al., 2005). Based on our own

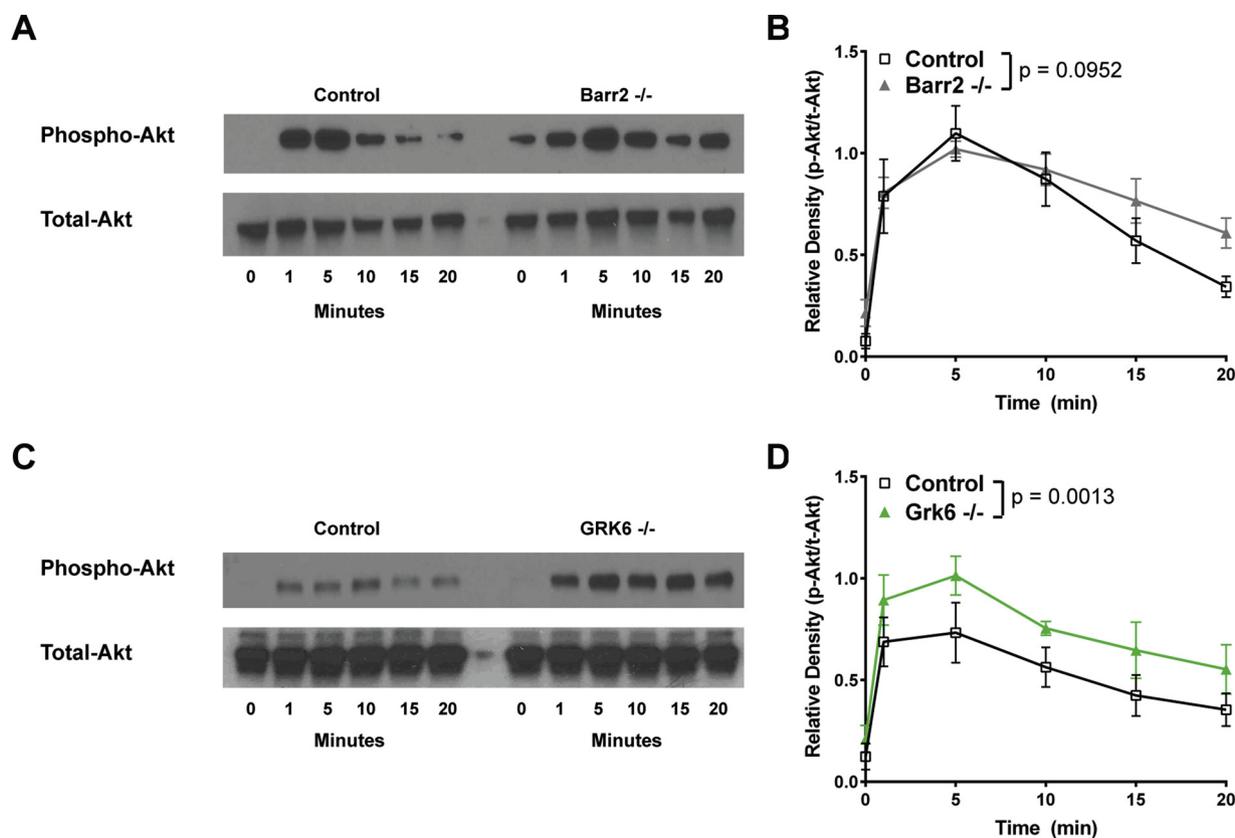


Fig. 6. GRK6^{-/-} pro-inflammatory macrophages show enhanced AKT phosphorylation after activation by chemerin. Control versus Barr2^{-/-} (A, B) or GRK6^{-/-} (C, D), C57BL/6 J peritoneal macrophages were either left unstimulated (0 min) or stimulated with 6.25 nM chemerin *ex vivo* for 1, 5, 10, 15, or 20 min. Total protein was normalized using a BCA assay. Western blot analysis was performed on cell lysates probed using Phospho-AKT (Ser473) and Total-AKT. Representative blots (A, C) of 4 independent experiments are shown. Quantification shown in (B, D) was done by densitometry using ImageJ software and is shown as a mean ratio of relative density \pm SEM (n = 4).

previously published results (Tarrant et al., 2013), we hypothesized that cells lacking either β -arrestin 2 or GRK6 would exhibit prolonged signaling due to sustained CMKLR1 surface expression and signaling after stimulation when compared to wild type control cells. Indeed, Barr2^{-/-} pro-inflammatory macrophages exhibited enhanced signaling of ERK1/2 compared to control cells with maximum ERK1/2 phosphorylation at 5 min (Fig. 5), similar to other published reports (Zhou et al., 2014). β -arrestin 2 is known to directly transduce signals through the ERK1/2 pathway following receptor ligation as part of a bifurcated GPCR signaling pathway (Shenoy et al., 2006); therefore, it is possible that the increased ERK1/2 signaling in Barr2^{-/-} cells is due to an indirect mechanism predicated on delayed CMKLR1 internalization in these deficient cells. Chemerin stimulation increased AKT signals in GRK6^{-/-} which could also be explained by the delayed internalization of CMKLR1 seen in these cells (Fig. 3A) or by another, unknown mechanism that impacts alternate signaling cascades. Taken together, these data suggest non-redundant or partially-overlapping roles for Barr2 and GRK6 in regulating chemerin/CMKLR1 signals to the ERK1/2 and AKT pathways. Our findings are consistent with results previously published by others (Luo et al., 2008) and recent work by Grundmann, et al. that elegantly dissect the dependence of arrestin-mediated ERK signaling on G protein function (Grundmann et al., 2018). Their study also highlights the importance of considering whether or not *absolute absence* of expression (“zero functional”) is achieved when interpreting results. While the macrophages used in our experiments are Grk6^{-/-} and Barr2^{-/-} genetic knockouts, we were unable to directly test the potential confounding effects of other Grk or arrestin isoforms that might be present in these cells.

In conclusion, this present study provides evidence that GRK6 and β -arrestin 2 are involved in desensitization, internalization, and

migration of primary inflammatory macrophages, which are relevant to RA pathogenesis. These functional outcomes are similar in Barr2^{-/-} and GRK6^{-/-} macrophages, but appear to be based on unique signaling characteristics. This work provides a foundation to better define the role of CMKLR1 in inflammatory arthritis, as well as highlights potential therapeutic targets for treatment within the chemerin/CMKLR1 signaling axis.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions

DSS performed and analyzed the experiments, prepared the figures, and wrote the paper. BA performed and analyzed experiments in Figs. 5 and 6. MFS provided technical assistance for Fig. 2. RGT provided technical assistance and performed experiments in Fig. 1. DM provided technical assistance and performed experiments in Fig. 2. JMB reviewed the manuscript. DPS reviewed the manuscript and provided technical assistance with the Tango assay. DE and YKT analyzed the experiments. TKT and MJB conceived and coordinated the study, analyzed results, contributed to the preparation of the figures, and wrote the paper. All authors reviewed the results and approve the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.molimm.2018.12.016>.

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