Ligand Bias by the Endogenous Agonists of CCR7

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
Immunology in the Graduate School
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ABSTRACT

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Abstract

Chemokine receptors are members of the seven transmembrane receptor (7TMR) superfamily and are regulated by the G-protein coupled Receptor Kinase (GRK)/β-arrestin system. CCL19 and CCL21 are endogenous agonists for the chemokine receptor CCR7. They are known to be equipotent in promoting Gi/o mediated calcium mobilization, chemotaxis and inhibition of adenylyl cyclase activity. Here we test the hypothesis that, relative to CCL19, CCL21 is a biased agonist for CCR7 that differentially activates the G-protein coupled Receptor Kinase (GRK)/β-arrestin system.

In order to test whether these ligands have distinct activity, murine T lymphocytes were used to compare the effects of CCL19 and CCL21 activation of CCR7 at endogenous expression levels. While each ligand stimulates similar chemotactic responses, we also find that CCR7 ligands lead to differential signaling. For instance, CCL19 is markedly more efficacious than CCL21 for the activation of ERK and JNK, but not AKT in these cells. Furthermore, ERK activation and chemotaxis are maintained as separate pathways, also distinguishable by their dependency upon PKC and PI3 kinase, respectively. Thus, CCL19 and CCL21 stimulate PI3 kinase, AKT, and chemotaxis equally, but differentially activate MAP kinase in murine T lymphocytes.

To determine the mechanism of CCR7 ligand bias, we used HEK-293 cells expressing CCR7 to compare the proximate signaling events following CCL19 and
CCL21 activation. We found striking differences in the activation of the GRK/β-arrestin system. CCL19 leads to robust CCR7 phosphorylation and β-arrestin2 recruitment catalyzed by both GRK3 and GRK6. CCL21 activates GRK6 alone. This differential GRK activation leads to distinct functional consequences. Only CCL19 leads to the recruitment of β-arrestin2-GFP into endocytic vesicles and classical receptor desensitization. In contrast, each agonist is fully capable of signaling to MAP kinase through β-arrestin2 in a GRK6 dependent fashion.

Therefore, CCR7 and its ligands represent a natural example of ligand bias whose mechanism involves differential GRK isoform utilization by CCL19 and CCL21 despite similar G-protein signaling. This study suggests that the GRK signatures of 7TMRs can determine the function of discrete pools of β-arrestin and thus guide its cellular effects.
Dedication

This thesis is dedicated to those who have so patiently supported me through the years. I thank my wife, Jennifer, for her enthusiasm, loyalty, unending love, humor, and encouragement. I thank my dad, Bernard Luke Zidar, the consummate “gentleman and scholar”, whose example as a family man, sportsman, and “hairy cell” aficionado I will continue to emulate. I thank my mom, Susan Zidar, for creating a home in which learning was made enjoyable, procrastination was acceptable (even when it led to her “proof reading” (AKA: typing) till dawn), and whose calming influence made our house a home and family my most treasured asset. My sibs… Lizzzzzzo and Andy, best man Bernie, little sister Kate… thanks for keeping me on my tippy toes. Okay, as a certain patriarch of ours would say, hope this “Blows ‘em Away”.
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Chapter 1. Introduction and Background

1.1 General Principles of 7 Transmembrane Receptor Signaling

1.1.1 G-protein Signaling

Traditional agonists of seven-transmembrane receptors (7TMRs) lead to the simultaneous activation of G-protein and β-arrestin dependent signaling pathways through a series of broadly conserved biochemical steps [1, 2]. Agonist binding to 7TMRs stabilize an “active” conformation of the receptor, capable of coupling to G-proteins. This interaction allows the exchange of GDP for GTP by the α subunit of G-proteins which results in the dissociation of the heterotrimeric complex into free Gα and Gβγ subunits. Dissociated G-protein subunits then lead to the activation or inhibition of numerous second messenger signaling programs. For instance, G-proteins of the Gαq subfamily activate phospholipase C, Gαs activate adenylate cyclase leading to increases in intracellular cAMP levels, and Gαi subunits inhibit adenylate cyclase to decrease cAMP levels.
1.1.2 The GRK/β-arrestin system

In addition to G protein activation, agonist activated 7TMRs also undergo phosphorylation by G-protein coupled receptor kinases (GRKs). The GRKs comprise a family of seven homologous serine/threonine kinases[3, 4] unique in their ability to specifically recognize and phosphorylate activated 7TMRs. The earliest GRKs discovered were rhodopsin kinase (GRK1)[5] and the β-adrenergic receptor kinase (β-ARK, GRK2)[6], using functional assays and purified receptors. The subsequent development of homology cloning approaches focusing on the conserved catalytic domain ultimately led to the discovery of the rest of the GRK family (GRKs 3-7)[7-10]. GRK2, GRK3, GRK5, and GRK6 are ubiquitously expressed, with GRK1 and GRK7 expression restricted to the eye, and GRK4 expressed primarily in the testes and peripheral nerves. Several have been shown to phosphorylate adrenergic receptors in an agonist dependent fashion[7-11].

GRKs can be further classified into GRK2 (GRK2 and GRK3) and GRK4 (GRK4, GRK5, GRK6) subfamilies based upon similarities in structure and mechanism of recruitment/membrane association. GRK2 and GRK3 contain pleckstrin homology (PH) domains that can interact with phosphatidylinositols of the cell membrane as well as free G_{βγ} subunits thus promoting their recruitment from the cytoplasm to the activated receptor [4, 12-14]. The GRK4 subfamily does not contain PH domains and their mode of recruitment is less conserved. For example, GRK 5 contains a small phospholipid
binding domain that is thought to promote constitutive plasma membrane localization, whereas for GRKs 4 and 6 cysteine palmitoylation, serves this purpose [15].

Canonically speaking the GRK mediated phosphorylation increases the affinity of the activated receptor for members of a small family of the multifunctional adaptor proteins termed arrestins[16]. There are four known arrestin isoforms, with expression of arrestins 1 and 4 being restricted to the eye, and arrestins 2 and 3, being expressed throughout the body. These ubiquitous arrestins are termed β-arrestin1 (arrestin 2) and β-arrestin2 (arrestin 3). The arrestins were discovered for their ability to desensitize rhodopsin and β-adrenergic receptors from further G-protein activation. As a result of their all but ubiquitous expression, β-arrestin1 and β-arrestin2 serve as proximate multifunctional regulators of 7TMRs. One consequence of β-arrestin binding is to promote agonist induced, homologous desensitization. Desensitization is accomplished via several mechanisms including direct steric hinderance of the receptor by β-arrestin attachment[17], recruitment of 2\textsuperscript{nd} messenger degrading enzymes[18-20], and by acting as a scaffold for proteins that facilitate receptor internalization[16, 21-23].

In addition to their role in regulating receptor stimulated G protein activation, the β-arrestins also initiate signaling programs through coordinated recruitment of signaling intermediates to the activated receptor[1, 2, 24, 25]. Proteomic studies have shown that β-arrestins interact with over a hundred binding partners[26]. β-arrestin dependent MAP kinase activation has been studied extensively as a model for β-
arrestin dependent signaling. For example, β-arrestin dependent ERK activation for the angiotensin 1A receptor (AT1aR) is fundamentally distinct from G-protein mediated ERK, with different kinetics, spatial distribution, and function[27-30]. β-arrestin dependent signaling has been similarly observed for other receptors and appears to be a general feature of 7TMRs[1, 25, 28, 31-38].

1.2 Biased Agonism

The concept of a singular “activated” receptor has been fundamentally altered in recent years due to biophysical and pharmacologic evidence that agonists and receptors oscillate among multiple “active” conformations and the recognition that this may lead to differential signaling[39-43]. Thus, signaling cascades that emerge from receptors activated by traditional agonists may be initiated in parallel by distinct receptor conformations. Further, these distinct receptor conformations and resultant downstream signals can be promoted or stabilized by different ligands. The term ligand bias refers to the observation that “biased agonists” activate some signals maximally and others submaximally[44, 45]. Unlike partial agonists, biased ligands may alter the equilibrium between “active” conformations and thus lead to differential signaling in a non-uniform fashion.
1.3 “β-arrestin Biased” Ligands

Recently, biased ligands have been discovered that differentiate G-protein pathways from GRK and β-arrestin signaling. These “β-arrestin biased” agonists include various synthetic agonist analogues that recruit β-arrestin but do not lead to activation of G-proteins. Ligands with this property have now been described for the AT1 angiotensin receptor (AT1aR) [28, 46], the β2-adrenergic receptor (β2AR)[47, 48], and the parathyroid hormone (PTH) receptor [38]. Each causes receptor phosphorylation, β-arrestin recruitment and β-arrestin mediated signaling in the absence of G-protein activation for their respective receptor. Thus, these studies suggest that the “activated” form of the receptor that leads to G-protein signaling may in fact be distinct from the conformation that undergoes phosphorylation and β-arrestin binding.

