THE ROLE OF G-PROTEIN COUPLED RECEPTOR KINASE 2 IN MUCOSAL INFLAMMATION

By

Michael Steury

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Physiology—Doctor of Philosophy

2017

ABSTRACT

THE ROLE OF G-PROTEIN COUPLED RECEPTOR KINASE 2 IN MUCOSAL INFLAMMATION

By

Michael Steury

G-protein coupled receptor kinases (GRKs) are a family of protein kinases comprised of seven serine/threonine kinases that were initially identified for their ability to phosphorylate G-protein coupled receptors (GPCRs). Furthermore, it has recently become evident that individual GRKs can interact in a kinase dependent or independent manner with non-receptor substrates and influence a variety of physiological functions and pathologies. This study focuses on the family member GRK2. GRK2 is expressed ubiquitously throughout the body and in addition to phosphorylating and regulating GPCR function, GRK2 is able to phosphorylate and/or interact with a large interactome of cellular proteins in a tissue - and context - specific manner. This combination of canonical and non-canonical roles of GRK2 is now attributed to a multitude of vital physiological functions including: cell migration, proliferation, metabolism, angiogenesis, and insulin resistance. This vast array of influence makes GRK2 a popular target of study for both diagnostic opportunities as well as therapeutic interventions and while GRK2 has been extensively studied in cardiac and immune cells its role in the intestine and the intestinal epithelium is not well understood.

Inflammatory bowel disease is characterized by damage to the intestinal epithelial barrier resulting in increased permeability and the resultant dissemination of the commensal microbiota. This translocation of the luminal contents into the lamina propria constantly stimulates the immune system leading to its hyper-activation and eventual damage to the intestine. Inflammatory bowel disease is associated with

increases in inflammatory cytokine production, namely TNFα and this study was performed to investigate the regulation of GRK2 on TNFα signaling in the intestinal epithelial cells and in a larger context its role in the regulation in onset and pathogenesis of acute colitis. We found that decreasing the levels of GRK2 in human epithelial cells influenced the induction of ROS production by TNFα that influences ERK1/2 signaling and the production of MMP9 to influence wound closure both in culture and in animal models. Furthermore, mice heterozygous for GRK2 were markedly protected from the onset and pathogenesis of acute DSS-induced colitis in the absence of any alterations in immune infiltration. Myeloid specific knockout studies showed this population to be in part responsible for the protection seen in the whole body knockout. Together these studies suggest that GRK2 may serve as a novel therapeutic option for the treatment of colitis.

ACKNOWLEDGEMENTS

I would like to extend my deepest gratitude to my mentor Narayanan Parameswaran for not only allowing me the opportunity to join his lab but also for his time, knowledge, and help with my project. Outside of the lab, I would like to thank him for his constant support in my career goals and his guidance that has helped prepare me for the future and allowed me to succeed in this competitive field as well as shaping me into the scientist that I am today.

I would also like to extend my thanks to the current and previous members of the Parameswaran and McCabe laboratories that have helped me with experiments and techniques and through scientific discussions about my project. I would also like to thank them for the general atmosphere of the lab making it such a memorable and enjoyable experience over these last years.

I would like to thank my committee members for their willingness and guidance to help with my development as a scientist and their insight into my work; Dr. Laura McCabe, Dr. Brian Gulbransen, Dr. L. Karl Olson and Dr. Cheryl Rockwell.

I would like to thank Amy Porter and Kathy Joseph (Investigative Histopathology Lab, Michigan State University, East Lansing, MI) for their expertise and their patience with me in my histology sample preparation.

I would like to thank all of the members and faculty of the Physiology Program at Michigan State University for the supportive atmosphere and for all of the research and teaching opportunities.

I would like to thank my wife, Sarah, my family (Doug, LuAnn, Robbie, Bekah, Abby, Seth, Kate and Jon) as well as all of my friends both near and far for all of their

support and love throughout my graduate career. Without their love and encouragement I could never achieve all that I have.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

AD: Alzheimer's disease

AVP: Arginine vasopressin

βARKct: C-terminal peptide of GRK2

BM: Bone marrow

CCR: CC chemokine receptor

cDNA: Complimentary DNA

cLP: Colonic lamina propria

CXCL: CXC chemokine ligand

CXCR: CXC chemokine receptor

DAI: Disease activity index

DMEM: Dulbecco's Modified Eagle's Medium

DNA: Deoxyribonucleic acid

DSS: Dextran sodium-sulfate

EGF: Epidermal growth factor

ERK: Extracellular signal-regulated kinases

FBS: Fetal bovine serum

FITC: Fluorescein isothiocyanate

GDP: Guanosine diphosphate

GIT1: GRK2-interacting factor

GM-CSF: Granulocyte macrophage colony stimulating factor

GPCR: G-protein coupled receptor

GRK: G-protein coupled receptor kinase

$GRK2^{+/-}$: GRK2 heterozygous mice

$GRK2^{LysM}$: GRK2 Myeloid Knockout mice

GTP: Guanosine triphosphate

HEK cells: Human embryonic kidney cells

Het: Heterozygous

HSP: Heat shock protein

IBD: Inflammatory bowel disease

IκB: Inhibitor of nuclear factor κB

IKK: IκB kinase complex

IL: Interleukin

JNK: Jun-N terminus kinase

KC: Keratinocyte chemokine

KO: Knockout

LPS: Lipopolysaccharide

LTB4: Leukotriene B4

MAPK: Mitogen-activated protein kinase

MCP-1: Monocyte chemoattractant protein 1

ml: Milliliter

MLN: Mesenteric lymph node

MMP: Matrix metalloproteinase

mRNA: Messenger RNA

MS: Multiple sclerosis

NAC: N-acetylcysteine

NFκB: Nuclear factor κB

ng: Nanograms

PBMC: Peripheral blood mononuclear cells

PBS: Phosphate buffered saline

PH Domain: Pleckstrin homology domain

PIP_2 : Phosphatidylinositol 4,5-bisphosphate

RH Domain: Regulator of G-protein signaling homology domain

RITC: Rhodamine B isothiocyanate

RNA: Ribonucleic acid

ROS: Reactive oxygen species

RT-PCR: Real Time polymerase chain reaction

RTK: Receptor tyrosine kinase

SDS PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM: Standard error of the mean

siRNA: Small interfering RNA

SSRI: Selective serotonin reuptake inhibitor

TLR: Toll-like receptor

TNBS: 2,4,6 trinitrobenzenesulfonic acid

TNFα: Tumor necrosis factor α

µl: Microliter

µM: Micromolar

uPa: Urokinase plasminogen activator

WT: Wild-type

CHAPTER 1: INTRODUCTION

This chapter represents an accepted manuscript ahead of print in the book “G Protein-Coupled Receptors in Immune Response and Regulation” published by Elsevier.

HISTORY

Cells have the remarkable ability to adapt and respond to changes in their environment through the recognition of extracellular signals from both the host as well as invading pathogens. These signals come in a variety of forms from the host environment including hormones, cytokines, and chemokines but also from microbial substances such as lipopolysaccharides and the breakdown of products from our dietary intake. Cells have developed sophisticated mechanisms for detecting and responding to these signals, largely through the development of both extracellular and intracellular receptors. Despite the abundance of receptor classes, one family represents a large proportion of the receptor population, the G-protein coupled receptors (GPCRs). This family of receptors is encoded by approximately 950 genes and transmits responses to a wide variety of stimuli including odorants, hormones, neurotransmitters, light, extracellular calcium, and many other stimuli regulating a wide variety of biological processes [1]. These receptors are characterized by their seven transmembrane domains that, when active, undergo a conformational change allowing them to activate nearby heterotrimeric GTP-binding proteins (G-proteins). These G-proteins are specialized proteins that have the ability to bind to nucleotides guanosine triphosphate (GTP) and guanosine diphosphate (GDP) and form a complex composed of α-subunits (Gα encoded by 16 genes) and βγ dimers (Gβ encoded by 5 genes and Gγ encoded by 12 genes). When active, GPCRs can serve as guanyl nucleotide exchange factors for these G-proteins, catalyzing the release of the bound GDP and exchanging it for GTP which in turn causes the dissociation of the heterotrimeric G protein in Gα and Gβγ subunits [2]. These dissociated G-proteins then interact with other effector proteins and secondary messengers to facilitate a large variety of

biological outcomes including both activation and inhibition. This activity is terminated when the intrinsic GTPase activity of the Gα subunit causes GTP hydrolysis and the formation Gα-GDP, leading to its re-association with Gβγ and the reformation of the heterotrimeric G-protein complex. For a comprehensive review of G-protein activation and regulation please see other excellent reviews [3–6].

Aside from the intrinsic GTPase activity of the G-proteins, G-protein mediated signaling is most often terminated by a conserved two-step mechanism: receptor phosphorylation by G-protein-coupled receptor kinases (GRKs) followed by arrestin recruitment and binding to the activated receptor ultimately leading the internalization and termination of the GPCR signal. Four genes encode arrestins; arrestin-1 and arrestin-4 that are restricted to the retina, whereas arrestin-2 (β-arrestin-1) and arrestin-3 (β-arrestin-2) are ubiquitously expressed throughout the body [7]. This process of receptor desensitization is responsible for the decreasing the magnitude of stimulation that GPCRs exhibit in response to prolonged exposure to agonist binding. This process is initiated by various GRKs that are able to phosphorylate the intracellular loops and/or the C-terminal domain of activated GPCRs and this phosphorylation directly leads to the recruitment of multifunctional adaptor proteins, arrestins. Interestingly, some receptors are regulated by a single GRK whereas other receptors can be regulated by multiple different GRKs with varying affinities. Recently it has been suggested that there are distinct phosphorylation patterns termed “barcodes” that determine β-arrestin functionality and contribute to the diverse responses witnessed in GPCR activity after phosphorylation [8]. This phosphorylation and subsequent binding of arrestins prevent further activation of G-proteins resulting in the cessation of G-protein signaling despite the continued presence of the agonist on the GPCR. Furthermore, this GRK-arrestin

interaction is able to then internalize the activated GPCR from the cellular surface through the formation of clathrin-coated pits. During this internalization, signaling can still occur through the binding of additional signaling proteins to the still attached arrestin proteins where they may serve as scaffolding proteins. Recently, it was shown that this interaction between β-arrestins and GPCRs form distinct conformational complexes and that these complexes are specialized to carry out different cellular functions depending on the relationship of their binding [9]. This converts the GPCR activation from heterotrimeric G-protein signaling pathways to G-protein-independent pathways [10]. More recently it has been shown that G-protein signaling can be sustained within the endosome through the formation of “megaplexes” indicating a possible new signaling role for these receptors during internalization [11]. These alternative G-protein-independent pathways are addressed in other reviews [7,12,13]. Because of this wide array of biological stimuli and functionality, the regulation and control of these receptors and their subsequent signaling is a potential target for pharmaceutical intervention. In fact, the GPCR family of receptors represent the largest family of drug targets for a wide variety of conditions including cardiovascular, metabolic, neurodegenerative, infectious, and oncologic diseases [14,15].

As studies on the regulation of GPCRs continues to unfold, new roles for GRKs have been emerging in addition to the part they play in GPCR phosphorylation. It is now apparent that GRKs (and arrestins) regulate GPCRs in a canonical fashion but also have GPCR-independent functions in cell signaling and biology. This non-canonical role for GRKs has now been shown to have critical regulatory roles in the context of inflammation and inflammatory diseases. In this review, we will investigate and discuss

the known and emerging roles for these GRKs in both GPCR-dependent and independent functions in the context of inflammatory processes and pathologies.

GRK STRUCTURE AND FAMILIES

The discovery and association between G proteins and receptor-based signal transduction developed through a progression of discoveries. Initially, it was discovered that GTP was necessary for signal transduction upon receptor activation and when the proteins were characterized that interacted with GTP to initiate the signaling cascade they were termed “G-proteins” [16,17]. Our understanding of the regulation of GPCRs then progressed through the identification of rhodopsin phosphorylation and that this phosphorylation contributed to the rapid desensitization of rhodopsin signaling. This desensitization was attributed to the “opsin kinase” now known as GRK1 [18]. It was later in the mid-1980’s through the cloning of the β-adrenergic receptor kinase (now named GRK2) that it was demonstrated that GRK1 was, in fact, a member of a family of related kinases that have a common mechanism that can recognize and regulate the active state of a variety of GPCRs [19]. During this period, another protein was discovered to have a regulatory impact and could dampen G-protein mediated retinal signaling by also associating with rhodopsin. This protein (termed S antigen because of its role in allergic uveitis) was then renamed “arrestin” because of its ‘arresting’ function in retinal signaling [20–22]. It was then through further characterization of the relationship between GRKs and arrestins that the canonical two-step inactivation process of desensitization of GPCRs was established. As studies progressed the family of GRKs expanded to include seven serine/threonine kinases including GRK3 [23], GRK4 [24], GRK5 [25,26], GRK6 [27] and GRK7 [28,29]. These kinases are now grouped based on their sequence similarities and are broken down into three subfamilies: GRK1, composed of GRK1 (rhodopsin kinase) and GRK7 (cone opsin

kinase); GRK2, including GRK2 and GRK3; and GRK4, made up of GRK4, GRK5, and GRK6.

Evolutionarily, GRKs are present in most vertebrate and invertebrates species as well as identified in non-metazoan species, with the kinase domain being the most evolutionarily conserved among the different GRKs [30]. All seven GRK members are ~500-700 amino acids long, multi-domain proteins containing a family specific N-terminal domain, followed by a regulator of G protein signaling homology domain (RH domain), an AGC protein kinase domain and a C-terminal domain [31]. The C-terminal is variable (both in length and structure) among the different subfamilies and contains structural elements that are partly responsible for the diversity of GRK function, regulation, and interaction. Specifically, members of the GRK1 family have C-terminal prenylation sites while GRK2 subfamily members have a pleckstrin homology domain in their C-termini, that can interact with Gβγ subunits [32]. The GRK4 family members have palmitoylation sites with the exception of GRK5 that contains a positively charged lipid-binding amphipathic helix in its C-terminus [33]. GRK5 is also slightly different than its family members due to the presence of a nuclear localization signal that allows it to accumulate in the nucleus where it can act as a histone deacetylase kinase (although it is predominantly cytoplasmic) [34]. Structurally, GRK1 and GRK7 genes (in humans) contain 4 exons, GRK2 and GRK3 contain 21 exons, and members of GRK4 subfamily have 16 exons. Compared to the differences and similarities in domains, tissue localization of these kinases varies drastically between subfamilies. GRK1 and GRK7 are termed the “visual kinases” and are localized in the eyes, whereas the non-visual kinases are distributed nearly ubiquitously throughout the body albeit at different expression levels. This distribution holds true for all non-visual GRKs with the exception

of GRK4 that is restricted to the testis, the proximal tubule of the kidney, and the cerebellum [35]. Although it was originally believed that all GRKs can phosphorylate GPCRs equally recent studies have shown that the GRK2/3 and the GRK5/6 subfamilies differ in their ability to phosphorylate inactive GPCRs with GRK5/6 being able to phosphorylate GPCRs in both the active and inactive conformations [36]. Since the GPCR superfamily represents the largest class of receptors and there are only four GRKs ubiquitously expressed throughout the body each GRK must be able to phosphorylate and regulate a wide variety of receptors, thus increasing the importance as well as the complexity of their role in signal transduction.

GRK REGULATION

GRKs play a vital role in the regulation and behavior of many biological processes through a variety of regulatory mechanisms. It is perhaps due to this variety of functions and interactions that they are so heavily conserved evolutionarily. One of the first proteins that were shown to interact with and activate these GRKs was the Gβγ-subunits [37]. In addition to G-protein activation of GRKs, lipids also have the capability to regulate GRKs as well as other proteins including calmodulin, caveolin-1, ERK and actin through direct binding [38,39]. Interestingly, these methods of regulation are not conserved among the various subfamilies of GRKs. For example, GRK2 family members are activated by phospholipids (phosphatidylinositol bisphosphate) through its interaction with the PH domain in the C-terminus, whereas GRK4-like members are activated by phosphatidylinositol bisphosphate through its binding to the N-terminal polybasic region [40].

Phosphorylation has differing regulatory effects (either activation or deactivation) on GRKs depending on exactly which kinase interacts with a given GRK or whether or not that GRK undergoes autophosphorylation. To illustrate this contrasting regulation in the case of the visual GRKs, phosphorylation by protein kinase-A or for GRK5 by protein kinase-C causes a decrease in kinase activity [41,42]. In contrast, phosphorylation of GRK2 by protein kinase-C enhances its activity [43]. Interestingly, where GRK2 and GRK5 can be phosphorylated by multiple kinases, there is no reported evidence of phosphorylation of GRK3, GRK4 or GRK6. In addition to phosphorylation, other post-translational modifications such as S-nitrosylation of GRK2 by nitric oxide synthase, has been shown to inhibit its activity [44]. These contrasting effects highlight that there are GRK specific regulation and suggests that there are

multiple pathways involved in fine tuning each GRK's response and ability to regulate GPCRs, non-GPCRs, and non-receptor biological functions. GRKs similar to other protein families that interact with GPCRs engage an inter-helical cavity that opens on the cytoplasmic side of the GPCR after activation [45] and it is probable that GRKs interact in a similar fashion to discern active versus inactive receptors [46]. In recent studies, the crystal structures for various GRKs including GRK1, GRK2 and GRK6 have been characterized and it was observed that the conserved 18-20 amino acids on the N-terminal become helical and form an α-helix stabilizing the closed (active) conformation allowing it to interact with both lobes of the kinase domain [47]. This suggests then, that GPCR binding helps to promote GRK activity by helping to align catalytic residues of the kinase domain indicating that GPCRs can serve as both the substrate and the allosteric activators [48,49]. Furthermore, the mutation of this N-terminus α-helix abolishes GPCR phosphorylation suggesting that this helix formation is indeed the primary step in the activation of GRKs by GPCRs [50]. This mechanism sets apart GRKs from other kinases that phosphorylate accessible residues (Ser, Thr, Tyr) that are in a specific sequence context. Instead, in GRKs phosphorylation of Ser and Thr residues are not strongly sequence context-specific and this context independence may help to explain the versatility and flexibility of GRKs as seen in the fact that a small family of proteins can interact with and regulate hundreds of different GPCR subtypes [51]. This allosteric binding of GRKs by GPCRs essentially ensures that GRKs, when bound, will phosphorylate nearby proteins with accessible loops, i.e. the intracellular loops and tails of the receptor molecules that they are currently bound to.

In the early years of their discovery, this phosphorylation of GPCRs was believed to be the only function of GRKs. With the identification of non-receptor substrates,

however, it has been shown that these non-receptor substrates can be phosphorylated more efficiently than peptides derived from cognate GPCRs [52]. This suggests that there must be alternative mechanisms of activation other than binding to GPCRs but these mechanisms still remain to be identified. One hypothesis about this non-GPCR activation of GRKs stems from the idea that any physiochemical environment that resembles the binding pocket found on GPCRs could favor GRK activation.

ANIMAL MODELS OF GRK DEFICIENCY

Studies using knockout and transgenic mice as well as using viral-mediated overexpression models have enabled researchers to begin to identify various pathophysiological roles for GRKs. However, due to the nature of GRK function involving the regulation of activated receptors, mice lacking these GRKs oftentimes appear to function normally without the addition of a stressor. As indicated before, GRKs also have dual functionality in various tissues by both suppressing G-protein signaling as well as promoting signaling independent of G-proteins. Because of this loss of regulation by any given GRK, knockouts can have differing effects that can either a) allow enhanced or unregulated receptor signaling because of loss of desensitization or b) alter the signaling cascade by preventing the switch from G protein signaling to non-G protein pathways or c) alter the functionality of biological processes in a receptor-independent fashion [53]. It is also possible that certain phenotypes don't present due to compensation and overlapping roles of other GRKs present in the animal. For animals that do have abnormal functionality, the most notable phenotype was in the GRK2 homozygous knockout animal that was embryonic lethal because of defective cardiac development [54]. Interestingly, recent work has shown that this lethality, in fact, stems from an undefined role of GRK2 in embryogenesis rather than a specific role in cardiac development since mice with cardiac myocyte-specific GRK2 ablation develop normally [55]. Intrigued by the extreme phenotype, GRK2 heterozygous mice were heavily studied and GRK2 was shown to be important for heart development, lymphocyte chemotaxis, experimental autoimmune encephalomyelitis, sepsis, atherosclerosis and others making GRK2 one of the most heavily researched of all the GRKs. Studies focusing on the other GRK knockout and over-expression animals have shown an

important role in several distinct phenotypes including GRK3 in olfaction [56]; GRK5 in cholinergic responses and inflammation; GRK6 in chemotaxis, behavioral responses, locomotor-stimulating effect of cocaine and others [57]. Interestingly, recent studies crossing heterozygous GRK5 and heterozygous GRK6 knockout mice in order to generate GRK5/GRK6 double knockout mice was found to be embryonic lethal though the mechanism is unknown [58]. A detailed summary of all the phenotypes is provided in Table 1. 1.

This culmination of research on the various GRKs ranging from the biochemical and molecular machinations and functionality to the whole body knockout phenotypes demonstrates a unique and essential role for this family of proteins. However, the role that these GRKs play in specific inflammatory responses is only beginning to be revealed. This review will focus mainly on our recent understanding of the roles that GRKs play in the

Table 1.1. Summary of GRKs and Associated Diseases

GRK	Genotype	Phenotype	Reference
GRK1	GRK1 KO	Photon responses are increased and longer-lasting, loss of phosphorylation of rhodopsin.	[59]
	GRK1 Overexpression	Increased damage to photoreceptors after intense light	[60]
GRK2	Homozygous KO (whole body)	Homozygous KO is embryonic lethal	[54]
	Heterozygous KO (whole body)	Susceptible to β-Arrestin stimulation; altered progression of experimental autoimmune encephalomyelitis through increased infiltration of CNS by lymphocytes and macrophages; increase in lymphocyte chemotaxis toward CCL4	[55,61,62]
	Cardiac Specific KO	Enhanced ionotropic sensitivity to isoproterenol; lusitropic, tachyphylaxis	[55]
	Cardiac Specific OE	Decreased β-arrestin signaling; attenuated ventricular contractility	[63]
	Cardiac Specific β-ARKct	Enhanced contractility	[63]
	Cardiac Specific β-ARKnt	Cardiac hypertrophy, enhanced β-AR density and signaling	[64]
	GRK2-C340S	Loss of nitrosylation-mediated cardioprotection from postischemic injury	[65]
	Myeloid Specific KO	Increased Inflammation in endotoxemia and polymicrobial sepsis; decreased atherosclerotic lesions in LDL-myeloid dual KO mice	[66–68]

Table 1.1. (cont'd)

GRK	Genotype	Phenotype	Reference
GRK2	Vascular Smooth Muscle OE	βAR signal attenuation; attenuation of isoproterenol induced vasodilation; elevation of resting mean blood pressure; hypertrophy	[69]
	Adrenal-specific KO	Attenuates heart failure progression and improves function postmyocardial infarction	[70]
GRK3	GRK3 KO	Enhanced sensitivity to olfactory stimuli; altered M2 muscarinic airway regulation; loss of neuropathic pain induced opioid tolerance	[56,71,72]
	Cardiac Specific OE	Normal β-AR signaling and hemodynamic function; decreased MAPK activity to thrombin	[73]
	Cardiac Specific GRK3ct	Hypertension; α-1 receptor hypersensitivity	[74]
GRK4	GRK4 KO	No differences detected in fertility or sperm function	[75]
	GRK4-γA142V	Hypertension, altered dopamine signaling	[76,77]
GRK5	Cardiac Specific KO	Cardioprotection posttransaortic constrtiction	[78,79]
	Vascular smooth muscle OE	VSM-specific OE of GRK5 increases blood pressure through β1AR and Ang II receptors	[64]
	Cardiac Specific OE	Increased β-adrenergic receptor desensitization attenuating contractility and heart rate	[80]

Table 1.1. (cont'd)

GRK	Genotype	Phenotype	Reference
GRK5	GRK5 KO	Muscarinic M2 supersensitivity and impaired desensitization; decreased LPS-induced neutrophil infiltration in lung; decreased cytokine chemokine levels; enhanced hypothermia, hypoactivity, tremor and salivation by oxotremorine; decreased NFκB activation in thioglycollate-induced peritoneal macrophages and cardiomyocytes; increased NFκB in endothelial cells; increased apoptotic response to genotoxic damage; decreased thymocyte apoptosis during sepsis	[67,78,80–84]
GRK6	GRK6 KO	Altered desensitization of D2-like dopamine receptors and supersensitivity to psychostimulants; deficient lymphocyte chemotaxis; increased acute inflammation and neutrophil chemotaxis	[57,85–87]
GRK5/6	Double KO	Embryonic Lethal	[58]

immune system, specifically their influence on inflammatory signaling pathways and in inflammatory diseases.

GRK1 SUBFAMILY

Biochemistry

The GRK1 subfamily is comprised of the retinal GRKs, GRK1 (rhodopsin kinase) and GRK7 (cone opsin kinase). Both of these proteins are expressed in the retina of vertebrates but the expression pattern within the retinal cells is species dependent, where both GRKs are not always present. Highlighting these differences is the fact that all rod cells in vertebrates express GRK1 but cone cells express either GRK1, GRK7 or both, depending on the species [88]. GRK1 and GRK7 share their major domains and sequence homology but much of the current work on this family has been done on GRK1; first, because GRK1 was discovered decades before GRK7 and second, because of the demonstration of the X-ray structure of GRK1. Functionally, after activation of rhodopsin by light, GRK1 facilitates desensitization by phosphorylating rhodopsin on Ser/Thr residues in the C-terminal domain, which facilitates arrestin recruitment and binding. In order to accomplish this GRK1 and GRK7 must be properly localized to the membrane, and these GRKs are post-translationally modified to ensure their proper localization within the cell. GRK1 differs from GRK7 in this regard in that GRK1 is post-translationally modified via farnesylation and blocking this markedly limits its ability to phosphorylate active rhodopsin [89]. In contrast, GRK7 is predicted to have a geranylgeranyl modification site on its C-terminus rather than the N-terminus like GRK1. These modifications are believed to serve as hydrophobic anchors that secure GRK1 and GRK7 to the cellular membranes [89] and allow for these kinases to “probe” for activated receptors in a two-dimensional rather than three-dimensional search which is essential for rapid signal termination.

Role of GRK1 and GRK7 in Inflammation

Due to the limited tissue expression of GRK1 and GRK7, little is known about the role they play in inflammatory conditions of the retina. They do play a critical role in photoreceptors cells and GRK1 null-mice show shorter outer segments and undergo apoptosis if exposed to constant dim light [59]. Over-activation of these GRKs can also be detrimental to photoreceptor health as well and hyper-phosphorylation of rhodopsin caused by GRK1 mutations can cause a retinal degenerative disease known as retinal pigmentosa [90]. GRK1 levels are regulated in rat diabetic models Brown Norway and Sprague-Dawley after 12 weeks but were unaffected in the Long Evans models [91]. What these altered levels mean in terms of GRK function and if they play a role in diseases such as diabetic retinopathy or other inflammatory conditions remain unknown. Despite their limited role in inflammation it is important to think of how other therapeutics can influence these GRKs as a side effect of other treatments. For example, HSP90 inhibitors are being developed for oncology, retinal pigmentosa, inherited retinal degeneration and other neurodegenerative diseases but HSP90 is critical for the sustained production of GRK1. Treatments that include inhibition of HSP90 for sustained periods of time run this risk of adversely affecting visual function similar to Oguchi's Disease (stationary night blindness), a disease that results from genetic mutations and low expression of GRK1 [59].

GRK2 SUBFAMILY

Biochemistry

The GRK2 subfamily is comprised of two GRKs, GRK2 and GRK3. These proteins are found ubiquitously throughout the body but show some variability in their localization and expression in different tissues and cell types. One of the fundamental differences that are unique to this subfamily is that their ability to phosphorylate GPCRs is restricted to GPCRs that are actively bound to a ligand [36]. Furthermore, the RGS homology domain (RH) in the N-terminus region of GRK2 family members are able to bind to $G\alpha_q$ and Gα11, inhibiting their downstream signaling capabilities [92]. Interestingly neither GRK2 nor GRK3 is able to interact with Gαs, Gαi, Gαo, or Gα12/13, indicating that this functional interaction is specific to those two G-proteins [92–94]. Their RH domain consists of nine α-helices that are analogous to other RGS domains and two additional α-helices derived from a region between the kinase and PH domain. This allows for the RH domain to interact with the PH domain indicating a possible role for regulation of the kinase activity of this subfamily. In addition to the unique binding capabilities of this subfamilies' RH domain, GRK2, and GRK3 contain a pleckstrin homology domain (PH domain) in the C-terminus region that is unique to only these two GRKs. The PH domain consists of seven β-strands and one C-terminal α-helix. This PH domain has the functional capability to bind to phosphatidylinositol 4,5-bisphosphate (PIP_2) as well as the Gβγ subunit of the heterotrimeric G protein. This binding to the Gβγ subunit is particularly important for cytoplasmic localization of this subfamily and the subsequent phosphorylation of activated GPCRs. It is believed that through the binding of GRK2 and GRK3 to Gβγ these GRKs can be shuttled to the activated GPCRs and is a potential mechanism for how GRK2 and GRK3 recognize active receptors [19].

A side effect of this interaction of Gβγ to GRK2 and GRK3 is that this high-affinity binding can sequester and interfere with downstream targets of Gβγ in a similar manner to $G\alpha_q$. With the discovery of GRK2's crystal structure in complex with Gβγ, we have been able to gain new insights into the regulation of GRK2 (and potentially GRK3). This structure places the three distinct domains at the vertices of a triangle and gives insight on how these proteins with multiple modular domains can act as an integrated signaling molecule [71]. Interestingly, GRK2 and GRK3 are the only isoforms that interact with the free Gβγ subunit and translocate to the membrane in this fashion. Lastly, this presence of the PH domain in combination with the cytoplasmic localization of these GRKs may provide a mechanism for this subfamily to influence a large number of non-receptor substrates both in the presence and absence of GPCR ligand activity.

Role of GRK2 in Inflammation

GRK2 is primarily located in the cytoplasm; although it is ubiquitously expressed and has the capability to interact with a variety of GPCRs, and while most research has been GRK2's ability to phosphorylate β-adrenergic and angiotensin II type 1 receptors [92] and GRK2 has also been shown to phosphorylate other non-GPCR receptors including the platelet-derived growth factor receptor-β, epithelial Na+ channel, and the downstream regulatory element antagonist modulator (DREAM) [52]. It has also been suggested that GRK2 and GRK3 are more efficient at clathrin-mediated endocytosis than GRK5 and GRK6 [95]. Outside of its role in phosphorylating and regulating GPCR functions, GRK2 has the ability to phosphorylate and/or interact with a large variety of cellular proteins in both tissue– and context- specific fashion. This ability to regulate cellular processes in both a canonical and non-canonical fashion leads to GRK2 having

an influence on a multitude of vital physiological functions including its role in inflammation and inflammatory diseases [96].

Among the inflammatory pathways, research from our lab and others have shown that GRKs are important regulators of NFκB signaling pathway. NFκB is a critical player in the regulation of expression of many inflammatory genes and therefore is a target for therapeutic manipulation in a number of inflammatory diseases. Under un-stimulated conditions, NFκB transcription factors (p65 (RelA), p50, RelB, cRel and p52) are sequestered in the cytoplasm by members of the IκB family of proteins (IκBα, IκBβ, IκBε, p105 (NFκB1) and p100 (NFκB2)). Upon stimulation, IκB is phosphorylated by the IκB kinase complex (IKK) and undergoes ubiquitination and degradation. This releases the sequestered NFκB transcription factors which then translocate to the nucleus and modulate gene transcription (reviewed in [97]). In particular, GRK2 and GRK5 most notably regulate this signaling pathway to influence the outcome of inflammation in immune and non-immune cell types. GRK2 has been shown to directly interact with proteins involved in this signaling pathway, IκBα, and p105. GRK2 is able to phosphorylate IκBα albeit at very low stoichiometry and able to modulate this pathway in response to TNFα in a macrophage cell line and in HEK293 cells. [67]. This relationship between GRK2 and NFκB signaling was recently shown in neonatal rat cardiac fibroblasts in response to arginine vasopressin (AVP). AVP increased mRNA and protein levels of IL6 in these cells and pharmacological inhibition of GRK2 ablated this increase and NFκB activation, linking GRK2 and inflammation in cardiac stress [98]. This biochemical regulation was also recently demonstrated in colon cancer epithelial cell line and this effect of GRK2 on IκBα was linked to its role in positively inducing some inflammatory genes in these cell lines. Interestingly, GRK2 has also been shown

to interact with p105 and modulate ERK pathway downstream of p105 in primary macrophages. In this case, GRK2 was shown to be a negative regulator of this pathway and downstream inflammatory genes [67]. Interestingly, Toll-like receptor ligands induce GRK2 expression in primary macrophages and neutrophils [99]. Additionally, GRK2 has also been shown to localize in the mitochondria after LPS stimulation and regulate the production of reactive oxygen species [100]. Furthermore, immune cells from septic patients also exhibit increased levels of GRK2 indicating a possible clinical connection between GRK2 levels and the associated signaling pathways and inflammatory diseases [101]. This was shown to be the case at least in an animal model of sepsis where a IL-33 mediated decrease in GRK2 expression was beneficial for neutrophil migration and therefore better clearance of bacteria and improvement in sepsis. Thus, GRK2 has been suggested as a possible target in sepsis pathogenesis [102].

Another common set of pathways activated in response to inflammatory stimuli are the mitogen-activated protein kinase pathways (MAPKs). These kinases are a family of serine/threonine protein kinases that respond and react to extracellular signals. These reactions typically mediate a variety of fundamental biological processes ending in the translocation of transcription factors from the cytoplasm to the nucleus in order to alter gene expression. These MAPK signaling cascades are generally broken down into three major groups: extracellular signal-regulated protein kinases 1 and 2 (ERK 1/2), p38 MAPK and Jun-N terminus kinase (JNK). These individual kinases are activated upstream by additional MAPKs, specifically: ERK is activated by MAPK kinase 1 (MKK1) and MKK2; p38 MAPK by MKK3, MKK4, and MKK6; and JNK by MKK4 and MKK7.

Similar to the NFκB pathway, GRK2 most notably regulates this family of signaling proteins in response to both GPCR ligands (i.e. CXCL8) and RTK ligands (TNFα). Because of the plethora of studies looking at the role of GRK2 in MAPK signaling in response to various GPCR ligands [103,104] here, we focus primarily on the role of GRK2 in MAPK signaling in the context of inflammation. When these ligands activate the ERK1/2 pathway they can induce a wide variety of inflammatory mediators such as TNFα, interleukin 1 (IL-1), IL-8, and prostaglandin E2. The effect of GRK on this pathway has been studied in a variety of contexts beginning with its role in macrophages where GRK2 was shown to negatively regulate LPS-induced ERK signaling. GRK2 also has been shown to bind to Raf1 and RhoA that can serve as a scaffolding protein for the ERK MAPK cascade enhancing its activation in response to EGF and in a similar study GRK2 inhibition in cardiomyocytes increased ERK activity through interaction with the RAF MAPK axis [105]. Another study focusing on the connection between the scaffolding ability of β-arrestin2 and GRKs found that in HEK cells overexpression of GRK2 completely mitigated the β-arrestin2 mediated increase in ERK1/2 activation [106]. These effects held true with a variety of receptors including the β2-adrenergic receptor, cannabinoid receptor-2 [107], angiotensin 1A receptor [83] and the insulin-like growth factor-1 receptor [108]. GRK2 also has the ability to regulate ERK signaling through direct association with MEK (MKK1) and increased levels or GRK2 inhibit ERK activation following chemokine induction [109]. Consistent with that, our lab also has recent data in colon epithelial cells that ERK1/2 activity is enhanced in GRK2 knockdown cells not through MKK1 regulation but rather through altering the levels of ROS produced in those cells (Steury, 2017). Interestingly, GRK2 is able to regulate ERK in a variety of fashions but ERK can also directly interact with and phosphorylate

GRK2. Agonist-induced GRK2 phosphorylation by ERK1/2 reduces GRK2's activation by Gβγ and its ability to phosphorylate receptors and marks it for degradation [110,111].