Ligand bias has offered important insights into the nature of β-arrestin dependent signaling since it provides an experimental system with which G-protein and β-arrestin mediated signaling can be isolated from one another. Thus, the use of biased agonists has become a useful tool for the study of the functional consequences of the GRK/β-arrestin system. However, it should be noted that, in these examples of biased agonism, determining the full functional spectrum of β-arrestin is limited due to the lack of G-protein signaling. For example, while various biased agonists lead to β-arrestin dependent MAP kinase, it is unclear if this β-arrestin is also able to cause homologous desensitization since G-protein signaling is absent.
1.4 GRK Specificity and Redundancy

Studies using purified proteins, overexpressed receptors, and inhibitors generally suggest that GRKs are able to interact broadly with 7TMRs. As such, GRKs do not bind to strict substrate consensus sequences, but rather have general preferences for acidic (GRK2) or basic (GRK5/6) residues at sites adjacent to phosphorylation. Yet, the degree of GRK specificity for activated receptors under physiologic conditions is largely unknown. Recently, siRNA has been used to knock-down the expression of individual GRK isoforms in cultured cells to further address the question of substrate specificity in live cells. These studies suggest that each of the ubiquitously expressed GRKs contribute at least in part to the phosphorylation of the β2AR and AT1a[49, 50]. Thus, receptors are generally able to utilize each of available GRKs for receptor phosphorylation. Yet, recent studies also seem to suggest that GRK isoforms may not be redundant for β-arrestin mediated processes such as signaling to MAP kinase. For instance, GRK5 and GRK6 appear to be uniquely required for β-arrestin mediated ERK activation via the AT1a and V2 vasopressin receptors while GRK2 and GRK3 appose this [36, 51].

Thus, to the extent that our understanding of the complex roles of β-arrestin has broadened, the traditional view of GRKs as redundant kinases is likely to be overly simplistic. Additional experimental approaches are therefore needed to better
understand the role of individual GRKs in promoting/modulating β-arrestin mediated functions.

### 1.5 Chemokine Receptors

#### 1.5.1 General Form and Function

Chemokine receptors are a family of homologous 7TMRs that function to cause chemotaxis, directed migration up a concentration gradient of ligand. Many receptors within this family have multiple ligands, with over forty seven chemokines currently recognized[52, 53]. Although sequence homology is variable among chemokines, they have a conserved three dimensional structure with the N-terminus believed to be particularly important for receptor activation[54-56]. By virtue of tight regulation of chemokine expression by tissues and unique receptor profiles on leukocytes, this receptor-ligand system aids in the selective recruitment of leukocytes from the circulation and spatial organization of complex immune responses.

Despite the complexity of this receptor/ligand system, and unlike that of the adrenergic system, the proximate signaling events as well as the overall cellular function of these receptors are similar. Chemokine receptors generally, if not exclusively, activate Gαi subfamily G-proteins, leading to decreased cAMP production, and promote cell migration[57-59]. Additionally, free Gβγ subunits lead to phospholipid mediated
signals including PLC and PI3 kinase activation. However, important variability does exist across receptors and cell types as to how these early proximate signals translate to migration/activity of a given cell.

Chemokine receptors are also known to be regulated by the GRK/β-arrestin system in a variety of experimental systems [60-63], but general principles regarding the role of the GRK/β-arrestin system for this family of 7TMRs have yet to emerge. For example, CXCR4 chemotaxis is positively regulated by GRK6 and β-arrestin2 in murine T lymphocytes[60]. In contrast, LTB4 mediated chemotaxis in the neutrophils of GRK6 null mice is enhanced[62]. Thus, a better understanding of the mechanism of GRK/β-arrestin system involvement in chemotaxis is required.

1.5.2 CCR7

The chemokine receptor CCR7 is particularly important for immune responses requiring the coordinated interaction of various cell types within lymphoid tissues[64-71]. For instance, CCR7 is highly expressed on naïve, mature T cells, and is vital for homeostatic recirculation of these cells through secondary lymphoid tissues[72]. In contrast, the expression of CCR7 is induced on dendritic cells (DCs) in response to inflammatory signals. The migration of DCs via CCR7 into T cell zones allows them to present antigen to nearby T cells[73-75]. After T lymphocyte activation, CCR7 is then down-regulated on T cells in conjunction with migration out of T cell zone and toward
adjacent B cell zone. Thus, tight regulation of CCR7 expression and signaling allows T cells to interact effectively with both DCs and B cells during an immune response[76]. This interactions are required for germinal center formation and all T cell dependent humoral responses. Consistent with this, mice lacking CCR7 have abnormal lymph node and splenic architecture as a result of impaired migration of DCs and T cells[65]. The delayed immune responses[77], impaired tolerance[78], and autoimmunity[79, 80] observed in these mice are consistent with the involvement of leukocyte trafficking via CCR7 in a wide variety of immunological processes.

1.5.3 CCR7 Ligands

CCR7 has two endogenous ligands, CCL19 and CCL21, which have evolved from a series of complex gene duplications and retain 32% amino acid sequence homology[81-83]. They are predicted to be structurally similar except that CCL21 has a conserved motif consisting of an additional 37 amino acids at its carboxyl-terminus[84]. This motif has a predominance of highly basic residues which mediates binding to glycosaminoglycans and is also required for triggering integrin activation[85, 86]. Each ligand is highly expressed in lymph node, spleen, and Peyer’s patch[87], but differential localization of these ligands within these organs has also been observed[88-90]. Although individual CCL19 or CCL21 knockout mice have not been described, a mutant strain, termed paucity of lymphoid tissue or plt, lacks expression of both CCL19 and
CCL21 in lymphoid tissue [91, 92]. Similar to the CCR7 null mouse, the plt phenotype includes defective T cell and DC trafficking and underscores the importance of CCR7 and its ligands for trafficking of various immune subsets and related immune responses[93].

CCL19 and CCL21 have similar binding affinities (Kd~100pM), equal efficacy for G-protein activation, calcium flux, and chemotactic responses in HEK, T cells and DCs [81-83, 94-96]. Each is also capable of promoting antigen activated cell death of T cells [97]. Despite the similarities between CCL19 and CCL21, some signaling characteristics appear to be unique to one or the other chemokine. For instance, CCL21 but not CCL19 induces activation of β2 integrins [88, 89]. In murine DCs, rapid dendrite formation occurs in response to CCL19 but not CCL21[98]. In human T lymphocytes, CCL19 but not CCL21 leads to CCR7 internalization despite equivalent calcium flux and chemotactic responses[99]. CCL19 but not CCL21 has also been shown to lead to receptor desensitization[81], β-arrestin recruitment[100], internalization [99, 101] and receptor degradation[102] in a number of cell types. The mechanism of these differences and the role of GRK function for CCR7 signaling has not been previously reported.

1.6 Central Aim

CCL19 and CCL21 are endogenous ligands for CCR7 and are generally believed to have overlapping functions, each leading to chemotaxis with similar efficacy and
potency. This work seeks to compare the ability of these ligands to activate unique signaling programs, and to determine whether these agonists differentially activate the GRK/β-arrestin system. The hypothesis is that these ligands are in fact biased agonists with similar ability to promote signaling via G-proteins, but with differential activity for CCR7 phosphorylation and β-arrestin recruitment. **The purpose of this thesis is to:** (1) Determine whether CCL19 and CCL21 lead to differential signaling in murine T lymphocytes, and (2) Determine whether CCL19 and CCL21 lead to differential activation of the GRK/β-arrestin system by CCR7.
Chapter 2. MATERIALS AND METHODS

2.1 Materials

Human CCL19 and CCL21 was purchased from R and D systems (Minneapolis, MN). \(^{[^{125}}]^{5}\) CCL19 was obtained from PerkinElmer Life Sciences (Waltham, MA).

Minimum essential Eagle's medium, 10% (v/v) fetal bovine serum, 1% (v/v) penicillin/streptomycin, G418, M2 anti-FLAG beads, and forskolin were from Sigma (St. Louis, MO). Pertussis toxin was purchased from List Biological Laboratories (Campbell, CA). 24-well Costar chemotaxis transwells (6.5-mm diameter, 5-μm pore size) were from Corning (Corning, NY). Anti-CD3 FITC and anti-CD4 PE were from BD biosciences (San Jose, CA). Anti-phosphoMAPK p42/44 antibody was purchased from Cell Signaling Technology (Danvers, MA). Anti-MAPK p44 antibody was from Upstate (Lake Placid, NY). Inhibitors of phosphatases (Microcystin-LF), PI3 kinase (LY94002, 10uM), PKC (GF 109203X, 2uM), and Rho kinase (Y-27632, 5uM) were from Calbiochem. Complete EDTA-free protease inhibitor mixture tablet were from Roche Applied Science. 4-20% Tris-glycineSDS-polyacrylamide gels were from Invitrogen.
2.2 Plasmids and Cell Culture

CCR7-Flag has been previously described[100]. $\beta$-arrestin2-mYFP has been previously described[49]. CCR7-mCFP was generated by PCR amplification of human CCR7 containing a diglycine linker in place of the stop codon, flanked by HindIII and XhoI restriction sites. This product was then subcloned into the pcDNA3.1-mCFP vector [103]. CCR7-pTRE2-hyg was generated by PCR amplification of human CCR7, flanked by BamHI and SalI sites. This product was then subcloned into pTRE2-hyg (Clontech). All plasmids were amplified in bacteria, kit purified (Qiagen), and validated by capillary electrophoresis sequencing.