The p38 MAPK pathway shares many similarities with the other MAPK signaling responses including inflammation, cell growth, cell differentiation, and death. Similar to ERK, the p38 MAPK pathway is stimulated by a wide array of stimuli including LPS and other TLR activators such as enterotoxin B and herpes simplex virus [112,113]. In the context of inflammation and the inflammatory response, p38 plays a critical role in the production of inflammatory mediators to initiate leukocyte recruitment and activation. It accomplishes this task through the regulation of inflammatory genes including TNFα, IL-1β, IL-6, IL-8, cyclooxygenase-2 (COX2) and collagenase 1 and 3 [114] all of which are diminished in monocytes/macrophages, neutrophils and T lymphocytes in the presence of p38 inhibitor SB203580 [115]. Previous studies have shown that GRK2 and p38 appear to oppose each other where GRK2 can inhibit p38 function by directly phosphorylating it whereas p38 inhibits GRK2 mediated GPCR desensitization [116,117]. It regulates GRK2 by directly phosphorylating GRK2 at Ser670 inhibiting its ability to translocate to the outer membrane and interact with activated GPCRs for desensitization. This specifically has been seen in response to MCP-1 and the prevention of the CC chemokine receptor2 (CCR2) [118]. GRK2 is able to phosphorylate p38 at its Thr123 residue, which interferes with the ability for p38 to bind to MKK6, therefore, preventing its activation [116]. Furthermore, altering the levels of GRK2 influences the activation of p38 and its functionality including differentiation and cytokine production. This alteration holds true in murine $GRK2^{+/-}$ macrophages and microglial cells; both of these cells exhibit increased p38 activation and TNFα production in response to LPS [119]. In contrast to this, complete GRK2 silencing in

mast cells decreases cytokine production (IL-6 and IL-13) in a p38 dependent manner during antigen-induced degranulation [120]. Lastly, GRK2 through its regulation of p38 has been connected to cytokine-induced pain by reducing the neuronal responsiveness to cytokines such as IL-1β and TNFα and thereby reducing cytokine-induced hyperalgesia [121,122].

Similar to ERK and p38 pathways, Jun-N terminus kinase (JNK) can also be activated by mitogens as well as a large variety of stimuli. The role of GRKs in JNK signaling in the immune system and inflammation is not very well characterized. GRK2 has a large and diverse regulatory capacity in the context of NFκB and MAPK signaling pathways that is dependent on expression level, cell type, stimulus, and many other factors. As we continue to study this role both *in vitro* and *in vivo* and our understanding of this regulation increases it remains a likely possibility that novel therapeutic targets and treatments for inflammatory diseases can develop from these interactions.

GRK2 Regulation of Immune Processes

An important component of an effective immune system is the ability for the immune cells to arrive at the site of inflammation. This is achieved through a process called chemotaxis, where cells at the site of inflammation or disease produce chemokines that act on the chemokine receptors (mostly GPCRs) on immune cells to initiate a chemotactic response. The amplitude of that response is dependent on the amount of chemokines produced and their gradient as well as the expression levels of the receptors that are responding to them. This process is also dependent on a series of different steps in the chemotactic process including receptor sensing, cell polarization, membrane protrusion, and adhesion/de-adhesion cycles all of which can be cell type and stimulus-specific [123]. Given that most of the chemokine receptors are GPCRs,

these receptors undergo desensitization with constant stimulus and it is not surprising that the GRKs regulating that process play a critical role in chemotaxis and in the immune response. Interestingly, GRK2, GRK3, GRK5 and GRK6 are all expressed at high levels in the immune cells suggesting that alteration or regulation of their expression levels or functionality will drastically influence immune cell chemotaxis and the pathological progression of disease.

Similar to signaling, GRK2 has been heavily studied in its role in chemotaxis; but looking at the regulation of cellular migration by this kinase is complex. Studies have shown that this regulation is both cell type and stimulus dependent, with GRK2 acting most often in a canonical manner negatively regulating GPCR signaling [113,118,124]. Overexpression of GRK2 by transfection in cell lines increases phosphorylation and/or desensitization of different chemokine receptors such as CCR2b [125], CCR5 [126], and CXCR1 [127]. Increased circulating neutrophils as well as increased macrophage localization to inflammatory sites have been observed in mice that are deficient for GRK2 in the myeloid cells or in bone marrow cells of low-density lipoprotein receptors knockout chimeric mice in an atherosclerosis model [66]. Consistently, both T lymphocytes and splenocytes isolated from GRK2 heterozygous mice had increased migration due to increased activation of ERK and PI3K/Akt pathways in response to CCL5 and CXCl12 compared to WT controls [62]. This, however, was not the case in the sepsis model in the myeloid-specific GRK2 knockout suggesting disease specific regulation [68]. Interestingly, a recent study has shown that acute mental stress significantly increased GRK2 levels in peripheral blood mononuclear cells (PBMCs) at both 30 and 60 minutes and had a positive correlation with levels of epinephrine that also increased after the stressor. This study suggests a link between stress and

intracellular inflammatory signaling and chemotaxis [128]. GRK2 levels are also reduced in cultured human T lymphocytes when exposed to oxidative stress or when co-cultured with activated neutrophils [129]. Additionally, GRK2 has been examined in the context of various human diseases and it was seen that neutrophils from patients with different disease conditions including malaria [130] and sepsis [101] have increased GRK2 levels that then have a decreased CXCR2 expression and reduced response to IL-8. When this phenomenon was studied in mice, exposure to IL-33 reversed this overexpression and restore CXCR2 expression leading to increased chemotaxis as well as increased bacterial clearance and survival [102].

In contrast to the above studies, GRK2 can also positively regulate chemotaxis in certain cell types. It's hypothesized that this bimodal role for GRK2 may depend on the cell type as well as the polarization state of the cells. In polarized cells, such as epithelial cells, GRK2 has been shown to positively regulate cellular migration independently of its catalytic activity thus supporting protein-protein interactions as a potential mechanism [131]. Further supporting this idea, membrane-targeted kinase mutants strongly enhance cell motility through interactions between GRK2 and GIT1 (GRK2-interacting factor) at the leading edge of polarized/migrating cells in wound healing assays [131]. This association between GRK2 and GIT1 is critical for proper ERK1/2 activation and efficient cellular migration. Recent work in our lab has shown in colon epithelial cells GRK2 knockdown enhances epithelial migration in wound healing assays also by critically regulating the activation of ERK1/2 in response to TNFα. These studies highlight the importance of ERK1/2 activation in polarized/migrating epithelial cells and the ability for GRK2 to influence migration and chemotaxis in a variety of manners [132]. GRK2 has also been shown to directly phosphorylate histone

deacetylase 6, a cytoplasmic histone deacetylase responsible for the deacetylation of tubulin and other substrates critical for cellular migration [133]. Additionally, GRK2 can interact with and phosphorylate ERM proteins ezrin and radixin, both of which contribute to F-actin polymerization-dependent motility [134,135]. These novel interactions and non-receptor substrates for GRK2 contribute to our understanding of GRK2s role in non-canonical regulation of GRK2 on cell motility.

GRK2 in Disease

In vitro experiments can give valuable insights into the mechanisms of GRK2 behavior but understanding the role of GRK2 in the context of human disease oftentimes is beyond the scope of an isolated cell line in culture. In mouse and human studies, GRK2 has been implicated in a variety of pathogenesis in multiple tissues and organ systems. GRK2 is a major player in several neurodegenerative diseases such as Alzheimer's disease (AD) [136], multiple sclerosis (MS) [61] and Parkinson's disease [137]. In AD, GRK2 was shown to serve as a biomarker for early hypoperfusion-induced brain damage, which is associated with mitochondrial damage seen in patients with AD [138]. GRK2 expression levels are also increased during the early stages of damage in aged human and in AD patients (observed postmortem) [138]. This increased expression may lead to more interactions with α and β-synuclein both of which are substrates for GRK2 and have been linked to both Parkinson's and Alzheimer's diseases [139]. Furthermore, GRK2 was shown to regulate metabotropic glutamate receptor function and expression, whose role has been implicated in AD and MS pathogenesis [140,141]. Also, studies have shown that GRK2s down-regulation of GRK2 following chronic inflammation sensitizes human and rodent neurons to excitotoxic neurodegeneration via over-action of group I mGluRs [141].

GRK2 has also been shown to play a prominent role in inflammatory hyperalgesia. GRK2 heterozygous mice suffer from chronic hyperalgesia due to continued microglial activation via p38-dependent TNFα production [121,142] and prolonged activation of prostaglandin E2-mediated pathway. This prolonged activation is mediated through interactions with EPAC1 (exchange protein directly activated cAMP) and activation of protein kinase Cε- and ERK-dependent pathways [143,144]. Through this same regulation of p38, GRK2 exacerbates brain damage in hypoxic-ischemic injury [119]. The importance of GRK2 in this system is highlighted in that even a transient decrease in GRK2 levels (by intrathecal injection of *GRK2* antisense oligodeoxynucleotides) is sufficient to produce long-lasting neuroplastic changes in nociceptor function that can lead to chronic pain [145].

Due to the cardiac developmental defects in the GRK2 homozygous knock out mice, GRK2 has been heavily studied in the context of cardiovascular diseases. GPCR signaling via the β-adrenergic receptor predominates during heart failure in an attempt to improve myocardial contractility and cardiac output. However, increased levels of GRK2 during heart failure causes an increase in β-adrenergic receptor desensitization reducing myocardial contractility and cardiac output worsening heart failure [146]. This reduced contractility triggers an influx of catecholamines, which leads to a cycle of activation and persistent desensitization of β-adrenergic receptors. Overexpression of GRK2 *in vivo* mimics this regulation and decreases myocardial contractility and cardiac output in response to adrenergic stimulation. These effects suggest impaired adrenergic receptor signaling [147,148]. Conversely, inhibition of GRK2 results in increased contractility and enhanced survival in heart failure models [74,149]. Furthermore, competitive inhibition through βARKct (C-terminal peptide of GRK2) prevented

cardiomyopathy and improved heart failure [150–153]. These benefits seen in the cardiovascular diseases have inspired researchers to try and develop GRK2 inhibitors as a therapeutic option in heart failure and has been met with success at least in preclinical models [154].

Changes in GRK2 expression has also been implicated in the pathogenesis of other diseases. Overexpression of GRK2 in vascular smooth muscle cells has been shown to lead to the development of hypertension [69,155] and this increase in GRK2 expression is also found in human patients with hypertension [156]. Also, GRK2 has been implicated in the development of atherosclerosis. Low-density lipoprotein receptor knockout mice with partial GRK2 deficiency in hematopoietic cells developed fewer atherosclerotic plaques [66]. This partial GRK2 deficiency led to increased infiltration of macrophages to inflammatory sites resulting in plaques with smaller necrotic cores. Finally, GRK2 has a significant role in the pathogenesis human and animal models of sepsis [101,102,68,82,157], polymicrobial sepsis [102,68,82], endotoxemia [67,157] and in the regulation of septic shock. Sepsis and a systemic inflammatory response syndrome are leading causes of mortality in intensive care units [158] and GPCRs play a major role in the pathophysiological events through regulation of the cardiovascular, immune and coagulatory responses. Given the role of GRK2 in the pathogenesis of sepsis, inhibition of GRK2 expression and/or activity may be a useful strategy for targeting human sepsis [102,68]. However given that GRK2 is ubiquitously expressed and its role varies in different tissues and cell types, inhibition of GRK2 may be beneficial in some diseases whereas it may be deleterious in others. Thus, while GRK2 inhibitors are being developed, it is important to keep in perspective the various roles of

GRK2 in different cell signaling pathways, the multitude of substrates/interactive partners and their potential involvement in the various diseases (**Figure 1.1.)**

GRK3: Biochemistry

GRK3, like GRK2, is ubiquitously expressed throughout the body but its expression levels are considered to be lower than those of GRK2 in all areas except for the central nervous system and the brain [159]. Despite these similarities in tissue expression, GRK3 has an independent role from GRK2 highlighted by its regulation in olfaction and neuronal functions. In the central nervous system, GRK3 is primarily located within the dorsal root ganglions and the olfaction neurons regulating the desensitization of odorant receptors and α2-adrenergic receptors [72].

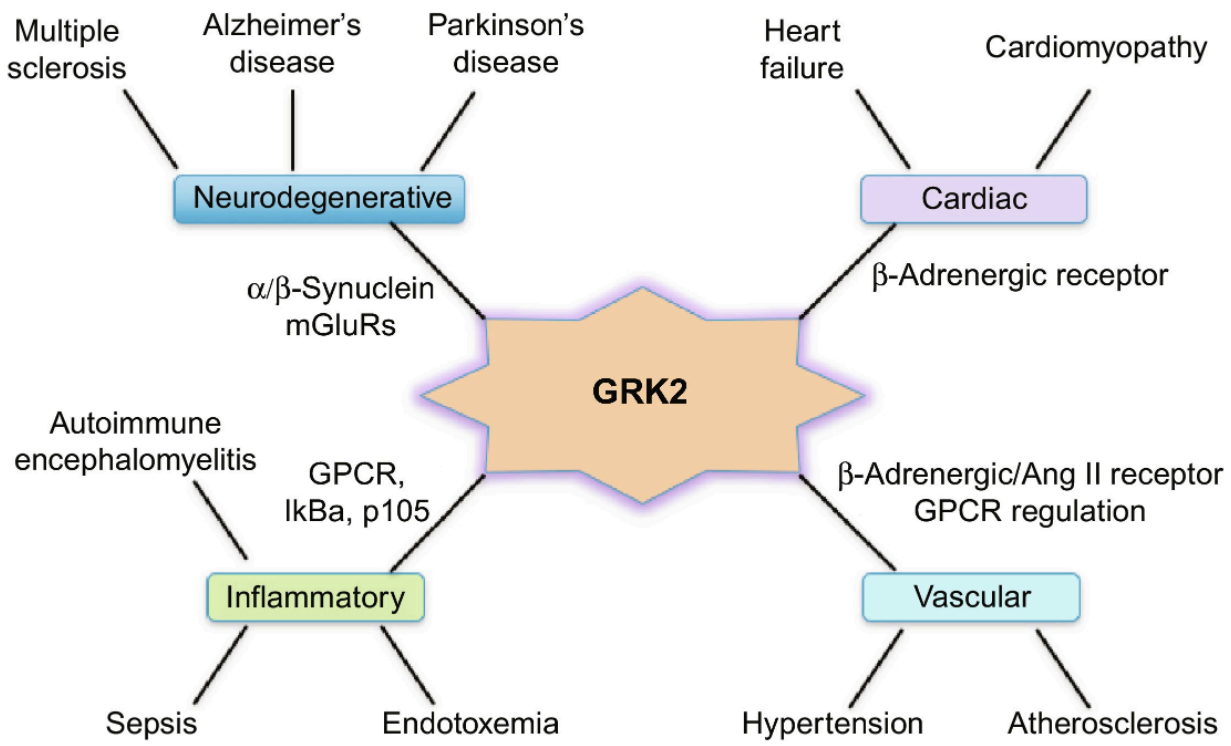
GRK3 in Inflammation

GRK3s role in inflammatory signaling has been less established than some of the other GRKs, such as GRK2 and GRK5. However, GRK3 has been shown to play a role in ERK1/2 signaling where overexpression of GRK3 abolished β-arrestin 2 mediated activation of ERK1/2. This was seen in response to activation of a variety receptors including the β2-adrenergic receptor, cannabinoid receptor-2 [107], angiotensin 1A receptor [83] and the insulin-like growth factor-1 receptor [108] (similar to GRK2). In support of this, bone marrow-derived leukocytes from GRK3 knockout mice exhibit enhanced ERK1/2 expression in response to CXCL12 [160]. GRK3 has the ability to interact with and modulate these pathways and so further study in this area is necessary to fully understand the regulatory capabilities of this GRK on these pathways.