2.3 Cell Culture

HEK-293 cells were purchased from ATCC and maintained in minimum essential medium (Sigma) plus 10% (v/v) fetal bovine serum (Sigma) and 1% (v/v) penicillin/streptomycin (Sigma). Cells were transfected with FuGENE 6 (Roche Applied Science) according to manufacture’s instructions. Where applicable, G418 (500 μg/ml; Sigma), hygromycin (250 μg/ml), or zeocin (300 μg/ml; Invitrogen) were added for selection and maintenance of stable clones.
2.4 siRNA Transfection

Chemically synthesized double-stranded siRNA duplexes (with 3’ dTdT overhangs) were chemically synthesized and purchased from Dharmacon (Lafayette, CO) or Xeragon (Germantown, MD) as described [27, 36, 51]. The siRNA sequences used in this study have been validated elsewhere [27, 31, 36, 38] and were as follows: GRK2: 5′-AAGAAGUACGAGAAGCUGGAG-3′ (NM_001619, position 268–288), GRK3: 5′-AAGCAAGCUGUAGACGUA-3′ (X69117, position 376–396), GRK 5: 5′-AAGCCGUGCAAAGAACUCUUU-3′ (NM_005308, position 406–426), GRK6: 5′-AACAGUAGGUUUGUAGUGAGC-3′ (AF040751, position 724–744), β-arrestin 1: 5′-AAAGCCUUCUGCGCGGAGAAU-3′ (NM_020251, position 439–459) β-arrestin 2: 5′-AAGGACCGAAAGUGUUUGUG-3′ (NM_004313, position 201–221), and simultaneous β-arrestin 1 and β-arrestin 2: 5′-ACCUGCGCCUUCGCCUAUG-3′ (positions 172–190 and 175–193, respectively). Indicated position numbers are relative to the start codon. A nonsilencing RNA duplex (5′-AAUUCUCCGAACGUGUCACGU-3′), as the manufacturer indicated, was used as a control. GeneSilencer was from Gene Therapy Systems (San Diego, CA).
2.5 CCR7 Phosphorylation

HEK293 cells stably expressing CCR7-FLAG were grown overnight on poly-D lysine coated plates, then serum-starved in phosphate-free DMEM for 1 h. Cells were then labeled with 200 μCi/ml $^{32}$P$_i$ (PerkinElmer Life Sciences) for 1 h. After 45 minutes, microcystin-LF (100 nM) was added. Cells were moved to a hot plate at 37 °C and stimulated with the appropriate ligand for 10 min, or left untreated. Cells were washed twice with ice cold PBS, and cell extracts were prepared by lysing cells in 500 μl of glycerol lysis buffer (50 mM HEPES, pH 7.4, 0.5% Nonidet P-40, 250 mM NaCl, 2 mM EDTA, 10% glycerol, 100 nm microcystin-LF, and protease inhibitors. The samples were clarified by centrifugation and protein concentrations were determined using the Bradford Assay reagent (Bio-Rad). When done in conjunction with GRK siRNA, radiolabelled ligand binding was also performed in parallel to allow equal amounts of surface receptor to be added for each immunoprecipitation. Equal numbers of receptor were added to 35 μl of M2 anti-FLAG-agarose beads for immunoprecipitation overnight at 4 °C with constant rotation. Immunoprecipitates were washed five times with glycerol buffer and eluted with 50 μl of 2x SDS sample buffer. Equal volumes of the immunoprecipitates were resolved by SDS-PAGE, dried, and $^{32}$P$_i$ incorporation was measured using a phosphorimaging device (Bio-Rad). Agonist-induced phosphorylation responses were quantified as the difference between stimulated and unstimulated samples, expressed as a percentage of CCL19 responses. When done in
conjunction with GRK siRNA, radiolabelled ligand binding was performed in parallel to allow equal amounts of surface receptor to be added for each immunoprecipitation.

### 2.6 ICUE2 cAMP Assay

The cAMP biosensor ICUE2 has been previously described[50, 104]. HEK-293 cells stably expressing CCR7-pTRE2-hyg and ICUE2 were stimulated with 50uM forskolin in the presence or absence of 100nM CCL19 or 100nM CCL21. cAMP accumulation was measured as the change in ICUE2 FRET ratio.

### 2.7 CCR7-β-arrestin FRET

HEK cells were transfected sequentially with β-arrestin2-YFP and CCR7-CFP. The measurement of β-arrestin2-YFP recruitment by FRET was done as previously described[49]. Cells were grown overnight, washed once, placed in imaging buffer (125 mM NaCl, 5 mM KCl, 1.5 mM MgCl2, 1.5 mM CaCl2, 10 mM glucose, 0.2% bovine serum albumin, 10 mM HEPES, pH 7.4), and imaged in the dark on a stage heated to 37 °C. Images were acquired on a Zeiss Axiovert 200M microscope (Carl Zeiss MicroImaging, Inc.) with a Roper Micromax cooled charge-coupled device camera (Photometrics) controlled by SlideBook 4.0 (Intelligent Imaging Innovations). All graphs display calculations based on intensity from whole cells or sets of cells.
2.8 **MAP Kinase Activation**

HEK cells stably expressing CCR7-pTRE2-hyg were grown at low confluence and split onto poly-D lysine coated 24 well plates. Cells were serum-starved for 2 h and stimulated with 100nM CCL19 and 100nM CCL21 for 5 min at 37 °C. Cells were washed once with PBS and harvested directly into 4x SDS sample buffer. Cell extracts were sonicated, heated at 55 °C for 5 min, and subjected to SDS-PAGE. Resolved proteins were transferred onto nitrocellulose membranes. The activated phosphorylated form of ERK1/2 was detected using an anti-phosphoMAPK p42/44 diluted at 1:1000 in 4% BSA in TBST. Total ERK1/2 was detected with the anti-ERK2 antibody at 1:3000 in 4% BSA in TBST. Chemiluminescent detection was performed with SuperSignal West Pico reagent (Pierce).

2.9 **β-arrestin translocation assay**

HEK cells were co-transfected with CCR7-Flag and β-arrestin2-GFP at a ratio of 5:1 using FuGENE 6 (Roche Applied Science) according to manufacture’s instructions. Cells were split and grown on dishes containing glass coverslips. Cells were stimulated
with 100nM CCL19 or 100nM CCL21 for 60 minutes and imaged by confocal microscopy as previously described[105].

2.10 CCR7 Expression

HEK cells were plated in 24 well, poly-D lysine coated plates (CoStar) and grown overnight. Cells were then washed three times with ice cold serum free media (MEM with 0.1% BSA). Cells were incubated at 4 degrees for 1 hour with 0.1nM I^{125}\text{-}-CCL19 (Perkin Elmer) in the presence or absence of 100nM CCL19. Cells with then washed three times with ice cold media. Cells were lysed with 0.5M NaOH/0.05% SDS and the solublized cells were transferred into 12 x 75mm tubes and the radioactivity measured in a gamma counter.

2.11 Chemotaxis assay

Chemotaxis assays were performed as previously described[60]. Splenic lymphocytes were isolated from male C57BL/6 mice at 8 weeks of age and homogenized in RPMI medium 1640 containing 10 mM Hepes (GIBCO/BRL). Cells were washed with RBC lysis buffer (0.14M NH4Cl/0.017M Tris), pH 7.2, and then resuspended in RPMI containing 10% FBS. Transwell chemotaxis assays were performed in 24-well transwells (6.5-mm diameter, 5-μm pore size, Costar) according to manufacturer’s instructions.
Cells were stained with fluorescent Abs for cell surface markers using anti-CD3 FITC and anti-CD4 PE and cell counts determined by flow cytometry with an EPICS XL flow cytometer (Beckman Coulter). All chemotaxis assays were performed in duplicate. The number of migrated cells as a percentage of total cells added was calculated and graphed as a dose response for each ligand.
Chapter 3. Differential Signaling by CCR7 ligands in T Lymphocytes.

3.1 Rationale and Hypothesis

Chemokine receptors are a remarkably homologous family of 7MSRs. The proximate steps in the signaling of these receptors appear to be well conserved. Activated receptors interact with pertussis toxin sensitive G-proteins (Gαi/0). Gαi subunits bind adenylate cyclase to inhibit cAMP production while Gβγ subunits are capable of activating various downstream effectors including PLC and PI3 kinase.