GRK3 Regulation of Immune Processes

GRK2, GRK3, GRK5 and GRK6 are all expressed at high levels in the immune cells suggesting that alteration or regulation of their expression levels or functionality will

Figure 1.1. GRK2 and its potential role in various diseases processes



drastically influence immune cell chemotaxis and the pathological progression of disease. Compared to the other GRKs relatively little work has been done on the role of GRK3 in these cells however recent work has shown GRK3s ability to critically regulate immune behavior. GRK3 was identified as a negative regulator of CXCL12 (CXC chemokine ligand 12)/CXCR4 signaling (a defective pathway in human WHIM syndrome) and GRK3s deficiency results in impaired CXCL12-mediated desensitization, enhanced response to CXCR signaling through ERK1/2 activation, altered granulocyte chemotaxis and mild myelokathexis (a form of chronic leukopenia) [160]. Work done on human skin cells and leukocytes indicate that over-expression of GRK3 is able to restore CXCL12-induced internalization and desensitization of CXCR4 and normalize chemotaxis [161]. GRK3 is also important in other granulocyte-dependent disease models such as inflammatory arthritis by mediating the retention of cells in the bone marrow resulting in fewer circulating granulocytes in the blood as well as in the joints during inflammation. In conjunction with regulation of CXCR4, GRK3 has also been reported to play an important role in oncology and influence the tumorigenicity, molecular subtype and metastatic potential of triple negative breast cancer, glioblastoma, ovarian tumors, medulloblastoma and malignant granulosa cells through dysregulation of GPCR signaling [162–164]. In a cultured leukemia cell line, GRK3-mediated receptor phosphorylation of the chemokine receptor CC3A and association with β-arrestins were essential for expression of the chemokine CCL2, supporting the hypothesis that β-arrestins and GRK3 may contribute to chemotaxis in vivo at multiple levels [165]. GRK3 has also been shown to play a critical role in opioid receptor signaling in that Mu-opioid receptor and kappa-opioid desensitization is significantly slower in the GRK3 knock out mice [166].

GRK3 in Disease

Unlike GRK2, homozygous knockout mice of GRK3 are viable thus demonstrating different physiological roles for the members of the GRK2 family. GRK2 and GRK5 are well known for their role in cardiovascular diseases but the effect of GRK3 in this tissue is less understood. A study in transgenic mice that expressed a cardiac-specific GRK3 inhibitor showed that cardiac myocytes had significantly enhanced ERK1/2 activation in response to α_1-adrenergic receptor stimulation. Additionally, these transgenic mice showed elevated systolic blood pressure in comparison to littermate controls causing hypertension due to hyperkinetic myocardium and hyper-responsiveness to α_1-adrenergic receptor stimulation [74]. GRK3 has also been shown to be overexpressed in human prostate cancer and found to promote tumor growth and metastasis by enhancing angiogenesis [167]. Finally, based on its chromosomal location and sequence, GRK3 is believed to be a potential biomarker for bipolar disorder susceptibility through potential alterations of dopamine receptor desensitization [168]. In mice, GRK3 deficiency plays no role in basal locomotor activity but interestingly, completely knocking out GRK3 significantly reduces locomotor and climbing responses to either cocaine or apomorphine [169] potentially through the desensitization of D3 dopamine autoreceptors [170].

GRK4 SUBFAMILY

The GRK4 subfamily is comprised of GRK4, GRK5, and GRK6 and is located ubiquitously throughout the body with the exception of GRK4, which is located primarily in the testes, kidney and cerebellum [35]. Structurally all members share the AGC kinase domain and a common N-terminus domain that expresses a PIP_2 binding site that is similar to the GRK2 subfamily. The C-terminal domain aids in membrane localization and is regulated through palmitoylation sites on GRK4 and GRK6 but GRK5 is regulated through positively charged lipid-binding amphipathic helices [2]. This regulation establishes an equilibrium between these GRKs and the membrane allowing for phosphorylation of both activated and inactivated GPCRs [75]. Interestingly, GRK4 has low sequence conservation with GRK5 and GRK6 in the C-terminal region and forms a different crystal structure. These differences between GRK4 and the rest of the subfamily have been shown to influence its ability to translocate to the plasma membrane [171]. It has been hypothesized that these changes in structure and membrane targeting capabilities may be a result of the tissue-specific expression of GRK4 and may account for some of the functional variability amongst this subfamily.

GRK4 in Inflammation and Disease

GRK4 can be regulated and alternatively spliced into four different splice variants that are all capable of independent functions including GCPR regulation. As indicated before, GRK4 has not been examined in detail in immune cells and its role in inflammatory disease is largely unknown. It is known that GRK4α constitutively phosphorylates the dopamine receptor in the proximal tubule reducing its responsiveness hinting at an essential role in the regulation of hypertension. In fact, constitutive phosphorylation of the dopamine receptor is problematic in people with

hypertension and polymorphisms in GRK4 can cause dysregulation of dopamine-stimulated salt and fluid excretion in the kidneys [76]. Similarly, GRK4γ is able to phosphorylate the dopamine receptor as well but not constitutively and only upon agonist activation. These differences between splice variants indicate areas of similarities but also variability amongst the splice variants leading to questions about the regulation of GRK4 translation that have yet to be answered [172]. GRK4's role in the other tissues where it is expressed is less clear and requires further study.

Expression of GRK4 in the cerebellum seems to be limited to the Purkinje cells and may be involved in combination with GRK2 in the regulation of metabotropic glutamate 1 receptors. The regulation of these receptors suggests a role for GRK4 in motor coordination as well as in learning but further work remains to be done in this area [173]. In a recent study, it was shown that GRK4 levels are upregulated in *Fmr*1-null mice, suggesting that RNA-binding protein (FMRP) negatively regulates the expression of GRK4. Fragile X syndrome is caused by the silencing of the *Fmr1* gene and lack of FMRP, and since FMRP regulates negatively regulates GRK4 it is hypothesized that this silencing results in increased GRK4 levels in Fragile-X syndrome and these increased levels contribute to cerebellum-dependent phenotypes due to deregulated desensitization of GABAB receptors [174]. Finally, due to its localization in the cerebellum and testes studies on the GRK4 knockout mouse were done investigating its role in those tissues. In these studies, GRK4 knockout animals showed no differences in basal levels or in response to cocaine for locomotor activity or motor coordination [169]. Furthermore, despite the high levels of GRK4 in the testes, there were no apparent changes in fertility or sperm function and no obvious phenotypes were detected in these animals [75]. Thus, the role of GRK4 in these tissues remains to

be elucidated and more work needs to be done to fully understand the role of this kinase.

GRK5: Biochemistry

GRK5 is unique from its family members due to the presence of the positively charged lipid-binding amphipathic helixes on its C-terminus and there is evidence supporting an interaction with the membrane through this C-terminus involving a region of the RH domain [171]. These motifs, in combination with the polybasic N-terminus regions predominantly localize GRK5 to the plasma membrane. This presence at the membrane allows for significant activation independent phosphorylation of several GPCRs [36]. GRK5 has recently been crystalized in two unique structures both of which crystalize GRK5 as a monomer with its C-termini forming consistent structures that pack closely to the RH domain. Disturbing this interface between the C-terminus and the RH domain significantly decreased the catalytic activity on GPCRs in both *in vitro* and in cells. This same study concluded that individual GRK5 subunits were insufficient for persistent membrane association [171]. Additionally, GRK5 also has a nuclear localization motif and can accumulate in the nucleus where it can interact with proteins including class II histone deacetylase and mediate gene transcription [34]. This localization is in competition with GRK5's membrane localization sequences on the C and N-termini, these termini are in fact key regulatory sites and phosphorylation by PKC on its C-terminus is inhibitory to its function. Calmodulin (CaM) is inhibitory to most GRKs but is especially preferential to the GRK4 subfamily. Ca^{2+}□CaM can bind to both the N- and C-terminal membrane phospholipid binding motifs and disrupt the membrane association of GRK5 allowing the nuclear localization motif to direct GRK5 to the nucleus [175]. As mentioned, GRK5 has ubiquitous expression but is especially

expressed at high levels in the heart, lungs, placenta, and kidney with lower expression in the brain (except for the limbic system). Interestingly, expression levels of GRK5 have been shown to increase two-fold during neuronal differentiation [35].

GRK5 Role in Inflammation

Similar to GRK2, GRK5 plays a major role in NFκB signaling and is able to directly interact with and phosphorylate NFκB p105 (one of the IκB members). This was shown to inhibit Toll-like receptor-4-induced IκB kinase β-mediated phosphorylation of p105, thus negatively regulating lipopolysaccharide-induced ERK activation in macrophages [176]. Furthermore, GRK5 was subsequently discovered to interact with an additional member of the IκB family, IκBα, to facilitate the nuclear accumulation of IκBα by masking its nuclear export signal. This nuclear accumulation of IκBα led directly to decreased NFκB activation in endothelial cells [83]. Both of these interactions were shown to be dependent on the RH domain of GRK5 and independent of its kinase domain. Further research has also shown non-canonical regulation of NFκB by GRK5. GRK5 was demonstrated to phosphorylate Ser32/36 – the same sites phosphorylated by IκB kinase β and IκBα kinase – and was supported by biochemical findings showing decreased levels of cytokines and chemokines in GRK5 knockout mice compared to wild-type in endotoxemia model [177,178]. Further evidence showed that IκBα phosphorylation and p65 nuclear translocation were significantly reduced in LPS-treated GRK5 deficient peritoneal macrophages. As these studies progressed, there have been contrasting results in different models of the GRK5 knockout mouse. Wherein mice generated by the Lefkowitz group [179] found that endothelial GRK5 stabilized IκBα similar to earlier studies by Sorriento [83], studies done in macrophages using a different GRK5 knock out mouse did not show any role for GRK5 in IκBα

phosphorylation or p65 translocation. More recent studies using mice from the Lefkowitz group, however, have shown that GRK5 positively regulates the NFκB pathway in cardiomyocytes [81]. It is unclear the exact reasoning behind these discrepancies in GRK5 behavior but it has also been shown that GRK5 itself can be regulated by the NFκB pathway, generating a potential positive feedback loop between GRK5 and NFκB [180]. However, in primary peritoneal macrophages, GRK5 was observed to be down regulated after TLR4 activation suggesting that both regulation of GRK5 expression and its influence on the NFκB pathway are distinct in different cell types and conditions. Interestingly, however, GRK5 was shown to be an evolutionarily conserved kinase in NFκB regulation in human cell lines as well as different species including *Drosophilia* and zebrafish [181] thus demonstrating an important role for this kinase in this pathway.

There are some studies examining the relationship between GRK5 and the MAPK signaling pathways. GRK5 has been shown to play a role in the support of β-arrestin 2 mediated ERK activation after the activation of the V2 vasopressin and angiotensin II receptors despite its smaller role in desensitization in comparison to GRK2 or GRK3 [95,106] and was later determined to independent of G-protein coupling [182]. Conversely, in macrophages through its regulation of p105 GRK5 negatively regulates LPS-stimulated ERK activation [176]. JNK can be activated by mitogens as well as a large variety of stimuli including: environmental stresses (heat shock, ionizing radiation and oxidants), genotoxins (topoisomerase inhibitors and alkylating agents), ischemic-reperfusion injury, mechanical shear stress, vasoactive peptides, proinflammatory cytokines and pathogen-associated molecular pattern molecules/danger-associated molecular patterns [183–187]. JNK activation induces the translocation of transcription factors AP-1, c-Jun, ATF-2 and ELK-1 to the nucleus to

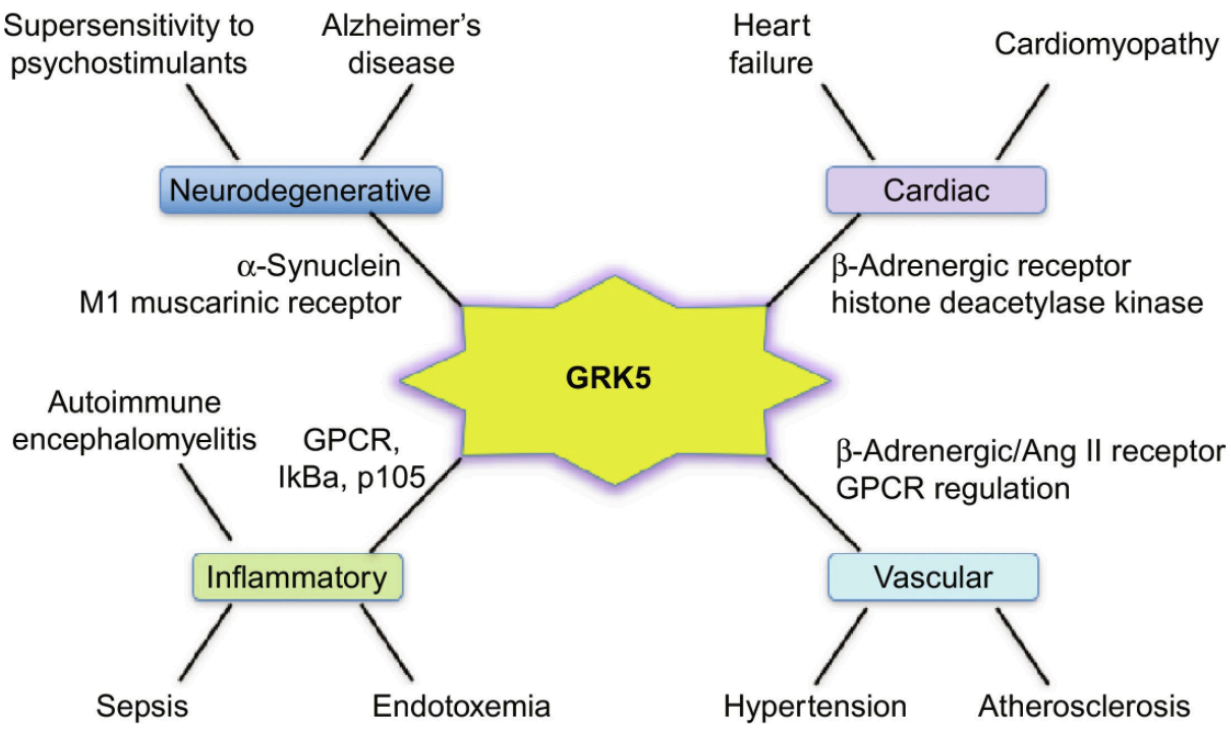
regulate inflammatory gene transcription [185]. JNK activation of AP-1 also plays a critical role in the synthesis of TNFα and for the proliferation and differentiation of lymphocytes, giving it a vital role in the regulation of the immune system [187,188]. In mice over-expressing GRK5 in cardiac myocytes they constitutively active mutants of α_{1B}AR exhibited attenuation of JNK activation in comparison to the controls. GRK5 also had an influence of α_{1B}AR signaling but the complexity of this interaction and regulation remains to be fully elucidated [189]. Finally, there is little known about the relationship between GRK5 and p38, highlighting an area of study that still requires further work to understand the role for this kinase in the MAPK processes.

It is clear that GRK5 plays a critical role in the regulation of NFκB and MAPK signaling in inflammation and immune signaling. With further understanding of this regulation, it may become clearer if these signaling pathways are targetable regulatory sites for therapeutics in inflammatory diseases (**Figure 1.2.)**.

GRK5 Regulation of Immune Processes

Similar to GRK2, GRK5 can also regulate GPCRs necessary for chemotaxis in both a canonical and non-canonical fashion. Unlike GRK2, GRK5 has been shown to regulate these processes in much fewer cell types, primarily in monocytes. GRK5 modulates chemotaxis in these cells by regulating CCR2, a GPCR for monocyte chemoattractant protein-1 [179]. GRK5 can also influence these cells in a non-canonical fashion through the regulation of colony-stimulating factor-1 receptor, a receptor tyrosine kinase [179]. Through this inhibition, desensitization of CCR2 and inhibition of monocyte migration, GRK5 has been shown to attenuate atherosclerosis [179]. Additionally, GRK5 can regulate CXCR4 desensitization via phosphorylation of HIP

Figure 1.2. GRK5 and its potential role in various disease processes



(HSP70-interacting protein) in an *in vitro* model [190]. Work in our lab, however, has shown that GRK5 has little to no role in immune chemotaxis in a polymicrobial septic peritonitis model [82] and similar findings were reported in an arthritic model [191]. This however, was again model specific since GRK5 did regulate neutrophil infiltration in *E.* Coli pneumonia model [192]. Similar to GRK3, GRK5 has been shown to have an influence on the migration and invasion of cancer cells. In prostate cancer, GRK5 has been shown to regulate cell migration and invasion through interactions with moesin (ERM (ezrin-radixin-moesin)) proteins. It accomplishes this primarily through phosphorylation of moesin on the Thr-66 residue regulating its cellular distribution [193]. Additionally, GRK5 may be involved in neutrophil granulocyte exocytosis, signal transduction pathways regulating this function are poorly defined although several proteins, including Src, are known to participate in this response. Proteomic studies first identified a potential Src tyrosine kinase motif on GRK5 and the phosphorylation of GRK5 by Src was then confirmed in an *in vitro* kinase assay. Furthermore, immunoprecipitation of phosphorylated GRK5 with Src in intact cells was decreased in the presence of a Src inhibitor possibly implicating it in the regulation of granulocyte exocytosis [194].

GRK5 in Disease

GRK5 also plays a prominent role in a multitude of pathologies. GRK5 canonically regulates muscarinic receptors but shows a preference for M2 and M4 receptors [195]. These receptors are present in the presynaptic cells and negatively regulate acetylcholine release in hippocampal memory circuits. Abnormal increases in presynaptic cholinergic activity causes a decrease in acetylcholine release, therefore, decreasing postsynaptic muscarinic M1 activity. M1 signaling has been shown to inhibit

β-amyloidogenic APP processing and decreased β-amyloid accumulation [196]. This connection might be the reason that an Alzheimer's disease (AD)-like pathology is observed in GRK5-deficient mice. Further connecting GRK5 to AD is its ability to phosphorylate α-synuclein and tubulin possibly influencing their polymerization and neuronal functions as seen in AD [197]. GRK5 has many similarities to GRK2 in its role in cardiovascular diseases. Expression and activity of GRK5 is increased during heart failure inducing β-adrenergic receptor desensitization reducing myocardial contractility and worsening heart failure [148]. Overexpression of GRK5 in these cells *in vivo* decreases myocardial contraction and cardiac output in response to adrenergic stimulation [147,148] and its inhibition results in increased cardiac contractility and enhanced survival in heart failure patients [74,149]. Inhibition of GRK5 with adGRK5-NT (N-terminal peptide of GRK5) prevented cardiomyopathy and improved heart failure [150–153]. Again similar to GRK2, overexpression of GRK5 in the vascular smooth muscle cells led to the development of hypertension through regulation of β-1adrenergic receptor and Angiotensin II receptors [69,155]. In addition to these canonical GPCR dependent roles, GRK5, through its nuclear localization signal, has been shown to accumulate in the nucleus of cardiomyocytes and function as a histone deacetylase kinase and promote cardiac hypertrophy [34]. GRK5 has also been implicated in the development of atherosclerosis and its deficiency in apolipoprotein E knockout mice leads to development more aortic atherosclerosis than their wild-type controls [179]. Investigating the role of GRK5 in endotoxemia and polymicrobial sepsis models, our lab has shown that GRK5 deficiency leads to diminished cytokine levels [82,157,178] likely through decreased NFκB activation in tissues and macrophages (as outlined above). In addition to these reduced cytokine levels, GRK5-deficient mice also showed decreased

thymocyte apoptosis and immune suppression [82]. This immune suppression was attributed to reduced plasma corticosterone levels in these mice suggesting regulation of the pituitary-adrenal axis by GRK5. With its diverse role in inflammatory signaling as well as its role in multiple diseases furthering our understanding of this kinase is critical for our ability to effectively design therapeutics and treatments targeting GRK5's role in these various pathologies.