We sought to compare CCL19 and CCL21 signaling in T lymphocytes to determine whether differential signaling exists and if so how these differential signals relate mechanistically to the overlapping aspects of CCR7 signaling. Our hypothesis was that, CCL19 and CCL21 are likely to lead to overlapping effects for some pathways such as chemotaxis, but that unique signaling pathways for each of these ligands might also be apparent, particularly for signaling programs that do not directly lead to chemotaxis.
3.2 Experimental System

CCR7 is endogenously expressed on naive T lymphocytes. Thus we used purified, splenic T cells from mice in cellular assays to assess the effects of CCL19 and CCL21 on chemotaxis and activation of P38, AKT, JNK, and ERK. C57BL/6 mice were maintained in pathogen free conditions and sacrificed after 2-3 months of age. Splenic T cells were isolated as described in the methods section. These cells were used for both chemotaxis and signaling assays. Inhibitors to canonical signaling pathways such as PI3 kinase and PKC as well as cells from β-arrestin2 -/- mice were also used to interrogate these pathways.

3.3 Results

3.3.1 Differential Signaling by CCL19 and CCL21 in Murine T Lymphocytes.

In order to compare the responsiveness of murine T lymphocytes to CCL19 and CCL21, chemotaxis and ERK activation were first determined for each ligand at various concentrations of agonist. Both CCL19 and CCL21 lead to robust chemotactic responses (Figure 1a). Importantly, chemotaxis to CCL19 was comparable to CCL21 across the dose response.

Next, T lymphocytes were stimulated with various concentrations of CCL19 and CCL21 for 2.5 minutes. The relative efficacy for CCR7 mediated ERK activation was
markedly greater for CCL19 than for CCL21 at all concentrations of agonist tested (Figure 1b).

Figure 1: CCL19 and CCL21 lead to similar chemotactic but differential ERK responses. (A) Murine splenocytes were washed added to the top chamber of modified Boyden chambers. Various concentrations of CCL19 and CCL21 were added to the bottom chamber. Cells were allowed to migrate for 90 minutes. Migration of CD3+/CD4+ cells is shown as a function of CCL19 and CCL21 concentration. (B) T lymphocytes were isolated from splenocytes by negative selection, washed with RPMI with 0.1% BSA and serum starved for 16 hours. Cells were then stimulated with various concentrations of CCL19 and CCL21 for 2.5 minutes. Lysates were sonicated and subjected to SDS-PAGE and immunoblotted for total and phospho-ERK. The graph represents the mean +/- SE of the dose dependent responses of CCL19 versus CCL21 for phospho-ERK/total ERK.

Next, a time course of CCL19 versus CCL21 stimulated intracellular signaling was examined. T cells were isolated by negative selection from splenocytes and stimulated with saturating concentrations (100nM) of CCL19 and CCL21 for 2.5, 5, 10, 20
and 40 minutes. CCL19 and CCL21 stimulated comparable levels of phospho-AKT (Figure 2). In contrast, phospho-ERK and phospho-JNK were preferentially activated by CCL19, with activation of phospho-ERK and phospho-JNK by CCL21 only 30% and 50% of CCL19, respectively.

Figure 2: Differential signaling after CCL19 and CCL21 stimulation in murine T Lymphocytes. Murine splenocytes were serum starved and stimulated with saturating concentrations (100nM) of CCL19 and CCL21 for 2.5, 5, 10, 20 and 40 minutes. The time course of AKT, JNK, and ERK activation after CCL19 and
CCL21 is shown. The graph represents the mean +/- SE (n=3-6) of the ligand dependent responses CCL19 and CCL21 as a percentage of maximal stimulation.

These results indicate that CCL19 and CCL21 lead to similar activation of some signaling pathways (e.g. AKT, chemotaxis), but to differential activation of ERK and JNK. This is consistent with our hypothesis that CCL19 and CCL21 are biased ligands with similar responsiveness for chemotaxis but differential signaling to MAP kinases including ERK and JNK.

### 3.3.2 The effect of β-arrestin2 on CCR7 signaling

Next, we sought to determine the mechanism whereby differential signaling to ERK is maintained despite equivalent chemotaxis responses. We hypothesized that chemotaxis and ERK activation result from divergent signals that remain distinct. Given that CCL19 but not CCL21 has been shown to cause internalization of CCR7 in human T cells, we sought to test the possibility that β-arrestin2 might be differentially activated by these ligands, and thus account for the additional signaling to ERK observed with CCL19. Thus, we compared the activation of ERK, AKT, and chemotaxis in wild type versus β-arrestin2 null T cells.

Knockout of β-arrestin2 did not significantly affect chemotaxis or AKT activation (Figure 3a, 3b). In addition, the differential ERK activation between CCL19 and CCL21
was also not significantly altered in cells devoid of β-arrestin2 (Figure 3c). Thus, β-arrestin2 mediated signaling does not appear to account for the additional ERK activation in response to CCL19.

Figure 3: The differential signaling to ERK in T lymphocytes is not affected by the loss of β-arrestin2. (A) Murine splenocytes were isolated and added to the top of modified Boyden chambers. Various concentrations of CCL19 and CCL21 were added to the bottom well. Chemotaxis is displayed as the percentage of migrated CD4+ T cells relative to the number of these cells initially added. T lymphocytes were isolated from murine splenocytes by negative selection, serum starved for 16 hours in RPMI/Hepes, and stimulated with CCL19 and CCL21. Phospho-AKT (B) and phospho-ERK (C) levels were quantified as the ratio of activated to total protein. Data represent the mean +/- SE of three to six experiments.
3.3.3 The effect of PKC and PI3 kinase on chemotaxis.

Next, we examined the signaling requirements for CCR7 mediated chemotaxis, hypothesizing that intermediates that were not required for chemotaxis might be required for ERK activation, and vice versa. Thus, we used inhibitors of canonical chemotactic pathways to determine the upstream intermediates leading to CCR7 chemotaxis. Splenocytes were incubated with either DMSO control or inhibitors of PI3 kinase, PKC, or Rho kinase at recommended concentrations for 30 minutes prior to chemotaxis assays. Cell migration to 100nM CCL19 for 90 minutes was assessed and the effect of each inhibitor compared to control cells. We found that CCR7 chemotaxis was impaired by inhibition of PI3 kinase and Rho kinase, but not PKC (Figure 4).
Figure 4: CCR7 Chemotaxis is PI3 kinase and Rho kinase dependent, but PKC independent. Splenocytes were isolated, washed, and resuspended in RPMI with 10% FBS. DMSO (0.1%) or inhibitors of PI3 kinase (LY94002, 10uM), PKC (GF 109203X, 2uM), and Rho kinase (Y-27632, 5uM) were added for 30 minutes prior to chemotaxis assays. Cells were then added to the top of modified Boyden chambers and allowed to migrate to 100nM of CCL19 for 90 minutes. Cell counts were determined by flow cytometry after labeling with anti-CD3 and anti-CD4 antibodies. Chemotaxis is displayed as the percentage of migrated CD4+ T cells relative to the number of these cells initially added. The effect of each inhibitor is reported as a percentage of untreated cells. The graph represents the mean +/- SE of these responses. Statistical significance was determined by paired two-tailed t-tests (*, p<0.05; **).
3.3.4 Effect of PKC and PI3Kinase on CCR7 stimulated ERK & AKT

Because PKC was not required for chemotaxis, we next sought to determine if it was involved in ERK activation. T lymphocytes were isolated and incubated alone or with inhibitors to PKC or PI3 kinase. Cells were then stimulated with CCL19 (100nM) or CCL21 (100nM) and the activation of ERK and AKT assessed by SDS-PAGE and phospho-specific antibodies.

We found that inhibition of PKC was associated with a marked decrease in ERK activation (Figure 5) in response to CCL19. PKC inhibition also decreased CCL21 mediated ERK, but to a lesser extent, which was not surprising given the reduced capacity of this ligand to stimulate ERK. Overall, the remaining, “PKC insensitive” ERK between CCL19 and CCL21 was similar. In contrast, PI3 kinase inhibition did not affect signaling to ERK, despite having an expected effect on AKT phosphorylation. These data suggest that the additional ERK response to CCL19 versus CCL21 is mediated by PKC.
Figure 5: The Differential P-ERK activated by CCL19 and CCL21 is regulated by PKC but not PI3 kinase. T lymphocytes were isolated from murine splenocytes by negative selection and serum starved for 16 hours in RPMI with 0.1% BSA. Cells were pretreated with inhibitors of G_{i/o} subunits (pertussis toxin: 200ng/ml x 2 hours), protein kinase C (GF 109203X: 2uM x 30 minutes), or PI3 kinase (LY94002: 10uM x 30 minutes) and stimulated with CCL19 and CCL21 for 2.5 minutes. Phospho-ERK and phospho-AKT levels were determined by immunoblotting. ERK activation is shown as a percentage of the CCL19 mediated response in the absence of inhibitors. Data represent the mean +/- SE from three experiments. Statistical significance was determined by paired two-tailed t tests (*, p<0.05; **, p<0.01; ***p<0.001).
Thus, CCR7 activation leads to at least two distinct and separable signaling networks. First, CCR7 mediated AKT and chemotaxis are activated in a manner that is PKC independent, PI3 kinase dependent, and equally activated by CCL19 and CCL21. In contrast, ERK activation in T lymphocytes is dependent upon PKC activation, independent of PI3 kinase, and preferentially activated by CCL19 bound CCR7.

Therefore, this comparison of CCL19 and CCL21 mediated CCR7 signaling in murine T lymphocytes reveals similar chemotactic responses but differential signaling to ERK. Remarkably, CCL19 appears to be able to uniquely access a pathway to ERK that is PKC dependent, β-arrestin2 and PI3 kinase independent, and notably distinct from the chemotaxis signaling network. Thus, CCL19 and CCL21 appear to be biased agonists, leading to differential signaling to ERK and JNK in murine T lymphocytes.

**3.4 Discussion**

**3.4.1 Biased signaling by CCR7 ligands in Murine T cells**

Despite the published accounts that CCL19 and CCL21 represent redundant chemokines for CCR7, recent studies strongly suggest that CCL19 and CCL21 may have differential activities on the receptor[88, 89, 98],[81, 99-102]. Thus, we sought to study the comparative effects of each ligand in an endogenous, physiologic setting, paying close attention to potential differential signaling. To compare and contrast CCL19 and
CCL21 signaling in these murine T lymphocytes, we first determined the relative effect of each of these ligands on a variety of endpoints.

**Figure 6: Distinct Chemotactic and MAP Kinase Pathways as Revealed by CCR7 Ligand Bias.** Chemotaxis through CCR7 is PI3 kinase sensitive. Chemotaxis and AKT phosphorylation are equally stimulated by CCL19 and CCL21. In contrast, ERK activation is PI3 kinase independent but PKC dependent. CCL19 preferentially activates ERK in comparison to CCL21. Since this additional ERK is also PKC dependent, CCL19 may uniquely activate this PKC mediated ERK pathway whereas either ligand appears able to activate PKC independent ERK.

Consistent with previous studies [81-83, 94-96], we found that CCL19 and CCL21 have similar efficacy for chemotaxis. Each ligand led to similarly robust responses.
across the entire dose response. In contrast, we found that CCL19 and CCL21 also lead to differential effects including preferential activation of both ERK and JNK.

### 3.4.2 Differential CCR7 mediated ERK activation in T lymphocytes

We next sought to identify a mechanism to account for the observed differences in CCR7 mediated ERK activation. Since CCR7 ligands differentially promote internalization in human T cells[99] and differentially recruit β-arrestin2[20], a known MAP kinase scaffold for ERK kinases, we tested whether the additional CCL19 mediated ERK activation was β-arrestin2 dependent. Comparing cells from wild type and β-arrestin 2 null mice, we found that the loss of β-arrestin 2 did not affect this enhanced signaling to ERK.

We next used inhibitors of canonical pathways to compare and contrast the upstream requirements for ERK activation versus chemotaxis. We found that ERK was highly dependent upon PKC. In the presence of PKC inhibition, CCL19 mediated ERK was comparable to CCL21 mediated ERK. Thus, differential activation of this PKC dependent pathway appears to account for the differential ERK activation. In contrast, PKC insensitive ERK is not differentially activated by CCR7 ligands.
3.4.3 Distinct pathways for Chemotaxis and ERK activation in T lymphocytes

These data show that at least two distinct signaling cascades are activated by CCR7. One pathway proceeds from G protein activation through PI3 kinase and controls chemotaxis. This arm is similarly activated by both CCL19 and CCL21 and is PKC independent. In contrast, CCR7 activation of ERK proceeds via a mechanism independent of PI kinase, but requiring Gi/o proteins and PKC activation. Thus, CCR7 mediated chemotaxis and ERK activation are distinct by virtue of differential requirements for PI3 kinase and PKC, respectively, but also distinguished by the differential activation of the later but not the former by CCL19 and CCL21 (figure 6). While additional studies are needed to determine the proximate mechanism of this biased signaling, these results clearly demonstrate that strict specificity and fidelity of these pathways is maintained at more distal steps.
Chapter 4. Differential activation of the GRK/β-arrestin system by CCR7 Ligands.

4.1 Rationale and Hypothesis

Chemokine receptors often bind multiple ligands[55, 94, 106, 107]. Generally, this promiscuity is perceived to offer redundancy for leukocyte trafficking. This notion is also supported by common functional consequences of chemokine stimulation (i.e. chemotaxis) and conserved signaling via Gi/o proteins. Yet, relatively little is known about the entirety of signaling possibilities and the regulation of these receptors by the GRK/β-arrestin system.

CCR7 has two endogenous ligands that have been well studied and shown to have similar biochemical properties, expression, and potency for CCR7 mediated canonical signaling through Gi/o and chemotaxis. Yet, CCL19 but not CCL21 has been shown to induce internalization[99]. Thus, we sought to test the hypothesis that relative to CCL19, CCL21 is a biased agonist for CCR7. Specifically, we sought to show whether CCL21 is a “negatively” β-arrestin biased ligand, equipotent for G-protein signaling but incapable of activating β-arrestin, in contrast to CCL19, a typical reference agonist, equally capable of both G-protein and GRK/β-arrestin activation.
4.2 Experimental System

Given that the GRK/β-arrestin system involves both cytosolic and cell membrane bound proteins, we utilized whole cell assays. To maintain a common cellular context, we also sought to perform all relevant assays in a single cell type. Heterologous expression in HEK-293 cells has been a preferred experimental system in recent studies of 7TMRs due to ease of transfection that allows for the efficient use of siRNA technology [108]. They also lend themselves well to imaging and FRET based assays [49]. In general, the GRK/β-arrestin system has been studied in the context of the β2 adrenergic and AT1 angiotensin receptors [109]. Thus, we sought to study CCR7 using similar systems so as to allow us to comparison and contrast with these archetypal receptors. HEK-293 cells were transfected with CCR7 constructs and experiments were performed as detailed in the materials and methods section (see above).

4.3 Results

4.3.1 G_{i/o} protein signaling by CCL19 and CCL21

CCL19 and CCL21 have previously been shown to have equal binding affinities (Kd~100pM) and equivalent efficacy and potency for activation of the G_{i/o} subfamily of G-proteins [82, 94, 95]. In order to confirm this in live cells, we designed an assay to study G_{i/o} mediated inhibition of adenylate cyclase using a recently described cAMP biosensor
ICUE2[50, 104]. HEK cells stably co-expressing CCR7 and ICUE2 were treated with forskolin (50uM) in the absence or presence of CCL19 (100nM) or CCL21 (100nM).

Forskolin (50uM) alone produces a dramatic increase in intracellular cAMP levels evidenced by a decrease in ICUE2 FRET (binding of cAMP to the ICUE2 FRET biosensor produces a conformational change resulting in a decrease in FRET), whereas co-incubation of forskolin with either CCR7 ligand led to a blunted response (Figure 7). Specifically, the FRET response of forskolin (50uM) with CCL19 (100nM) was 51.1 +/- 8.8% of the integrated response of forskolin alone and with CCL21 (100nM) resulted in

Figure 7: Figure 1: εactivation and chemotaxis stimulated by CCL19 and CCL21. HEK-293 cells stably expressing CCR7 and the cAMP biosensor ICUE2 were stimulated with 50uM forskolin in the presence or absence of 100nM CCL19 or 100nM CCL21. cAMP accumulation was measured as the change in ICUE2 FRET ratio. Leftward and middle panels show the time course of responses for each ligand with stimulation occurring at 30 seconds. The bar graft compares the integrated responses. Data in both panels represent the mean +/- SE from 4 independent experiments.
49.4 +/- 9.9% of forskolin alone. There was no statistical difference between either
CCL19 or CCL21 stimulated inhibition of forskolin mediated cAMP responses.