GRK6: Biochemistry

GRK6 is present ubiquitously throughout the body but is one of the most prominent GRKs in the striatum and other dopaminergic brain areas, particularly in the GABAergic and cholinergic areas. In these tissues, GRK6 localizes primarily in the plasma membrane although GRK6α, one of its four splice variants, can translocate to the nucleus although its function there is unclear [53]. GRK6s subcellular localization is regulated through both its C-terminus which can be palmitoylated which can help anchor this GRK to the plasma membrane [198]. Interestingly, in the brain GRK6 is primarily localized to synaptic membranes [199], suggesting that there must be neuronal specific mechanisms for altering the localization of GRK6 although these specific protein partners remain unknown. For its role in GPCR regulation and desensitization GRK6 has been shown to phosphorylate GPCRs in both an activated and an inactivated state with some notable exceptions such as the D_1 dopamine receptor [36]. Structurally, GRK6 is currently the only member of the family that has been successfully crystallized in a conformation that is expected to be an active conformation and is one of the only two structures that were able to detect the N-terminal region (along with GRK1). It was shown in these structures that the extreme N-terminal amino acids become helical and are able to interact with both lobes of the kinase domain helping to align the various

catalytic residues contributed by each lobe [47]. Mutational studies examining the role of these helical N-terminal motifs supported the model where the exposed hydrophobic residues at the apex of the helix are critical for GPCR phosphorylation [47].

GRK6 in Inflammation

GRK6 has been shown to be involved in NFκB signaling in the manner that it can phosphorylate IκBα at Ser32/36, similar to the role of GRK5. This phosphorylation of IκBα by GRK6 regulates inflammation in peritoneal macrophages when stimulated by TNFα by inducing a conformational change in GRK6 influencing its activity [200]. GRK6 also plays a role in the regulation of mitogen-activated protein kinases (MAPKs) by interacting with the p38 pathway. GRK6 is able to interact with and regulate p38 and down-regulate its activation. This down-regulation has been connected to cytokine-induced pain by reducing the neuronal responsiveness to cytokines such as IL-1β and TNFα and thereby reducing cytokine-induced hyperalgesia [121,122]. Furthermore, GRK6 can play a role in the dysregulated signaling of ERK and p38 after L-DOPA therapy for Parkinson's disease. This therapy results in a side effect termed L-DOPA induced dyskinesia (LID) that causes decreased expression of GRKs resulting in defective desensitization of dopamine receptors. Overexpressing GRK6 ameliorated supersensitive ERK and p38 MAP kinase responses to L-DOPA challenges normalizing the responses of these signaling pathways to control animals [201]. Investigating the role of GRK6 in C-kit positive cardiac stem cells (CSCs) it was shown that cells treated with GRK6 siRNA and stimulated with SCF had a significant decrease in the phosphorylation of p38 and ERK1/2 through alterations in the phosphorylation state of CXCR4-serine 339. This alteration in MAPK signaling influenced these cells ability to regulate c-kit ligand stem cell factor (SCF) induced migration during myocardial

infarction [202]. GRK6 has been shown to play a critical role in β-arrestin 2 mediated ERK activation in correlation with the V2 vasopressin and angiotensin II receptors despite its diminished role in desensitization in comparison to GRK2 or GRK3 [95,106] and was later discovered that this was independent of G-protein activation [182]. Alterations in this βarrestin mediated ERK activation can be seen in pathological conditions and receptor mutations. For example, in the immunodeficiency syndrome WHIM mutations in the CXCR4 receptor cause impairs GRK6's ability to associate and phosphorylate these receptors leading to a lack of βarrestin-2 recruitment and subsequent ERK phosphorylation [203]. GRK6 has a clear role in the regulation of p38 and ERK signaling it however remains unclear for its role in JNK signaling and further work in this area is needed to fully appreciate GRK6's role in MAPK signaling.

Taken together this signaling data shows that GRK6 plays a role in NFκB and MAPK signaling although the specific regulation of this pathway largely remains less understood. More work in this area will help elucidate the relationship between GRK6 and these inflammatory pathways in the immune system.

GRK6 Regulation of Immune Processes

GRK6 is also expressed at high levels in immune cells and GRK6 has been shown to regulate receptors CXCR2 [127], CXCR4 [87] and LTB4 [85] influencing immune trafficking. This regulation and desensitization influences neutrophil and lymphocyte recruitment to sites of pathogenesis in several different disease models including inflammatory arthritis and DSS-induced colitis [191,86,204]. In epithelial cells, GRK6 has been identified as a critical mediator in integrin-mediated cell adhesion and migration of tumor cells [205] and GRK6 deficiency promotes CXCR2-mediated progression and metastasis in a lung carcinoma model [206]. These effects are

primarily through GRK6s canonical regulation of GPCRs with little evidence for a non-canonical role in immune cell chemotaxis. GRK6 can also influence the hematopoietic process and mice lacking GRK6 have been demonstrated to exhibit lymphocytopenia, a loss of hematopoietic stem cells (HSC), and multiple progenitor populations. This deficiency leads to impaired lymphoid differentiation due to impairment of HSC self-renewal. A possible mechanism for these effects come from transcriptome and proteomic analysis suggesting that GRK6 alters these functions through its involvement in reactive oxygen species signaling and/or through its interaction with DNA-PKcs [207].

GRK6 in Disease

Recent studies have implicated GRK6 in regulating the clearance of apoptotic cells through interaction with GIT1 to activate Rac1, promoting apoptotic engulfment [208]. GRK6 was critical in the removal of apoptotic B cells by splenic white pulp macrophages and in removing senescent red blood cells by splenic red pulp macrophages. In a different study in GRK6-deficient mice F4/80+ macrophages were seen to have this same effect on senescent red blood cell clearance possibly as a result of increased iron stores in the splenic red pulp. In response to this uptake of apoptotic cells, macrophages, in general induce production and release of immunosuppressive cytokines such as IL-10, tumor growth factor-β, prostaglandin E2 and platelet-activating factor [209] while suppressing the production of proinflammatory cytokines IL1-β, IL-6, IL-12 and TNFα [210]. Because of these connections, apoptotic cells have a significant impact on the functionality of these phagocytes that can, in turn, modulate inflammatory disease pathogenesis. GRK6 has been shown to modulate these processes in macrophages by phosphorylation of radixin and moesin, both of which are essential for cytoskeleton reorganization and immune cell chemotaxis and phagocytosis. Indeed,

GRK6-deficient mice were shown to develop autoimmune disease possibly because of alterations in the response to immune cell phagocytosis. Additionally, GRK6 has been implicated in other diseases and has been shown to modulate arthritis and colitis by regulating immune cell chemotaxis and infiltration [191,204]. Specifically, in DSS-induced colitis model, GRK6 deficient mice produced more keratinocyte chemokine (the murine equivalent of IL-8), caused increased infiltration of immune cells and enhanced severity of disease manifestations. Similar to the colitis model, GRK6-deficient mice mimicked the increased weight loss and severity of disease in the arthritis model. Finally, studies focusing on the role of GRK6 in the brain have shown that D2 dopamine receptors are direct targets for GRK6 and GRK6 knock out mice, unlike all other GRK mouse models, show significantly enhanced responsiveness to psychostimulants and other dopamine agonists [57]. Due to this enhanced response GRK6 has been linked to Parkinson's disorder and potentially could influence other diseases related to dopamine supersensitivity including addiction, schizophrenia and Tourette's syndrome though these have not been thoroughly investigated.

CONCLUSION

GPCRs comprise the largest class of receptors in the human genome and represent one of the most popular targets for modern therapeutics. With only four universally present GRKs and three others in specific subsets of tissues, understanding their numerous physiological roles in maintaining homeostasis is critical in our continued and future use in targeting these kinases for treatments. This task has been greatly aided by the discovery of crystal structures for various GRKs and the pathophysiological role of GRKs in both their canonical GPCR-dependent function as well as their non-canonical functions in numerous diseases. Despite their limited expression, it is clear that these isoforms are not entirely interchangeable and these GRKs have very defined roles in certain tissues. The use of transgenic and knockout animals has greatly aided our progress in these studies but with new information, we see overlapping functionalities and compensatory mechanisms from other GRKs and other associated proteins adding to the complexity of these studies. As new functions and substrates are unveiled for GRKs it is critical to take into consideration GRKs substrate diversity and the role they will play when targeted for therapeutic purposes.

REFERENCES

REFERENCES

1 Takeda, S., Kadowaki, S., Haga, T., Takaesu, H. and Mitaku, S. (2002) Identification of G protein-coupled receptor genes from the human genome sequence. FEBS Lett. **520**, 97–101.

2 Cabrera-Vera, T. M., Vanhauwe, J., Thomas, T. O., Medkova, M., Preininger, A., Mazzoni, M. R. and Hamm, H. E. (2003) Insights into G protein structure, function, and regulation. Endocr. Rev. **24**, 765–81.

3 Ritter, S. L. and Hall, R. A. (2009) Fine-tuning of GPCR activity by receptor-interacting proteins. Nat. Rev. Mol. Cell Biol., Nature Publishing Group **10**, 819–30.

4 Hewavitharana, T. and Wedegaertner, P. B. (2012) Non-canonical signaling and localizations of heterotrimeric G proteins. Cell. Signal. **24**, 25–34.

5 Oldham, W. M. and Hamm, H. E. (2008) Heterotrimeric G protein activation by G-protein-coupled receptors. Nat. Rev. Mol. Cell Biol. **9**, 60–71.

6 Preininger, A. M. and Hamm, H. E. (2004) G protein signaling: insights from new structures. Sci. STKE **2004**, re3.

7 Lefkowitz, R. J. and Shenoy, S. K. (2005) Transduction of receptor signals by beta-arrestins. Science **308**, 512–7.

8 Nobles, K. N., Xiao, K., Ahn, S., Shukla, A. K., Lam, C. M., Rajagopal, S., Strachan, R. T., Huang, T.-Y., Bressler, E. A., Hara, M. R., et al. (2011) Distinct phosphorylation sites on the β(2)-adrenergic receptor establish a barcode that encodes differential functions of β-arrestin. Sci. Signal. **4**, ra51.

9 Cahill, T. J., Thomsen, A. R. B., Tarrasch, J. T., Plouffe, B., Nguyen, A. H., Yang, F., Huang, L.-Y., Kahsai, A. W., Bassoni, D. L., Gavino, B. J., et al. (2017) Distinct conformations of GPCR-β-arrestin complexes mediate desensitization, signaling, and endocytosis. Proc. Natl. Acad. Sci. U. S. A. **114**, 2562–2567.

10 Lefkowitz, R. J. (2013) A brief history of G-protein coupled receptors (Nobel Lecture). Angew. Chem. Int. Ed. Engl. **52**, 6366–78.

11 Thomsen, A. R. B., Plouffe, B., Cahill, T. J., Shukla, A. K., Tarrasch, J. T., Dosey, A. M., Kahsai, A. W., Strachan, R. T., Pani, B., Mahoney, J. P., et al. (2016) GPCR-G Protein-β-Arrestin Super-Complex Mediates Sustained G Protein Signaling. Cell, Elsevier Inc. **166**, 907–19.

12 Gurevich, V. V and Gurevich, E. V. (2004) The molecular acrobatics of arrestin activation. Trends Pharmacol. Sci. **25**, 105–11.

13 Luttrell, L. M. (2005) Composition and function of g protein-coupled receptor signalsomes controlling mitogen-activated protein kinase activity. J. Mol. Neurosci. **26**, 253–64.

14 Howard, A. D., McAllister, G., Feighner, S. D., Liu, Q., Nargund, R. P., Van der Ploeg, L. H. and Patchett, A. A. (2001) Orphan G-protein-coupled receptors and natural ligand discovery. Trends Pharmacol. Sci. **22**, 132–40.

15 Thompson, M. D., Burnham, W. M. and Cole, D. E. C. (2005) The G protein-coupled receptors: pharmacogenetics and disease. Crit. Rev. Clin. Lab. Sci. **42**, 311–92.

16 Rodbell, M., Krans, H. M., Pohl, S. L. and Birnbaumer, L. (1971) The glucagon-sensitive adenyl cyclase system in plasma membranes of rat liver. IV. Effects of guanylnucleotides on binding of 125I-glucagon. J. Biol. Chem. **246**, 1872–6.

17 Ross, E. M. and Gilman, A. G. (1977) Resolution of some components of adenylate cyclase necessary for catalytic activity. J. Biol. Chem. **252**, 6966–9.

18 Bownds, D., Dawes, J., Miller, J. and Stahlman, M. (1972) Phosphorylation of frog photoreceptor membranes induced by light. Nat. New Biol. **237**, 125–7.

19 Benovic, J. L., Deblasi, A., Stone, W. C., Caron, M. G. and Lefkowitz, R. J. (1989) Beta-Adrenergic Receptor Kinase: Primary Structure Delineates a Multigene Family. Science (80-.). **246**, 235–240.

20 Wacker, W. B., Donoso, L. A., Kalsow, C. M., Yankeelov, J. A. and Organisciak, D. T. (1977) Experimental allergic uveitis. Isolation, characterization, and localization of a soluble uveitopathogenic antigen from bovine retina. J. Immunol. **119**, 1949–58.

21 Zuckerman, R. and Cheasty, J. E. (1986) A 48 kDa protein arrests cGMP phosphodiesterase activation in retinal rod disk membranes. FEBS Lett. **207**, 35–41.

22 Wilden, U., Hall, S. W. and Kühn, H. (1986) Phosphodiesterase activation by photoexcited rhodopsin is quenched when rhodopsin is phosphorylated and binds the intrinsic 48-kDa protein of rod outer segments. Proc. Natl. Acad. Sci. U. S. A. **83**, 1174–8.

23 Benovic, J. L., Onorato, J. J., Arriza, J. L., Stone, W. C., Lohse, M., Jenkins, N. a, Gilbert, D. J., Copeland, N. G., Caron, M. G. and Lefkowitz, R. J. (1991) Cloning, expression, and chromosomal localization of beta-adrenergic receptor kinase 2. A new member of the receptor kinase family. J. Biol. Chem. **266**, 14939–46.

24 Ambrose, C., James, M., Barnes, G., Lin, C., Bates, G., Altherr, M., Duyao, M., Groot, N., Church, D. and Wasmuth, J. J. (1992) A novel G protein-coupled receptor kinase gene cloned from 4p16.3. Hum. Mol. Genet. **1**, 697–703.

25 Kunapuli, P. and Benovic, J. L. (1993) Cloning and expression of GRK5: a member of the G protein-coupled receptor kinase family. Proc. Natl. Acad. Sci. U. S. A. **90**, 5588–92.

26 Premont, R. T., Macrae, A. D., Stoffel, R. H., Chung, N., Pitcher, J. A., Ambrose, C., Inglese, J., MacDonald, M. E. and Lefkowitz, R. J. (1996) Characterization of the G protein-coupled receptor kinase GRK4. Identification of four splice variants. J. Biol. Chem. **271**, 6403–10.

27 Benovic, J. L. and Gomez, J. (1993) Molecular cloning and expression of GRK6. A new member of the G protein-coupled receptor kinase family. J. Biol. Chem. **268**, 19521–7.

28 Hisatomi, O., Matsuda, S., Satoh, T., Kotaka, S., Imanishi, Y. and Tokunaga, F. (1998) A novel subtype of G-protein-coupled receptor kinase, GRK7, in teleost cone photoreceptors. FEBS Lett. **424**, 159–64.

29 Weiss, E. R., Raman, D., Shirakawa, S., Ducceschi, M. H., Bertram, P. T., Wong, F., Kraft, T. W. and Osawa, S. (1998) The cloning of GRK7, a candidate cone opsin kinase, from cone- and rod-dominant mammalian retinas. Mol. Vis. **4**, 27.

30 Mushegian, A., Gurevich, V. V. and Gurevich, E. V. (2012) The origin and evolution of G protein-coupled receptor kinases. PLoS One **7**, e33806.

31 Siderovski, D. P., Hessel, A., Chung, S., Mak, T. W. and Tyers, M. (1996) A new family of regulators of G-protein-coupled receptors? Curr. Biol. **6**, 211–2.

32 Touhara, K., Inglese, J., Pitcher, J. A., Shaw, G. and Lefkowitz, R. J. (1994) Binding of G protein beta gamma-subunits to pleckstrin homology domains. J. Biol. Chem. **269**, 10217–20.

33 Premont, R. T., Macrae, A. D., Aparicio, S. A., Kendall, H. E., Welch, J. E. and Lefkowitz, R. J. (1999) The GRK4 subfamily of G protein-coupled receptor kinases. Alternative splicing, gene organization, and sequence conservation. J. Biol. Chem. **274**, 29381–9.