4.3.2 Agonist Induced CCR7 Phosphorylation and β-arrestin2
recruitment.

The ability to undergo agonist-induced phosphorylation is a hallmark of 7TMRs.
Therefore, we analyzed the incorporation of $^{32}$P$_i$ by CCR7-Flag after 10 minutes of
stimulation by either CCL19 or CCL21. CCL19 (100nM) led to robust CCR7
phosphorylation (Figure 8a), whereas CCL21 (100nM) produced a much smaller but
consistent level of CCR7 phosphorylation (21.6 +/- 6.1% of that induced by CCL19)
(Figure 8a).
Figure 8: Differential CCR7 Phosphorylation and β-arrestin2 recruitment by CCL19 and CCL21. A) HEK-293 cells stably expressing CCR7-Flag were labeled with $^{32}$P and stimulated at 37 °C for 10 minutes with 100nM CCL19 or 100nM CCL21. CCR7-Flag was immunoprecipitated, separated by SDS-PAGE, and quantitated via phosphoimager analysis. A representative image showing $^{32}$Pi incorporation is shown in the top panel. The bottom panel shows a bar graph with agonist induced phosphorylation expressed as a percentage of CCL19 mediated phosphorylation. The bar graph represents the mean +/- SE from six independent experiments. Statistical significance was determined using paired two-tailed t tests (*, p<0.05; **, p<0.01; ***p<0.001)

B) Live HEK-293 cells stably expressing CCR7-CFP and β-arrestin2-YFP were stimulated with 100nM CCL19, 100nM CCL21, or 100nM CXCL12 and the recruitment of β-arrestin2 measured by FRET. The time course of FRET responses is shown after agonist was added at 30 seconds. The bar graph shows the integrated responses as a percentage of CCL19 mediated recruitment. Data represent the mean +/- SE from three independent experiments each done in duplicate. Statistical significance was determined by using paired two-tailed t tests (*, p<0.05; **, p<0.01; ***p<0.001)
Next, we sought to compare β-arrestin2 recruitment to CCR7 in response to CCL19 and CCL21. Live HEK cells stably expressing CCR7-CFP and β-arrestin2-YFP were stimulated with 100nM CCL19 or 100nM CCL21 and the recruitment of β-arrestin2 to the receptor was analyzed by fluorescence resonance energy transfer (FRET) (figure 8b). CCL19 promoted a significant interaction between the receptor and β-arrestin2 whereby comparison CCL21 had less of an effect.

4.3.3 The Effect of Pertussis Toxin on G-protein Activation and β-arrestin2 recruitment.

Pertussis toxin (PTX) ADP ribosylates the alpha subunit of Gi/o, and thereby inhibits chemotactic signaling in response to 7TMR stimulation[57]. Thus, we used PTX to determine if G-protein activation and β-arrestin recruitment are in fact distinct responses to receptor activation.
To confirm the expected activity of PTX in our live cell assay, we incubated HEK-293 cells stably expressing CCR7 and the cAMP biosensor ICUE2 with pertussis toxin (200ng/ml) for 2hrs, washed and stimulated with 50uM forskolin in the presence or absence of 100nM CCL19. CCR7 mediated inhibition of cAMP accumulation was found to be effectively blocked by PTX pre-treatment (figure 9a). Next, we examined whether...
β-arrestin recruitment was dependent on Gi/o protein activation. Live HEK-293 cells stably expressing CCR7-CFP and β-arrestin2-YFP were pre-incubated with PTX (200ng/ml) for 2hrs where indicated. Cells were then washed and stimulated with 100nM CCL19 or 100nM CCL21 and the recruitment of β-arrestin2 measured by FRET (figure 9b). In contrast to the effect of PTX on CCR7 stimulated decreases in forskolin mediated increases in cAMP, PTX had no effect affect on β-arrestin recruitment to the receptor in response to either ligand.

Taken together, these findings demonstrate that CCR7 activation leads to two distinct sets of receptor based signaling consequences. First, ligand binding of CCR7 leads to G-protein activation and for this activity CCL19 and CCL21 are comparable. Second, the agonist occupied receptor can act as a substrate for GRK phosphorylation and β-arrestin binding. Remarkably, for this activity, the two CCR7 ligands are not equally effective. Thus, CCL19 and CCL21 are biased agonists with equivalent potency and activity toward Gi/o, but differential activity for the GRK/β-arrestin system. Importantly, however, we also note that the observed bias is not absolute, and that CCL21 does lead to a small but significant amount of bulk phosphorylation and β-arrestin recruitment as determined by FRET.
4.3.4 β-arrestin2 Trafficking and Desensitization

β-arrestin recruitment to 7TMRs is generally described in one of two patterns, referred to as classes A and B [110]. Class A receptors recruit β-arrestins transiently and form puncta at the cell membrane, whereas class B receptors associate more stably with the receptor, trafficking together into early endosomes, observed as ring like structures (doughnuts, bagels) under confocal microscopy. We compared the effects of both CCL19 and CCL21 on the pattern of β-arrestin recruitment to the CCR7 receptor, using HEK cells transfected with CCR7 and β-arrestin2-GFP. Cells were stimulated with CCL19 (100nM) or CCL21 (100nM) for sixty minutes and live cells were visualized by confocal microscopy. Representative images are shown in figure 10a. Treatment with CCL19 promoted β-arrestin2-GFP redistribution in a typical class B pattern. Interestingly, treatment with CCL21 showed no evidence of β-arrestin2-GFP redistribution.
Figure 10: CCL19 mediates β-arrestin2 trafficking to early endosomes and desensitization. A) HEK-293 cells were transiently transfected with CCR7 and β-arrestin2-GFP. Cells were left unstimulated or stimulated with 100nM CCL19 or 100nM CCL21 for 1 hour. Live cells were imaged by confocal microscopy and images representative of three independent experiments are shown. B) HEK-293 cells stably expressing CCR7 and the cAMP biosensor ICUE2 were stimulated with (1) 100uM forskolin alone, (2) 100nM CCL21 + forskolin, (3) 100nM CCL21 + 100uM forskolin after pretreatment with 100nM CCL19 for 1 hour, or (4) 100nM CCL21 + 100uM forskolin after pretreatment with 100nM CCL21 for 1 hour. The bar graph shows the integrated cAMP responses for each condition. Statistical significance was determined by paired two tailed t tests. (*, p<0.05; **, p<0.01; ***p<0.001)
β-arrestin recruitment is classically involved in agonist-induced receptor desensitization. In order to compare the ability of both CCR7 ligands to induce desensitization, HEK cells stably expressing CCR7 and ICUE2 were preincubated with either CCL19 (100nM) or CCL21 (100nM) for 60 minutes. Cells were then washed and G_{i/o} activity was assessed by re-stimulation with CCL21 (100nM) and forskolin (50uM). We found that preincubation with CCL19 leads to complete desensitization of CCR7 toward subsequent activation by CCL21 (figure 10b). In contrast, desensitization was not observed after preincubation with CCL21. Similar results were obtained when CCL19 was used to assess CCR7 responsiveness after pre-stimulation (data not shown). These results indicate that CCL19, but not CCL21, leads to both β-arrestin2 trafficking to early endosomes and agonist-induced desensitization.

4.3.5 β-arrestin2 Mediated MAPK Signaling

In addition to desensitization, β-arrestins also function as scaffolds or adaptors facilitating signaling pathways such as ERK activation. Next, we sought to compare these ligands for their ability to signal to MAP kinase in a β-arrestin2 dependant fashion. HEK cells stably expressing CCR7 were transfected with control siRNA or siRNA specific for β-arrestin1, β-arrestin 2, or both β-arrestins. Loss of protein expression was
generally above 85% and a representative blot is shown (figure 11a). Cells were then stimulated with CCL19 (100nM) or CCL21 (100nM) for 5 minutes and the activation of ERK was determined by immunoblotting for phospho-ERK.

Figure 11: Both CCL19 and CCL21 lead to β-arrestin2 dependent MAP kinase. A) HEK-293 cells stably expressing CCR7 were preincubated with pertussis toxin (200ng/ml) for 2hrs where indicated. Cells were stimulated with 100nM CCL19 or 100nM CCL21 for 5 minutes and analyzed for ERK phosphorylation by SDS-PAGE and immunoblotting. B) HEK-293 cells stably expressing CCR7 were transfected with either control siRNA or siRNA specific to β-arrestin1, β-arrestin2, or both β-arrestin1+2. β-arrestin expression was detected by SDS-PAGE and immunoblotting as shown. C) Cells were serum starved for 2 hours and then stimulated with 100nM CCL19 or 100nM CCL21 at 37° C for 5 minutes. P-ERK levels were normalized to total ERK levels and expressed as a percentage of CCL19 mediated ERK in control siRNA treated cells. The bar graph shows the mean +/- SE from three experiments and a representative blot is shown. Statistical significance was determined by paired two tailed t tests. (*, p<0.05; **, p<0.01; ***p<0.001)
CCL19 and CCL21 stimulate equivalent phospho-ERK which is highly dependent upon β-arrestin expression (figure 11b,11c). Loss of β-arrestin2 or both β-arrestins lead to a marked reduction in phospho-ERK in response to both CCL19 and CCL21. Whereas, siRNA mediated silencing of β-arrestin1 actually lead to an increase in phospho-ERK to either of these ligands; a pattern of β-arrestin dependence consistent with 7TMRs such as the AT1R [31, 111]. Thus, despite differential activation of the GRK/β-arrestin system by CCL19 and CCL21, these ligands were equally capable of activating phospho-ERK in a β-arrestin-dependent manner.

The fact that we did not observe differential β-arrestin mediated phospho-ERK between CCL19 and CCL21 was unexpected in light of the preferential GRK/β-arrestin activation seen with CCL19. These data strongly suggest that β-arrestin signaling was intact for CCL21 activated CCR7 despite weak bulk phosphorylation, modest β-arrestin recruitment, and lack of desensitization. Thus we speculated that this phenomenon might be the result of engaging different GRK isoforms. This led us to further examine more proximate steps of CCR7 signaling to determine the contribution of individual GRK isoforms to CCR7 phosphorylation, β-arrestin recruitment, and β-arrestin mediated signaling.
4.3.6 GRK Specificity for CCR7 Phosphorylation

Next, we examined the effect of silencing the expression of individual GRKs on CCR7 phosphorylation. HEK cells stably expressing CCR7-Flag were transfected with control siRNA or siRNA specific for GRK2, GRK3, or GRK6 (GRK5 expression could not be reliably assessed using available antibodies and was therefore omitted). The effect of siRNA on the expression of individual GRKs was specific and resulted in a greater than 90% reduction in protein expression for each kinase as determined by immunoblotting (figure 12a).
Figure 12: Differential GRK specificity for CCR7 Phosphorylation.
A) HEK-293 cells stably expressing CCR7-Flag transfected with control siRNA or siRNA specific for GRK2, GRK3, or GRK6. Cells were then split for both phosphorylation and receptor binding studies. Surface receptor expression was determined in parallel for each siRNA condition by competition binding with I125-CCL19. Receptor phosphorylation dishes were labeled with 32Pi and stimulated at 37°C for 10 minutes with 100nM CCL19 or 100nM CCL21. Lysates were prepared and protein concentrations were quantified. Using the calculated expression levels, equal amounts of receptor was added to the anti-Flag beads for immunoprecipitations. Samples were then separated by SDS-PAGE, and analyzed by phosphoimaging. A) A representative images showing the effect of
GRK siRNA is shown in the top panel. B) A representative image showing $^{32}$P$_i$ incorporation is shown in the middle panel. Agonist induced phosphorylation is expressed as a percentage of CCL19 mediated phosphorylation of the control siRNA treated cells. The bar graph represents the mean +/- SE from four to nine independent experiments. Statistical significance was determined by using paired two-tailed t tests (*, p<0.05; **, p<0.01; ***p<0.001)

Agonist-induced receptor phosphorylation in response to CCL19 (100nM) and CCL21 (100nM) was measured by analyzing the incorporation of $^{32}$P$_i$ into CCR7-Flag after 10 minutes of stimulation. Cells with individual GRKs knocked-down using siRNAs were similarly studied and compared to control siRNA transfected cells analyzed in parallel. Knock-down of GRK2 had no appreciable effect on either CCL19 or CCL21 mediated phosphorylation despite robust expression in these cells. Interestingly, loss of GRK3 lead to a significant reduction in CCL19 mediated phosphorylation, but no loss of CCL21 mediated phosphorylation. Finally, loss of GRK6 led to a significant reduction in both CCL19 and CCL21 stimulated phosphorylation.

4.3.7 GRK Specificity for $\beta$-arrestin recruitment

Next, we examined the effect of individual GRKs on agonist-induced $\beta$-arrestin recruitment in live cells. HEK cells stably expressing CCR7-CFP and $\beta$-arrestin2-YFP
were transfected with control siRNA or siRNA specific for GRK2, GRK3, or GRK6. Cells were then stimulated with CCL19 (100nM) or CCL21 (100nM) and the recruitment of β-arrestin2 to CCR7 was measured by FRET. Time courses were determined for each condition and normalized to maximal FRET from control siRNA treated cells stimulated with CCL19 (figure 13). The integrated responses were then calculated and are shown as bar graphs for each condition. Loss of GRK2 had no appreciable effect on either CCL19 or CCL21 mediated β-arrestin recruitment. Surprisingly, β-arrestin recruitment in the absence of GRK3 after CCL19 stimulation was significantly reduced whereas CCL21 stimulation in the absence of GRK3 was not. In the absence of GRK6, the FRET response to both CCL19 and CCL21 was reduced.
Figure 13: Differential GRK Specificity for β-arrestin2 Recruitment. Live HEK-293 cells stably expressing CCR7-CFP and β-arrestin2-YFP were transfected with control siRNA or siRNA specific for GRK2, GRK3, or GRK6. Cells were stimulated with 100nM CCL19 or 100nM CCL21 and the recruitment of β-arrestin2 measured by FRET. For GRK siRNA treated cells, CCR7/β-arrestin2 FRET is expressed as a percentage of CCL19 mediated FRET of the control siRNA treated cells done in parallel. The top panel shows the time course of FRET responses after agonist was added at 30 seconds. The bottom panel is a bar graph showing the integrated FRET responses for each condition. Data represent the mean ± SE from four to seven independent experiments each done in duplicate. Statistical significance was determined by paired two-tailed t tests (NS, not significant; *, p<0.05; **, p<0.01; ***p<0.001). These data are striking in that they demonstrate differential GRK specificity for CCR7 when stimulated by CCL19 or CCL21. Therefore, the differential bulk
phosphorylation and β-arrestin recruitment between CCR7 ligands is not due to a
generic GRK preference for the CCL19 activated receptor. Rather, GRK6 activity is
observed in response to either ligand, with the additional bulk phosphorylation and β-
arrestin recruitment observed with CCL19 potentially due to additional GRK3 mediated
phosphorylation this ligand generates.

4.3.8 GRK6 but not GRK3 is required for MAPK Signaling

We next sought to directly test whether GRK isoforms can specifically promote
β-arrestin2 dependent MAP kinase activation in a nonredundant fashion. HEK cells
expressing CCR7 were treated with GRK specific siRNAs, stimulated with CCL19
(100nM) and CCL21 (100nM), and ERK activation was determined by immunoblotting.
Figure 14: GRK6 is required for MAP kinase signaling by both CCL19 and CCL21. HEK cells stably expressing CCR7 were transfected with control siRNA or siRNA specific for GRK2, GRK3, or GRK6. Cells were serum starved and then stimulated with 100nM CCL19 or 100nM CCL21 at 37°C for 5 minutes. Lysates were prepared, separated by SDS-PAGE, and immunoblotted for phospho-ERK and total ERK. P-ERK levels were normalized to total ERK levels and expressed as a percentage CCL19 mediated ERK at 5 minutes after subtraction of basal ERK. The bar graph shows the CCL19 and CCL21 mediated ERK activation in the presence or absence of GRK specific siRNA. Data represent the mean +/- SE from four independent experiments. Statistical significance was determined by paired two-tailed t tests (*, p<0.05; **, p<0.01; ***p<0.001).

We found that the activation of ERK in response to CCR7 stimulation is highly GRK dependent (figure 8). Knock-down of GRK6 almost completely eliminated ERK
activation, and this GRK6 dependency was equally apparent for CCL19 and CCL21. This is consistent with our earlier observations that both ligands lead to GRK6 dependent CCR7 phosphorylation and β-arrestin recruitment, and β-arrestin dependent MAP kinase signaling. In addition, we observed that loss of GRK2 or GRK3 led to an increase in CCL19 stimulated P-ERK. Whether this is due to unopposed GRK6 activity or implies a negative regulatory function by these enzymes remains unknown. Overall, these data provide further evidence that GRK3 and GRK6 are not functionally redundant enzymes and only the latter leads to CCR7 stimulated MAP kinase signaling.
Figure 15: CCR7 Ligand Bias is Transmitted as Differential GRK Barcodes that Direct β-arrestin Function.
4.4 Discussion

4.4.1 CCR7 Ligand Bias

The purpose of this study was to investigate the differential activation of the GRK/β-arrestin system by the endogenous ligands of CCR7, CCL19 and CCL21. We found that CCL19 behaves as a traditional agonist, leading to robust G-protein activation, CCR7 phosphorylation, β-arrestin recruitment, CCR7 desensitization, and β-arrestin2 dependent MAP kinase activation. In contrast, activation of CCR7 by CCL21 leads to equivalent G-protein activity, but markedly less bulk phosphorylation and β-arrestin2 recruitment, and little to no desensitization. Thus, we were surprised to find that CCL21 was fully capable of activating ERK in a β-arrestin-dependent manner.