34 Martini, J. S., Raake, P., Vinge, L. E., DeGeorge, B. R., DeGeorge, B., Chuprun, J. K., Harris, D. M., Gao, E., Eckhart, A. D., Pitcher, J. A., et al. (2008) Uncovering G protein-coupled receptor kinase-5 as a histone deacetylase kinase in the nucleus of cardiomyocytes. Proc. Natl. Acad. Sci. U. S. A. **105**, 12457–62.

35 Inglese, J., Freedman, N. J., Koch, W. J. and Lefkowitz, R. J. (1993) Structure and mechanism of the G protein-coupled receptor kinases. J. Biol. Chem. **268**, 23735–8.

36 Li, L., Homan, K. T., Vishnivetskiy, S. A., Manglik, A., Tesmer, J. J. G., Gurevich, V. V. and Gurevich, E. V. (2015) G Protein-coupled Receptor Kinases of the GRK4 Protein Subfamily Phosphorylate Inactive G Protein-coupled Receptors (GPCRs). J. Biol. Chem. **290**, 10775–90.

37 DebBurman, S. K., Ptasienski, J., Benovic, J. L. and Hosey, M. M. (1996) G protein-coupled receptor kinase GRK2 is a phospholipid-dependent enzyme that can be conditionally activated by G protein betagamma subunits. J. Biol. Chem. **271**, 22552–62.

38 Chuang, T. T., Paolucci, L. and De Blasi, A. (1996) Inhibition of G protein-coupled receptor kinase subtypes by Ca2+/calmodulin. J. Biol. Chem. **271**, 28691–6.

39 Carman, C. V, Lisanti, M. P. and Benovic, J. L. (1999) Regulation of G protein-coupled receptor kinases by caveolin. J. Biol. Chem. **274**, 8858–64.

40 Pitcher, J. A., Fredericks, Z. L., Stone, W. C., Premont, R. T., Stoffel, R. H., Koch, W. J. and Lefkowitz, R. J. (1996) Phosphatidylinositol 4,5-bisphosphate (PIP2)-enhanced G protein-coupled receptor kinase (GRK) activity. Location, structure, and regulation of the PIP2 binding site distinguishes the GRK subfamilies. J. Biol. Chem. **271**, 24907–13.

41 Horner, T. J., Osawa, S., Schaller, M. D. and Weiss, E. R. (2005) Phosphorylation of GRK1 and GRK7 by cAMP-dependent protein kinase attenuates their enzymatic activities. J. Biol. Chem. **280**, 28241–50.

42 Pronin, A. N. and Benovic, J. L. (1997) Regulation of the G protein-coupled receptor kinase GRK5 by protein kinase C. J. Biol. Chem. **272**, 3806–12.

43 Chuang, T. T., LeVine, H. and De Blasi, A. (1995) Phosphorylation and activation of beta-adrenergic receptor kinase by protein kinase C. J. Biol. Chem. **270**, 18660–5.

44 Whalen, E. J., Foster, M. W., Matsumoto, A., Ozawa, K., Violin, J. D., Que, L. G., Nelson, C. D., Benhar, M., Keys, J. R., Rockman, H. A., et al. (2007) Regulation of beta-adrenergic receptor signaling by S-nitrosylation of G-protein-coupled receptor kinase 2. Cell **129**, 511–22.

45 Farrens, D. L., Altenbach, C., Yang, K., Hubbell, W. L. and Khorana, H. G. (1996) Requirement of rigid-body motion of transmembrane helices for light activation of rhodopsin. Science **274**, 768–70.

46 Homan, K. T. and Tesmer, J. J. G. (2014) Structural insights into G protein-coupled receptor kinase function. Curr. Opin. Cell Biol., Elsevier Ltd **27**, 25–31.

47 Boguth, C. A., Singh, P., Huang, C. and Tesmer, J. J. G. (2010) Molecular basis for activation of G protein-coupled receptor kinases. EMBO J., Nature Publishing Group **29**, 3249–59.

48 Palczewski, K., Buczyłko, J., Kaplan, M. W., Polans, A. S. and Crabb, J. W. (1991) Mechanism of rhodopsin kinase activation. J. Biol. Chem. **266**, 12949–55.

49 Chen, C. Y., Dion, S. B., Kim, C. M. and Benovic, J. L. (1993) Beta-adrenergic receptor kinase. Agonist-dependent receptor binding promotes kinase activation. J. Biol. Chem. **268**, 7825–31.

50 Pao, C. S., Barker, B. L. and Benovic, J. L. (2009) Role of the amino terminus of G protein-coupled receptor kinase 2 in receptor phosphorylation. Biochemistry **48**, 7325–33.

51 Vishnivetskiy, S. A., Paz, C. L., Schubert, C., Hirsch, J. A., Sigler, P. B. and Gurevich, V. V. (1999) How does arrestin respond to the phosphorylated state of rhodopsin? J. Biol. Chem. **274**, 11451–4.

52 Gurevich, E. V., Tesmer, J. J. G., Mushegian, A. and Gurevich, V. V. (2012) G protein-coupled receptor kinases: more than just kinases and not only for GPCRs. Pharmacol. Ther., Elsevier Inc. **133**, 40–69.

53 Premont, R. T. and Gainetdinov, R. R. (2007) Physiological roles of G protein-coupled receptor kinases and arrestins. Annu. Rev. Physiol. **69**, 511–34.

54 Jaber, M., Koch, W. J., Rockman, H., Smith, B., Bond, R. a, Sulik, K. K., Ross, J., Lefkowitz, R. J., Caron, M. G. and Giros, B. (1996) Essential role of beta-adrenergic receptor kinase 1 in cardiac development and function. Proc. Natl. Acad. Sci. U. S. A. **93**, 12974–9.

55 Matkovich, S. J., Diwan, A., Klanke, J. L., Hammer, D. J., Marreez, Y., Odley, A. M., Brunskill, E. W., Koch, W. J., Schwartz, R. J. and Dorn, G. W. (2006) Cardiac-specific ablation of G-protein receptor kinase 2 redefines its roles in heart development and beta-adrenergic signaling. Circ. Res. **99**, 996–1003.

56 Peppel, K., Boekhoff, I., McDonald, P., Breer, H., Caron, M. G. and Lefkowitz, R. J. (1997) G protein-coupled receptor kinase 3 (GRK3) gene disruption leads to loss of odorant receptor desensitization. J. Biol. Chem. **272**, 25425–8.

57 Gainetdinov, R. R., Bohn, L. M., Sotnikova, T. D., Cyr, M., Laakso, A., Macrae, A. D., Torres, G. E., Kim, K. M., Lefkowitz, R. J., Caron, M. G., et al. (2003) Dopaminergic supersensitivity in G protein-coupled receptor kinase 6-deficient mice. Neuron **38**, 291–303.

58 Burkhalter, M. D., Fralish, G. B., Premont, R. T., Caron, M. G. and Philipp, M. (2013) Grk5l controls heart development by limiting mTOR signaling during symmetry breaking. Cell Rep. **4**, 625–32.

59 Chen, C. K., Burns, M. E., Spencer, M., Niemi, G. A., Chen, J., Hurley, J. B., Baylor, D. A. and Simon, M. I. (1999) Abnormal photoresponses and light-induced apoptosis in rods lacking rhodopsin kinase. Proc. Natl. Acad. Sci. U. S. A. **96**, 3718–22.

60 Whitcomb, T., Sakurai, K., Brown, B. M., Young, J. E., Sheflin, L., Dlugos, C., Craft, C. M., Kefalov, V. J. and Khani, S. C. (2010) Effect of g protein-coupled receptor kinase 1 (Grk1) overexpression on rod photoreceptor cell viability. Invest. Ophthalmol. Vis. Sci. **51**, 1728–37.

61 Vroon, A., Kavelaars, A., Limmroth, V., Lombardi, M. S., Goebel, M. U., Van Dam, A.-M., Caron, M. G., Schedlowski, M. and Heijnen, C. J. (2005) G protein-coupled receptor kinase 2 in multiple sclerosis and experimental autoimmune encephalomyelitis. J. Immunol. **174**, 4400–6.

62 Vroon, A., Heijnen, C. J., Lombardi, M. S., Cobelens, P. M., Mayor, F., Caron, M. G. and Kavelaars, A. (2004) Reduced GRK2 level in T cells potentiates chemotaxis and signaling in response to CCL4. J. Leukoc. Biol. **75**, 901–9.

63 Koch, W. J., Rockman, H. A., Samama, P., Hamilton, R. A., Bond, R. A., Milano, C. A. and Lefkowitz, R. J. (1995) Cardiac function in mice overexpressing the beta-adrenergic receptor kinase or a beta ARK inhibitor. Science **268**, 1350–3.

64 Keys, J. R., Greene, E. A., Cooper, C. J., Naga Prasad, S. V, Rockman, H. A. and Koch, W. J. (2003) Cardiac hypertrophy and altered beta-adrenergic signaling in transgenic mice that express the amino terminus of beta-ARK1. Am. J. Physiol. Heart Circ. Physiol. **285**, H2201-11.

65 Huang, Z. M., Gao, E., Fonseca, F. V., Hayashi, H., Shang, X., Hoffman, N. E., Chuprun, J. K., Tian, X., Tilley, D. G., Madesh, M., et al. (2013) Convergence of G protein-coupled receptor and S-nitrosylation signaling determines the outcome to cardiac ischemic injury. Sci. Signal. **6**, ra95.

66 Otten, J. J. T., de Jager, S. C. A., Kavelaars, A., Seijkens, T., Bot, I., Wijnands, E., Beckers, L., Westra, M. M., Bot, M., Busch, M., et al. (2013) Hematopoietic G-protein-coupled receptor kinase 2 deficiency decreases atherosclerotic lesion formation in LDL receptor-knockout mice. FASEB J. **27**, 265–76.

67 Patial, S., Saini, Y., Parvataneni, S., Appledorn, D. M., Dorn, G. W., Lapres, J. J., Amalfitano, A., Senagore, P. and Parameswaran, N. (2011) Myeloid-specific GPCR kinase-2 negatively regulates NFkB1p105-ERK pathway and limits endotoxemic shock in mice. J. cell Physiol. **226**, 627–637.

68 Parvataneni, S., Gonipeta, B., Packiriswamy, N., Lee, T., Durairaj, H. and Parameswaran, N. (2011) Role of myeloid-specific G-protein coupled receptor kinase-2 in sepsis. Int. J. Clin. Exp. Med. **4**, 320–30.

69 Eckhart, A. D., Ozaki, T., Tevaearai, H., Rockman, H. A. and Koch, W. J. (2002) Vascular-targeted overexpression of G protein-coupled receptor kinase-2 in transgenic mice attenuates beta-adrenergic receptor signaling and increases resting blood pressure. Mol. Pharmacol. **61**, 749–58.

70 Lymperopoulos, A., Rengo, G., Gao, E., Ebert, S. N., Dorn, G. W. and Koch, W. J. (2010) Reduction of sympathetic activity via adrenal-targeted GRK2 gene deletion attenuates heart failure progression and improves cardiac function after myocardial infarction. J. Biol. Chem. **285**, 16378–86.

71 Lodowski, D. T., Pitcher, J. A., Capel, W. D., Lefkowitz, R. J. and Tesmer, J. J. G. (2003) Keeping G proteins at bay: a complex between G protein-coupled receptor kinase 2 and Gbetagamma. Science **300**, 1256–62.

72 Walker, J. K., Peppel, K., Lefkowitz, R. J., Caron, M. G. and Fisher, J. T. (1999) Altered airway and cardiac responses in mice lacking G protein-coupled receptor kinase 3. Am. J. Physiol. **276**, R1214-21.

73 Iaccarino, G., Rockman, H. A., Shotwell, K. F., Tomhave, E. D. and Koch, W. J. (1998) Myocardial overexpression of GRK3 in transgenic mice: evidence for in vivo selectivity of GRKs. Am. J. Physiol. **275**, H1298-306.

74 Vinge, L. E., von Lueder, T. G., Aasum, E., Qvigstad, E., Gravning, J. A., How, O., Edvardsen, T., Bjørnerheim, R., Ahmed, M. S., Mikkelsen, B. W., et al. (2008) Cardiac-restricted expression of the carboxyl-terminal fragment of GRK3 Uncovers Distinct Functions of GRK3 in regulation of cardiac contractility and growth: GRK3 controls cardiac alpha1-adrenergic receptor responsiveness. J. Biol. Chem. **283**, 10601–10.

75 Virlon, B., Firsov, D., Cheval, L., Reiter, E., Troispoux, C., Guillou, F. and Elalouf, J. M. (1998) Rat G protein-coupled receptor kinase GRK4: identification, functional expression, and differential tissue distribution of two splice variants. Endocrinology **139**, 2784–95.

76 Felder, R. A., Sanada, H., Xu, J., Yu, P.-Y., Wang, Z., Watanabe, H., Asico, L. D., Wang, W., Zheng, S., Yamaguchi, I., et al. (2002) G protein-coupled receptor kinase 4 gene variants in human essential hypertension. Proc. Natl. Acad. Sci. U. S. A. **99**, 3872–7.

77 Wang, Z., Armando, I., Asico, L. D., Escano, C., Wang, X., Lu, Q., Felder, R. A., Schnackenberg, C. G., Sibley, D. R., Eisner, G. M., et al. (2007) The elevated blood pressure of human GRK4gamma A142V transgenic mice is not associated with increased ROS production. Am. J. Physiol. Heart Circ. Physiol. **292**, H2083-92.

78 Gainetdinov, R. R., Bohn, L. M., Walker, J. K., Laporte, S. A., Macrae, A. D., Caron, M. G., Lefkowitz, R. J. and Premont, R. T. (1999) Muscarinic supersensitivity and impaired receptor desensitization in G protein-coupled receptor kinase 5-deficient mice. Neuron **24**, 1029–36.

79 Gold, J. I., Gao, E., Shang, X., Premont, R. T. and Koch, W. J. (2012) Determining the absolute requirement of G protein-coupled receptor kinase 5 for pathological cardiac hypertrophy: short communication. Circ. Res. **111**, 1048–53.

80 Rockman, H. A., Choi, D. J., Rahman, N. U., Akhter, S. A., Lefkowitz, R. J. and Koch, W. J. (1996) Receptor-specific in vivo desensitization by the G protein-coupled receptor kinase-5 in transgenic mice. Proc. Natl. Acad. Sci. U. S. A. **93**, 9954–9.

81 Islam, K. N., Bae, J.-W., Gao, E. and Koch, W. J. (2013) Regulation of nuclear factor κB (NF-κB) in the nucleus of cardiomyocytes by G protein-coupled receptor kinase 5 (GRK5). J. Biol. Chem. **288**, 35683–9.

82 Packiriswamy, N., Lee, T., Raghavendra, P. B., Durairaj, H., Wang, H. and Parameswaran, N. (2013) G-protein-coupled receptor kinase-5 mediates inflammation but does not regulate cellular infiltration or bacterial load in a polymicrobial sepsis model in mice. J. Innate Immun. **5**, 401–13.

83 Sorriento, D., Ciccarelli, M., Santulli, G., Campanile, A., Altobelli, G. G., Cimini, V., Galasso, G., Astone, D., Piscione, F., Pastore, L., et al. (2008) The G-protein-coupled receptor kinase 5 inhibits NFkappaB transcriptional activity by inducing nuclear accumulation of IkappaB alpha. Proc. Natl. Acad. Sci. U. S. A. **105**, 17818–23.

84 Chen, X., Zhu, H., Yuan, M., Fu, J., Zhou, Y. and Ma, L. (2010) G-protein-coupled receptor kinase 5 phosphorylates p53 and inhibits DNA damage-induced apoptosis. J. Biol. Chem. **285**, 12823–30.

85 Kavelaars, A., Vroon, A., Raatgever, R. P., Fong, A. M., Premont, R. T., Patel, D. D., Lefkowitz, R. J. and Heijnen, C. J. (2003) Increased acute inflammation, leukotriene B4-induced chemotaxis, and signaling in mice deficient for G protein-coupled receptor kinase 6. J. Immunol. **171**, 6128–34.

86 Fong, A. M., Premont, R. T., Richardson, R. M., Yu, Y. A., Lefkowitz, R. J. and Patel, D. D. (2002) Defective lymphocyte chemotaxis in beta-arrestin2- and GRK6-deficient mice. Proc. Natl. Acad. Sci. U. S. A. **99**, 7478–83.

87 Vroon, A., Heijnen, C. J., Raatgever, R., Touw, I. P., Ploemacher, R. E., Premont, R. T. and Kavelaars, A. (2004) GRK6 deficiency is associated with enhanced CXCR4-mediated neutrophil chemotaxis in vitro and impaired responsiveness to G-CSF in vivo. J. Leukoc. Biol. **75**, 698–704.

88 Osawa, S. and Weiss, E. R. (2012) A tale of two kinases in rods and cones. Adv. Exp. Med. Biol. **723**, 821–7.

89 Inglese, J., Koch, W. J., Caron, M. G. and Lefkowitz, R. J. (1992) Isoprenylation in regulation of signal transduction by G-protein-coupled receptor kinases. Nature **359**, 147–50.

90 Shi, G. W., Chen, J., Concepcion, F., Motamedchaboki, K., Marjoram, P., Langen, R. and Chen, J. (2005) Light causes phosphorylation of nonactivated visual pigments in intact mouse rod photoreceptor cells. J. Biol. Chem. **280**, 41184–91.

91 Kim, Y. H., Kim, Y. S., Noh, H. S., Kang, S. S., Cheon, E. W., Park, S. K., Lee, B. J., Choi, W. S. and Cho, G. J. (2005) Changes in rhodopsin kinase and transducin in the rat retina in early-stage diabetes. Exp. Eye Res. **80**, 753–60.