The apparent paradox of a ligand capable of β-arrestin-dependent signaling to MAP kinase but incapable of desensitization led us to investigate the effects of individual GRKs on CCR7 signaling. We sought to address two interrelated issues pertaining to GRK redundancy: First, whether substrate specificity exists among the ubiquitously expressed GRKs for CCR7 phosphorylation, and second whether GRK isoforms that are differentially specific for a receptor affect the eventual function of β-arrestin.
4.4.2 The Discovery of GRK Bias

We silenced the expression of individual GRK isoforms with siRNA to determine the relative contributions of each kinase to CCL19 and CCL21 mediated CCR7 phosphorylation. GRK2 did not appear to phosphorylate CCR7 in response to either ligand despite robust expression in HEK-293 cells. However, loss of GRK3 leads to a substantial reduction in bulk receptor phosphorylation and β-arrestin recruitment but only in response to CCL19, and not CCL21. On the other hand, GRK6 was active in response to either ligand and contributed equally to bulk phosphorylation in response to CCL21 as well as CCL19. Therefore, despite similar \( G_{\text{o}} \) protein activation with these ligands, GRK3 activity is unique to CCL19 and leads to additional bulk phosphorylation and β-arrestin recruitment. Conversely, GRK6 is catalytically active in a relatively unbiased manner for each of these ligands. Thus, we found marked GRK specificity for activated CCR7, but also biased GRK utilization by its endogenous ligands. Differential GRK phosphorylation despite equal G-protein activation, as observed here by CCR7 ligands, provides a novel mechanism of ligand bias. We speculate that other examples of GRK and β-arrestin bias will emerge, particularly among receptors with multiple ligands.
4.4.3 GRK isoforms determine β-arrestin function.

The GRK specificity and bias of CCR7 provides an attractive model to compare the functional consequences of individual GRKs. Thus, we examined whether GRKs differentially affected β-arrestin activity. We found that total β-arrestin recruitment, as measured by FRET, roughly correlated with the extent of bulk phosphorylation. However, assays of β-arrestin function did not directly correlate with these bulk phosphorylation responses. For instance, although both GRK3 and GRK6 lead to β-arrestin recruitment in response to CCL19 activation of the receptor, only the loss of GRK6, and not GRK3, abrogated MAP kinase signaling. Yet, while CCL21 mediated receptor phosphorylation and β-arrestin recruitment, and was fully sufficient for β-arrestin dependent MAP kinase signaling, it did not induce desensitization. Thus, desensitization and β-arrestin mediated signaling are not activated proportionately to each other, but instead are promoted independently by GRK3 and GRK6, respectively.

The effects of GRK bias observed in this study add important insight to our understanding of β-arrestins as multifunctional proteins. An expanding number of β-arrestin binding partners and cellular functions have been identified. Yet, less is known about how these processes are coordinated at the molecular level. The data presented here reveal that 7TMRs are capable of a remarkable degree of GRK specificity, even in response to ligands with equivalent G-protein responses. These data also highlight the emerging concept that the functions of β-arrestins may be pre-specified by the
particulars of the GRK-receptor interaction. Thus, individual GRK isoforms may lead to functionally distinct pools of β-arrestin, presumably by virtue of phosphorylating distinct sites on the receptor. These GRK specific phosphorylation patterns may establish a “bar code” of sorts which instructs the β-arrestin partners as to which functions to perform, and/or which conformation to adopt.

The GRK bias of CCR7 involves ligands that are endogenously expressed. Species therefore appear to have evolved the capacity to control the balance of distinct β-arrestin dependent functions by regulating the cellular GRK profile and/or the relative concentration of biased ligands in the extracellular milieu. Thus, this study reveals a novel mechanism of biased agonism and shows that ligands are able to selectively engage the GRK/β-arrestin system with more precision and functional distinction than previously appreciated.
Chapter 5. Concluding Remarks

The chemokine receptor family shares structural features, conserved G protein usage, and functional redundancy for chemotaxis; unlike the adrenergic system, whose receptors couple to a wide range of G-protein isoforms with a diverse host of cellular consequences. Although exceptions exist, in general, chemokine receptors interact with PTX sensitive G proteins to affect cell migration. However, in spite of these generalized characteristics, the activation of the GRK/β-arrestin system by these receptors appears to be remarkably specialized, leading to a complexity that stands in stark contrast to their receptor/G-protein relationships.

In Chapter 3, we show in murine T lymphocytes that the endogenous CCR7 ligands are in fact biased agonists. Despite similar chemotactic responses and AKT activation, CCL19 is preferentially capable of signaling to ERK and JNK in comparison to CCL21. The physiological significance of this natural biased agonism will be the subject of future studies. In light of previous observations that CCR7 ligands are differentially expressed [88-90], we speculate that CCL21 may support T cell transit through tissues. In contrast, destinations marked by CCL19 expression may cause retention within tissues via desensitization of CCR7. In addition, signaling pathways unique to CCL19 such as the PKC mediated ERK described in Chapter 3 may be required for programmatic changes that may accompany this retention for the purpose
of activation or differentiation. The creation of individual CCL19 and CCL21 knockout mice will also be instructive to test the degree to which these ligands are redundant, complementary, or even dependent on one another for proper CCR7 mediated trafficking \textit{in vivo}.

CCL21 contains an extra 3kDa motif at its C-terminus that is conserved in both mice and humans. This motif could account for the differential signaling described here. Alternatively, the first six amino acids of the N-terminus of CCL19 and CCL21 have been shown to regulate receptor activation\cite{112-114}. Thus, additional studies using chimeric ligands could test the relative importance of these motifs for CCR7 ligand bias.

In chapter 4, we investigate the mechanism by which CCL19 and CCL21 differentially activate the GRK/β-arrestin system. Surprisingly, unlike the other examples of biased agonism, we find that CCR7 bias is not an “all or none” activation of the GRK/β-arrestin system. Rather, each ligand is capable of engaging β-arrestin through GRK6. However, GRK3 mediated phosphorylation and β-arrestin recruitment is unique to CCL19.

Given this GRK bias, CCR7 is a unique model system to compare the effects of individual GRK isoforms, with the advantage of the inherent internal controls afforded by ligands with equal G-protein activation.

We found that individual GRKs lead to qualitative, not just quantitative, differences in β-arrestin function. β-arrestin function was not proportionate to total β-
arrestin recruitment but rather determined by the upstream GRK leading to the recruitment. GRK6 coded for β-arrestin signaling to ERK whether this was due to CCL19 or CCL21. In contrast, GRK3 activity did not lead to additional ERK signaling for CCL19, nor did its silencing reduce ERK activation. In contrast, GRK6 activation, even in isolation as activated by CCL21, was fully sufficient for signaling but did not lead to desensitization.

Thus, the lesson of CCR7 GRK bias is that the GRK isoforms are not simply redundant kinases. First, sufficient substrate selectivity exists such that the various isoforms are not activated in an “all or none” fashion. Second, although multiple GRK isoforms contribute to bulk phosphorylation and nominally to β-arrestin recruitment, they appear to have differential downstream effects.

These observations provide insight into the mechanics of β-arrestin multifunctionality. This study suggests that, at the single receptor level, β-arrestin gets programmed for a subset of its potential functions by the GRK barcode it detects. In contrast, at the cellular level, β-arrestin may accounts for the sum of these functions. Thus, these functions may not occur simultaneously for each receptor, but rather the β-arrestin functionality may be tailored according to the specifics of the receptor such as its GRK barcode. For chemotactic receptors, this raises the possibility that functions of β-arrestin may be spatially arranged in a manner dependent upon the concentration gradient outside the cell.
Physiologically, these data reveal that the balance of desensitization and β-arrestin mediated signaling of CCR7 on immune cells is highly regulated. The function of β-arrestin for CCR7 in a particular context appears dependent on: 1) The balance of ligands expressed in the extracellular milieu, 2) The mixture of cellular GRK isoform expression, and 3) β-arrestin1 and β-arrestin2 expression. Thus, the effects of the GRK/β-arrestin system are tightly controlled by multiple parameters and likely depend on the cellular context in vivo.

Lastly, it is worthwhile to speculate why endogenous ligand bias might have evolved within the chemokine system. One obvious factor is the sheer number of promiscuous receptors and ligands. Unlike the adrenergic receptor system which is confined to several endogenous catecholamines, chemokines have ten times the number of ligands and therefore more chances at ligand bias occurring by chance. Another factor may be the higher degree of Darwinian pressure applied to immune related processes and thus more force to optimize and specialize 7TMR plasticity. Another factor may be the biochemical requirements of the chemotactic process itself. Chemotaxis first demands that a cell recognize the polarity of ligand concentration. Then the cell must migrate up this concentration gradient, maintaining its sensitivity to the ligand despite the ability of 7TMRs to undergo homologous desensitization. Thus ligand bias may have emerged from the demands placed upon 7TMRs by the chemotactic process itself. We speculate that additional examples of ligand bias will
emerge from the chemokine family. As such, we expect that the study of chemokines will continue to shed light on novel aspects of 7TMR signaling.
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Biography:

I was born July 7th, 1972 in Pittsburgh, PA. I graduated from the University of Notre Dame with a BS in physics. I then attended medical school at Duke University School of Medicine from 1994 through 1998. After completing internship and residency training at the Johns Hopkins Hospital in Baltimore, MD, I returned to Duke as a cardiology fellow. My research interest became the mechanisms of chemokine receptor signaling and the role of inflammation in atherosclerotic disease.

My wife, Jennifer Zidar, and I enjoy all manner of sports, arts, cooking, and superbowl victories by the Pittsburgh Steelers.