92 Carman, C. V, Parent, J. L., Day, P. W., Pronin, a N., Sternweis, P. M., Wedegaertner, P. B., Gilman, a G., Benovic, J. L. and Kozasa, T. (1999) Selective regulation of Galpha(q/11) by an RGS domain in the G protein-coupled receptor kinase, GRK2. J. Biol. Chem. **274**, 34483–92.

93 Sallese, M., Mariggiò, S., D'Urbano, E., Iacovelli, L. and De Blasi, A. (2000) Selective regulation of Gq signaling by G protein-coupled receptor kinase 2: direct interaction of kinase N terminus with activated galphaq. Mol. Pharmacol. **57**, 826–31.

94 Day, P. W., Carman, C. V, Sterne-Marr, R., Benovic, J. L. and Wedegaertner, P. B. (2003) Differential interaction of GRK2 with members of the G alpha q family. Biochemistry **42**, 9176–84.

95 Ren, X., Reiter, E., Ahn, S., Kim, J., Chen, W. and Lefkowitz, R. J. (2005) Different G protein-coupled receptor kinases govern G protein and beta-arrestin-mediated signaling of V2 vasopressin receptor. Proc. Natl. Acad. Sci. U. S. A. **102**, 1448–53.

96 Packiriswamy, N. and Parameswaran, N. (2015) G-protein-coupled receptor kinases in inflammation and disease. Genes Immun. **16**, 367–77.

97 Jobin, C. and Sartor, R. B. (2000) The I kappa B/NF-kappa B system: a key determinant of mucosalinflammation and protection. Am. J. Physiol. Cell Physiol. **278**, C451-62.

98 Xu, F., Sun, S., Wang, X., Ni, E., Zhao, L. and Zhu, W. (2017) GRK2 mediates arginine vasopressin-induced IL-6 production via NF-kB signaling in neonatal rat cardiac fibroblast. Mol. Pharmacol. mol.116.107698.

99 Loniewski, K., Shi, Y., Pestka, J. and Parameswaran, N. (2008) Toll-like receptors differentially regulate GPCR kinases and arrestins in primary macrophages. Mol. Immunol. **45**, 2312–2322.

100 Sorriento, D., Fusco, A., Ciccarelli, M., Rungi, A., Anastasio, A., Carillo, A., Dorn, G. W., Trimarco, B. and Iaccarino, G. (2013) Mitochondrial G protein coupled receptor kinase 2 regulates proinflammatory responses in macrophages. FEBS Lett., Federation of European Biochemical Societies **587**, 3487–94.

101 Arraes, S. M. a, Freitas, M. S., da Silva, S. V, de Paula Neto, H. A., Alves-Filho, J. C., Auxiliadora Martins, M., Basile-Filho, A., Tavares-Murta, B. M., Barja-Fidalgo, C. and Cunha, F. Q. (2006) Impaired neutrophil chemotaxis in sepsis associates with GRK expression and inhibition of actin assembly and tyrosine phosphorylation. Blood **108**, 2906–13.

102 Alves-Filho, J. C., Sônego, F., Souto, F. O., Freitas, A., Verri, W. A., Auxiliadora-Martins, M., Basile-Filho, A., McKenzie, A. N., Xu, D., Cunha, F. Q., et al. (2010) Interleukin-33 attenuates sepsis by enhancing neutrophil influx to the site of infection. Nat. Med. **16**, 708–12.

103 Penela, P., Ribas, C., Aymerich, I. and Mayor, F. (2009) New roles of G protein-coupled receptor kinase 2 (GRK2) in cell migration. Cell Adh. Migr. **3**, 19–23.

104 Evron, T., Daigle, T. L. and Caron, M. G. (2012) GRK2: Multiple roles beyond G protein-coupled receptor desensitization. Trends Pharmacol. Sci., Elsevier Ltd **33**, 154–164.

105 Fu, X., Koller, S., Abd Alla, J. and Quitterer, U. (2013) Inhibition of G-protein-coupled receptor kinase 2 (GRK2) triggers the growth-promoting mitogen-activated protein kinase (MAPK) pathway. J. Biol. Chem. **288**, 7738–55.

106 Kim, J., Ahn, S., Ren, X.-R., Whalen, E. J., Reiter, E., Wei, H. and Lefkowitz, R. J. (2005) Functional antagonism of different G protein-coupled receptor kinases for beta-arrestin-mediated angiotensin II receptor signaling. Proc. Natl. Acad. Sci. U. S. A. **102**, 1442–7.

107 Franklin, J. M. and Carrasco, G. A. (2013) G-protein receptor kinase 5 regulates the cannabinoid receptor 2-induced up-regulation of serotonin 2A receptors. J. Biol. Chem. **288**, 15712–24.

108 Zheng, H., Worrall, C., Shen, H., Issad, T., Seregard, S., Girnita, A. and Girnita, L. (2012) Selective recruitment of G protein-coupled receptor kinases (GRKs) controls signaling of the insulin-like growth factor 1 receptor. Proc. Natl. Acad. Sci. U. S. A. **109**, 7055–60.

109 Jiménez-Sainz, M. C., Murga, C., Kavelaars, A., Jurado-Pueyo, M., Krakstad, B. F., Heijnen, C. J., Mayor, F. and Aragay, A. M. (2006) G protein-coupled receptor kinase 2 negatively regulates chemokine signaling at a level downstream from G protein subunits. Mol. Biol. Cell **17**, 25–31.

110 Pitcher, J. A., Tesmer, J. J., Freeman, J. L., Capel, W. D., Stone, W. C. and Lefkowitz, R. J. (1999) Feedback inhibition of G protein-coupled receptor kinase 2 (GRK2) activity by extracellular signal-regulated kinases. J. Biol. Chem. **274**, 34531–4.

111 Elorza, A., Sarnago, S. and Mayor, F. (2000) Agonist-dependent modulation of G protein-coupled receptor kinase 2 by mitogen-activated protein kinases. Mol. Pharmacol. **57**, 778–83.

112 Nick, J. A., Avdi, N. J., Gerwins, P., Johnson, G. L. and Worthen, G. S. (1996) Activation of a p38 mitogen-activated protein kinase in human neutrophils by lipopolysaccharide. J. Immunol. **156**, 4867–75.

113 Zachos, G., Clements, B. and Conner, J. (1999) Herpes simplex virus type 1 infection stimulates p38/c-Jun N-terminal mitogen-activated protein kinase pathways and activates transcription factor AP-1. J. Biol. Chem. **274**, 5097–103.

114 Lee, J. C., Laydon, J. T., McDonnell, P. C., Gallagher, T. F., Kumar, S., Green, D., McNulty, D., Blumenthal, M. J., Heys, J. R. and Landvatter, S. W. (1994) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature **372**, 739–46.

115 Marriott, J. B., Clarke, I. A. and Dalgleish, A. G. (2001) Inhibition of p38 MAP kinase during cellular activation results in IFN-gamma-dependent augmentation of IL-12 production by human monocytes/macrophages. Clin. Exp. Immunol. **125**, 64–70.

116 Peregrin, S., Jurado-Pueyo, M., Campos, P. M., Sanz-Moreno, V., Ruiz-Gomez, A., Crespo, P., Mayor, F. and Murga, C. (2006) Phosphorylation of p38 by GRK2 at the docking groove unveils a novel mechanism for inactivating p38MAPK. Curr. Biol. **16**, 2042–7.

117 Liu, X., Ma, B., Malik, A. B., Tang, H., Yang, T., Sun, B., Wang, G., Minshall, R. D., Li, Y., Zhao, Y., et al. (2012) Bidirectional regulation of neutrophil migration by mitogen-activated protein kinases. Nat. Immunol. **13**, 457–64.

118 Liu, Z., Jiang, Y., Li, Y., Wang, J., Fan, L., Scott, M. J., Xiao, G., Li, S., Billiar, T. R., Wilson, M. a, et al. (2013) TLR4 Signaling augments monocyte chemotaxis by regulating G protein-coupled receptor kinase 2 translocation. J. Immunol. **191**, 857–64.

119 Nijboer, C. H., Heijnen, C. J., Willemen, H. L. D. M., Groenendaal, F., Dorn, G. W., van Bel, F. and Kavelaars, A. (2010) Cell-specific roles of GRK2 in onset and severity of hypoxic-ischemic brain damage in neonatal mice. Brain. Behav. Immun. **24**, 420–6.

120 Subramanian, H., Gupta, K., Parameswaran, N. and Ali, H. (2014) Regulation of Fc∈RI signaling in mast cells by G protein-coupled receptor kinase 2 and its RH domain. J. Biol. Chem. **289**, 20917–27.

121 Willemen, H. L. D. M., Eijkelkamp, N., Wang, H., Dantzer, R., Dorn, G. W., Kelley, K. W., Heijnen, C. J. and Kavelaars, A. (2010) Microglial/macrophage GRK2 determines duration of peripheral IL-1beta-induced hyperalgesia: contribution of spinal cord CX3CR1, p38 and IL-1 signaling. Pain **150**, 550–60.

122 Eijkelkamp, N., Heijnen, C. J., Carbajal, A. G., Willemen, H. L. D. M., Wang, H., Minett, M. S., Wood, J. N., Schedlowski, M., Dantzer, R., Kelley, K. W., et al. (2012) G protein-coupled receptor kinase 6 acts as a critical regulator of cytokine-induced hyperalgesia by promoting phosphatidylinositol 3-kinase and inhibiting p38 signaling. Mol. Med. **18**, 556–64.

123 Insall, R. (2013) The interaction between pseudopods and extracellular signalling during chemotaxis and directed migration. Curr. Opin. Cell Biol., Elsevier Ltd **25**, 526–31.

124 Arnon, T. I., Xu, Y., Lo, C., Pham, T., An, J., Coughlin, S., Dorn, G. W. and Cyster, J. G. (2011) GRK2-dependent S1PR1 desensitization is required for lymphocytes to overcome their attraction to blood. Science **333**, 1898–903.

125 Aragay, A. M., Mellado, M., Frade, J. M., Martin, A. M., Jimenez-Sainz, M. C., Martinez-A, C. and Mayor, F. (1998) Monocyte chemoattractant protein-1-induced CCR2B receptor desensitization mediated by the G protein-coupled receptor kinase 2. Proc. Natl. Acad. Sci. U. S. A. **95**, 2985–90.

126 Olbrich, H., Proudfoot, A. E. and Oppermann, M. (1999) Chemokine-induced phosphorylation of CC chemokine receptor 5 (CCR5). J. Leukoc. Biol. **65**, 281–5.

127 Raghuwanshi, S. K., Su, Y., Singh, V., Haynes, K., Richmond, A. and Richardson, R. M. (2012) The chemokine receptors CXCR1 and CXCR2 couple to distinct G protein-coupled receptor kinases to mediate and regulate leukocyte functions. J. Immunol. **189**, 2824–32.

128 Blake Crabb, E., Franco, R. L., Bowen, M. K., Huang, C., Caslin, H. L. and Acevedo, E. O. (2016) G protein-coupled receptor kinase-2 in peripheral blood mononuclear cells following acute mental stress. Life Sci., Elsevier Inc. **145**, 184–9.

129 Lombardi, M. S., Kavelaars, A., Penela, P., Scholtens, E. J., Roccio, M., Schmidt, R. E., Schedlowski, M., Mayor, F. and Heijnen, C. J. (2002) Oxidative stress decreases G protein-coupled receptor kinase 2 in lymphocytes via a calpain-dependent mechanism. Mol. Pharmacol. **62**, 379–88.

130 Leoratti, F. M. de S., Trevelin, S. C., Cunha, F. Q., Rocha, B. C., Costa, P. A. C., Gravina, H. D., Tada, M. S., Pereira, D. B., Golenbock, D. T., Antonelli, L. R. do V., et al. (2012) Neutrophil paralysis in Plasmodium vivax malaria. PLoS Negl. Trop. Dis. **6**, e1710.

131 Penela, P., Ribas, C., Aymerich, I., Eijkelkamp, N., Barreiro, O., Heijnen, C. J., Kavelaars, A., Sánchez-Madrid, F. and Mayor, F. (2008) G protein-coupled receptor kinase 2 positively regulates epithelial cell migration. EMBO J. **27**, 1206–18.

132 Steury, M. D., Lucas, P. C., McCabe, L. R. and Parameswaran, N. (2017) G-Protein Coupled Receptor Kinase-2 is a Critical Regulator of TNFα Signaling in Colon Epithelial Cells. Biochem. J. **Accepted Manuscript**.

133 Lafarga, V., Mayor, F. and Penela, P. (2012) The interplay between G protein-coupled receptor kinase 2 (GRK2) and histone deacetylase 6 (HDAC6) at the crossroads of epithelial cell motility. Cell Adh. Migr. **6**, 495–501.

134 Cant, S. H. and Pitcher, J. A. (2005) G protein-coupled receptor kinase 2-mediated phosphorylation of ezrin is required for G protein-coupled receptor-dependent reorganization of the actin cytoskeleton. Mol. Biol. Cell **16**, 3088–99.

135 Kahsai, A. W., Zhu, S. and Fenteany, G. (2010) G protein-coupled receptor kinase 2 activates radixin, regulating membrane protrusion and motility in epithelial cells. Biochim. Biophys. Acta **1803**, 300–10.

136 Suo, W. Z. and Li, L. (2010) Dysfunction of G protein-coupled receptor kinases in Alzheimer's disease. ScientificWorldJournal. **10**, 1667–78.

137 Bychkov, E. R., Gurevich, V. V, Joyce, J. N., Benovic, J. L. and Gurevich, E. V. (2008) Arrestins and two receptor kinases are upregulated in Parkinson's disease with dementia. Neurobiol. Aging **29**, 379–96.

138 Obrenovich, M. E., Palacios, H. H., Gasimov, E., Leszek, J. and Aliev, G. (2009) The GRK2 Overexpression Is a Primary Hallmark of Mitochondrial Lesions during Early Alzheimer Disease. Cardiovasc. Psychiatry Neurol. **2009**, 327360.

139 Surguchov, A. (2008) Molecular and cellular biology of synucleins. Int. Rev. Cell Mol. Biol. **270**, 225–317.

140 Sorensen, S. D. and Conn, P. J. (2003) G protein-coupled receptor kinases regulate metabotropic glutamate receptor 5 function and expression. Neuropharmacology **44**, 699–706.

141 Degos, V., Peineau, S., Nijboer, C., Kaindl, A. M., Sigaut, S., Favrais, G., Plaisant, F., Teissier, N., Gouadon, E., Lombet, A., et al. (2013) G protein-coupled receptor kinase 2 and group I metabotropic glutamate receptors mediate inflammation-induced sensitization to excitotoxic neurodegeneration. Ann. Neurol. **73**, 667–78.

142 Eijkelkamp, N., Heijnen, C. J., Willemen, H. L. D. M., Deumens, R., Joosten, E. A. J., Kleibeuker, W., den Hartog, I. J. M., van Velthoven, C. T. J., Nijboer, C., Nassar, M. A., et al. (2010) GRK2: a novel cell-specific regulator of severity and duration of inflammatory pain. J. Neurosci. **30**, 2138–49.

143 Eijkelkamp, N., Wang, H., Garza-Carbajal, A., Willemen, H. L. D. M., Zwartkruis, F. J., Wood, J. N., Dantzer, R., Kelley, K. W., Heijnen, C. J. and Kavelaars, A. (2010) Low nociceptor GRK2 prolongs prostaglandin E2 hyperalgesia via biased cAMP signaling to Epac/Rap1, protein kinase Cepsilon, and MEK/ERK. J. Neurosci. **30**, 12806–15.

144 Wang, H., Heijnen, C. J., van Velthoven, C. T. J., Willemen, H. L. D. M., Ishikawa, Y., Zhang, X., Sood, A. K., Vroon, A., Eijkelkamp, N. and Kavelaars, A. (2013) Balancing GRK2 and EPAC1 levels prevents and relieves chronic pain. J. Clin. Invest. **123**, 5023–34.

145 Ferrari, L. F., Bogen, O., Alessandri-Haber, N., Levine, E., Gear, R. W. and Levine, J. D. (2012) Transient decrease in nociceptor GRK2 expression produces long-term enhancement in inflammatory pain. Neuroscience **222**, 392–403.

146 Iaccarino, G., Barbato, E., Cipolletta, E., De Amicis, V., Margulies, K. B., Leosco, D., Trimarco, B. and Koch, W. J. (2005) Elevated myocardial and lymphocyte GRK2 expression and activity in human heart failure. Eur. Heart J. **26**, 1752–8.

147 Brinks, H., Boucher, M., Gao, E., Chuprun, J. K., Pesant, S., Raake, P. W., Huang, Z. M., Wang, X., Qiu, G., Gumpert, A., et al. (2010) Level of G protein-coupled receptor kinase-2 determines myocardial ischemia/reperfusion injury via pro- and anti-apoptotic mechanisms. Circ. Res. **107**, 1140–9.

148 Chen, E. P., Bittner, H. B., Akhter, S. A., Koch, W. J. and Davis, R. D. (2001) Myocardial function in hearts with transgenic overexpression of the G protein-coupled receptor kinase 5. Ann. Thorac. Surg. **71**, 1320–4.

149 Raake, P. W., Vinge, L. E., Gao, E., Boucher, M., Rengo, G., Chen, X., DeGeorge, B. R., Matkovich, S., Houser, S. R., Most, P., et al. (2008) G protein-coupled receptor kinase 2 ablation in cardiac myocytes before or after myocardial infarction prevents heart failure. Circ. Res. **103**, 413–22.

150 Shah, A. S., White, D. C., Emani, S., Kypson, A. P., Lilly, R. E., Wilson, K., Glower, D. D., Lefkowitz, R. J. and Koch, W. J. (2001) In vivo ventricular gene delivery of a beta-adrenergic receptor kinase inhibitor to the failing heart reverses cardiac dysfunction. Circulation **103**, 1311–6.

151 Rengo, G., Lymperopoulos, A., Zincarelli, C., Donniacuo, M., Soltys, S., Rabinowitz, J. E. and Koch, W. J. (2009) Myocardial adeno-associated virus serotype 6-betaARKct gene therapy improves cardiac function and normalizes the neurohormonal axis in chronic heart failure. Circulation **119**, 89–98.

152 Harding, V. B., Jones, L. R., Lefkowitz, R. J., Koch, W. J. and Rockman, H. A. (2001) Cardiac beta ARK1 inhibition prolongs survival and augments beta blocker therapy in a mouse model of severe heart failure. Proc. Natl. Acad. Sci. U. S. A. **98**, 5809–14.

153 Sorriento, D., Santulli, G., Fusco, A., Anastasio, A., Trimarco, B. and Iaccarino, G. (2010) Intracardiac injection of AdGRK5-NT reduces left ventricular hypertrophy by inhibiting NF-kappaB-dependent hypertrophic gene expression. Hypertens. (Dallas, Tex. 1979) **56**, 696–704.

154 Sorriento, D., Ciccarelli, M., Cipolletta, E., Trimarco, B. and Iaccarino, G. (2016) “Freeze, Don’t Move”: How to Arrest a Suspect in Heart Failure – A Review on Available GRK2 Inhibitors. Front. Cardiovasc. Med. **3**, 48.

155 Keys, J. R., Zhou, R., Harris, D. M., Druckman, C. A. and Eckhart, A. D. (2005) Vascular smooth muscle overexpression of G protein-coupled receptor kinase 5 elevates blood pressure, which segregates with sex and is dependent on Gi-mediated signaling. Circulation **112**, 1145–53.

156 Gros, R., Tan, C. M., Chorazyczewski, J., Kelvin, D. J., Benovic, J. L. and Feldman, R. D. (1999) G-protein-coupled receptor kinase expression in hypertension. Clin. Pharmacol. Ther. **65**, 545–51.

157 Packiriswamy, N., Parvataneni, S. and Parameswaran, N. (2012) Overlapping and distinct roles of GRK5 in TLR2-, and TLR3-induced inflammatory response in vivo. Cell. Immunol. **272**, 107–11.

158 Melamed, A. and Sorvillo, F. J. (2009) The burden of sepsis-associated mortality in the United States from 1999 to 2005: an analysis of multiple-cause-of-death data. Crit. Care **13**, R28.

159 Erdtmann-Vourliotis, M., Mayer, P., Ammon, S., Riechert, U. and Höllt, V. (2001) Distribution of G-protein-coupled receptor kinase (GRK) isoforms 2, 3, 5 and 6 mRNA in the rat brain. Brain Res. Mol. Brain Res. **95**, 129–37.

160 Tarrant, T. K., Billard, M. J., Timoshchenko, R. G., McGinnis, M. W., Serafin, D. S., Foreman, O., Esserman, D. A., Chao, N. J., Lento, W. E., Lee, D. M., et al. (2013) G protein-coupled receptor kinase-3-deficient mice exhibit WHIM syndrome features and attenuated inflammatory responses. J. Leukoc. Biol. **94**, 1243–51.

161 Balabanian, K., Levoye, A., Klemm, L., Lagane, B., Hermine, O., Harriague, J., Baleux, F., Arenzana-Seisdedos, F. and Bachelerie, F. (2008) Leukocyte analysis from WHIM syndrome patients reveals a pivotal role for GRK3 in CXCR4 signaling. J. Clin. Invest. **118**, 1074–84.

162 Billard, M. J., Fitzhugh, D. J., Parker, J. S., Brozowski, J. M., McGinnis, M. W., Timoshchenko, R. G., Serafin, D. S., Lininger, R., Klauber-Demore, N., Sahagian, G., et al. (2016) G Protein Coupled Receptor Kinase 3 Regulates Breast Cancer Migration, Invasion, and Metastasis. PLoS One **11**, e0152856.

163 King, D. W., Steinmetz, R., Wagoner, H. A., Hannon, T. S., Chen, L. Y., Eugster, E. A. and Pescovitz, O. H. (2003) Differential expression of GRK isoforms in nonmalignant and malignant human granulosa cells. Endocrine **22**, 135–42.

164 Woerner, B. M., Luo, J., Brown, K. R., Jackson, E., Dahiya, S. M., Mischel, P., Benovic, J. L., Piwnica-Worms, D. and Rubin, J. B. (2012) Suppression of G-protein-coupled receptor kinase 3 expression is a feature of classical GBM that is required for maximal growth. Mol. Cancer Res. **10**, 156–66.

165 DeFea, K. A. (2013) Arrestins in actin reorganization and cell migration. Prog. Mol. Biol. Transl. Sci., Elsevier Inc. **118**, 205–22.

166 Stacey, M., Lin, H. H., Gordon, S. and McKnight, A. J. (2000) LNB-TM7, a group of seven-transmembrane proteins related to family-B G-protein-coupled receptors. Trends Biochem. Sci. **25**, 284–9.

167 Li, W., Ai, N., Wang, S., Bhattacharya, N., Vrbanac, V., Collins, M., Signoretti, S., Hu, Y., Boyce, F. M., Gravdal, K., et al. (2014) GRK3 is essential for metastatic cells and promotes prostate tumor progression. Proc. Natl. Acad. Sci. U. S. A. **111**, 1521–6.

168 Niculescu, a B., Segal, D. S., Kuczenski, R., Barrett, T., Hauger, R. L. and Kelsoe, J. R. (2000) Identifying a series of candidate genes for mania and psychosis: a convergent functional genomics approach. Physiol. Genomics **4**, 83–91.

169 Gainetdinov, R. R., Premont, R. T., Bohn, L. M., Lefkowitz, R. J. and Caron, M. G. (2004) Desensitization of G protein-coupled receptors and neuronal functions. Annu. Rev. Neurosci. **27**, 107–44.

170 Kim, K.-M., Gainetdinov, R. R., Laporte, S. A., Caron, M. G. and Barak, L. S. (2005) G protein-coupled receptor kinase regulates dopamine D3 receptor signaling by modulating the stability of a receptor-filamin-beta-arrestin complex. A case of autoreceptor regulation. J. Biol. Chem. **280**, 12774–80.

171 Xu, H., Jiang, X., Shen, K., Fischer, C. C. and Wedegaertner, P. B. (2014) The regulator of G protein signaling (RGS) domain of G protein-coupled receptor kinase 5 (GRK5) regulates plasma membrane localization and function. Mol. Biol. Cell **25**, 2105–15.

172 Villar, V. A. M., Jones, J. E., Armando, I., Palmes-Saloma, C., Yu, P., Pascua, A. M., Keever, L., Arnaldo, F. B., Wang, Z., Luo, Y., et al. (2009) G protein-coupled receptor kinase 4 (GRK4) regulates the phosphorylation and function of the dopamine D3 receptor. J. Biol. Chem. **284**, 21425–34.

173 Iacovelli, L., Salvatore, L., Capobianco, L., Picascia, A., Barletta, E., Storto, M., Mariggiò, S., Sallese, M., Porcellini, A., Nicoletti, F., et al. (2003) Role of G protein-coupled receptor kinase 4 and beta-arrestin 1 in agonist-stimulated metabotropic glutamate receptor 1 internalization and activation of mitogen-activated protein kinases. J. Biol. Chem. **278**, 12433–42.

174 Maurin, T., Melko, M., Abekhoukh, S., Khalfallah, O., Davidovic, L., Jarjat, M., D'Antoni, S., Catania, M. V., Moine, H., Bechara, E., et al. (2015) The FMRP/GRK4 mRNA interaction uncovers a new mode of binding of the Fragile X mental retardation protein in cerebellum. Nucleic Acids Res. **43**, 8540–50.

175 Gold, J. I., Martini, J. S., Hullmann, J., Gao, E., Chuprun, J. K., Lee, L., Tilley, D. G., Rabinowitz, J. E., Bossuyt, J., Bers, D. M., et al. (2013) Nuclear Translocation of Cardiac G Protein-Coupled Receptor Kinase 5 Downstream of Select Gq-Activating Hypertrophic Ligands Is a Calmodulin-Dependent Process. PLoS One **8**.

176 Parameswaran, N., Pao, C. S., Leonhard, K. S., Kang, D. S., Kratz, M., Ley, S. C. and Benovic, J. L. (2006) Arrestin-2 and G protein-coupled receptor kinase 5 interact with NFkappaB1 p105 and negatively regulate lipopolysaccharide-stimulated ERK1/2 activation in macrophages. J. Biol. Chem. **281**, 34159–70.

177 Patial, S., Luo, J., Porter, K. J., Benovic, J. L. and Parameswaran, N. (2009) G-protein-coupled-receptor kinases mediate TNFα-induced NFκB signalling via direct interaction with and phosphorylation of IκBα. Biochem. J. **425**, 169–78.

178 Patial, S., Shahi, S., Saini, Y., Lee, T., Packiriswamy, N., Appledorn, D. M., Lapres, J. J., Amalfitano, A. and Parameswaran, N. (2011) G-protein coupled receptor kinase 5 mediates lipopolysaccharide-induced NFκB activation in primary macrophages and modulates inflammation in vivo in mice. J. Cell. Physiol. **226**, 1323–33.

179 Wu, J.-H., Zhang, L., Fanaroff, A. C., Cai, X., Sharma, K. C., Brian, L., Exum, S. T., Shenoy, S. K., Peppel, K. and Freedman, N. J. (2012) G protein-coupled receptor kinase-5 attenuates atherosclerosis by regulating receptor tyrosine kinases and 7-transmembrane receptors. Arterioscler. Thromb. Vasc. Biol. **32**, 308–16.

180 Islam, K. N. and Koch, W. J. (2012) Involvement of nuclear factor κB (NF-κB) signaling pathway in regulation of cardiac G protein-coupled receptor kinase 5 (GRK5) expression. J. Biol. Chem. **287**, 12771–8.

181 Valanne, S., Myllymäki, H., Kallio, J., Schmid, M. R., Kleino, A., Murumägi, A., Airaksinen, L., Kotipelto, T., Kaustio, M., Ulvila, J., et al. (2010) Genome-wide RNA interference in Drosophila cells identifies G protein-coupled receptor kinase 2 as a conserved regulator of NF-kappaB signaling. J. Immunol. **184**, 6188–98.

182 Shenoy, S. K., Drake, M. T., Nelson, C. D., Houtz, D. A., Xiao, K., Madabushi, S., Reiter, E., Premont, R. T., Lichtarge, O. and Lefkowitz, R. J. (2006) beta-arrestin-dependent, G protein-independent ERK1/2 activation by the beta2 adrenergic receptor. J. Biol. Chem. **281**, 1261–73.

183 Knight, R. J. and Buxton, D. B. (1996) Stimulation of c-Jun kinase and mitogen-activated protein kinase by ischemia and reperfusion in the perfused rat heart. Biochem. Biophys. Res. Commun. **218**, 83–8.

184 Komuro, I., Kudo, S., Yamazaki, T., Zou, Y., Shiojima, I. and Yazaki, Y. (1996) Mechanical stretch activates the stress-activated protein kinases in cardiac myocytes. FASEB J. **10**, 631–6.

185 Liu, Y., Gorospe, M., Yang, C. and Holbrook, N. J. (1995) Role of mitogen-activated protein kinase phosphatase during the cellular response to genotoxic stress. Inhibition of c-Jun N-terminal kinase activity and AP-1-dependent gene activation. J. Biol. Chem. **270**, 8377–80.

186 Dabrowski, A., Grady, T., Logsdon, C. D. and Williams, J. A. (1996) Jun kinases are rapidly activated by cholecystokinin in rat pancreas both in vitro and in vivo. J. Biol. Chem. **271**, 5686–90.

187 Gómez del Arco, P., Martínez-Martínez, S., Calvo, V., Armesilla, A. L. and Redondo, J. M. (1996) JNK (c-Jun NH2-terminal kinase) is a target for antioxidants in T lymphocytes. J. Biol. Chem. **271**, 26335–40.

188 Jung, S., Yaron, A., Alkalay, I., Hatzubai, A., Avraham, A. and Ben-Neriah, Y. (1995) Costimulation requirement for AP-1 and NF-kappa B transcription factor activation in T cells. Ann. N. Y. Acad. Sci. **766**, 245–52.

189 Eckhart, A. D., Duncan, S. J., Penn, R. B., Benovic, J. L., Lefkowitz, R. J. and Koch, W. J. (2000) Hybrid transgenic mice reveal in vivo specificity of G protein-coupled receptor kinases in the heart. Circ. Res. **86**, 43–50.

190 Barker, B. L. and Benovic, J. L. (2011) G protein-coupled receptor kinase 5 phosphorylation of hip regulates internalization of the chemokine receptor CXCR4. Biochemistry **50**, 6933–41.

191 Tarrant, T. K., Rampersad, R. R., Esserman, D., Rothlein, L. R., Liu, P., Premont, R. T., Lefkowitz, R. J., Lee, D. M. and Patel, D. D. (2008) Granulocyte chemotaxis and disease expression are differentially regulated by GRK subtype in an acute inflammatory arthritis model (K/BxN). Clin. Immunol. **129**, 115–22.

192 Packiriswamy, N., Steury, M., McCabe, I. C., Fitzgerald, S. D. and Parameswaran, N. (2016) Bacterial Dose-Dependent Role of G Protein-Coupled Receptor Kinase 5 in Escherichia coli-Induced Pneumonia. Infect. Immun. **84**, 1633–1641.

193 Chakraborty, P. K., Zhang, Y., Coomes, A. S., Kim, W.-J., Stupay, R., Lynch, L. D., Atkinson, T., Kim, J. I., Nie, Z. and Daaka, Y. (2014) G protein-coupled receptor kinase GRK5 phosphorylates moesin and regulates metastasis in prostate cancer. Cancer Res. **74**, 3489–500.

194 Luerman, G. C., Powell, D. W., Uriarte, S. M., Cummins, T. D., Merchant, M. L., Ward, R. a and McLeish, K. R. (2011) Identification of phosphoproteins associated with human neutrophil granules following chemotactic peptide stimulation. Mol. Cell. Proteomics **10**, M110.001552.

195 Liu, J., Rasul, I., Sun, Y., Wu, G., Li, L., Premont, R. T. and Suo, W. Z. (2009) GRK5 deficiency leads to reduced hippocampal acetylcholine level via impaired presynaptic M2/M4 autoreceptor desensitization. J. Biol. Chem. **284**, 19564–71.

196 Sadot, E., Gurwitz, D., Barg, J., Behar, L., Ginzburg, I. and Fisher, A. (1996) Activation of m1 muscarinic acetylcholine receptor regulates tau phosphorylation in transfected PC12 cells. J. Neurochem. **66**, 877–80.

197 Carman, C. V, Som, T., Kim, C. M. and Benovic, J. L. (1998) Binding and phosphorylation of tubulin by G protein-coupled receptor kinases. J. Biol. Chem. **273**, 20308–16.

198 Loudon, R. P. and Benovic, J. L. (1997) Altered activity of palmitoylation-deficient and isoprenylated forms of the G protein-coupled receptor kinase GRK6. J. Biol. Chem. **272**, 27422–27427.

199 Ahmed, M. R., Bychkov, E., Gurevich, V. V, Benovic, J. L. and Gurevich, E. V. (2008) Altered expression and subcellular distribution of GRK subtypes in the dopamine-depleted rat basal ganglia is not normalized by l-DOPA treatment. J. Neurochem. **104**, 1622–36.

200 Ohba, Y., Nakaya, M., Watari, K., Nagasaka, A. and Kurose, H. (2015) GRK6 phosphorylates IκBα at Ser(32)/Ser(36) and enhances TNF-α-induced inflammation. Biochem. Biophys. Res. Commun. **461**, 307–13.

201 Ahmed, M. R., Bychkov, E., Kook, S., Zurkovsky, L., Dalby, K. N. and Gurevich, E. V. (2015) Overexpression of GRK6 rescues L-DOPA-induced signaling abnormalities in the dopamine-depleted striatum of hemiparkinsonian rats. Exp. Neurol. **266**, 42–54.

202 Zuo, K., Kuang, D., Wang, Y., Xia, Y., Tong, W., Wang, X., Chen, Y., Duan, Y. and Wang, G. (2016) SCF/c-kit transactivates CXCR4-serine 339 phosphorylation through G protein-coupled receptor kinase 6 and regulates cardiac stem cell migration. Sci. Rep., Nature Publishing Group **6**, 26812.

203 McCormick, P. J., Segarra, M., Gasperini, P., Gulino, A. V. and Tosato, G. (2009) Impaired recruitment of Grk6 and beta-Arrestin 2 causes delayed internalization and desensitization of a WHIM syndrome-associated CXCR4 mutant receptor. PLoS One **4**, e8102.

204 Eijkelkamp, N., Heijnen, C. J., Lucas, A., Premont, R. T., Elsenbruch, S., Schedlowski, M. and Kavelaars, A. (2007) G protein-coupled receptor kinase 6 controls chronicity and severity of dextran sodium sulphate-induced colitis in mice. Gut **56**, 847–54.

205 Chen, Y., Lu, B., Yang, Q., Fearns, C., Yates, J. R. and Lee, J. (2009) Combined integrin phosphoproteomic analyses and small interfering RNA--based functional screening identify key regulators for cancer cell adhesion and migration. Cancer Res. **69**, 3713–20.

206 Raghuwanshi, S. K., Smith, N., Rivers, E. J., Thomas, A. J., Sutton, N., Hu, Y., Mukhopadhyay, S., Chen, X. L., Leung, T. and Richardson, R. M. (2013) G protein-coupled receptor kinase 6 deficiency promotes angiogenesis, tumor progression, and metastasis. J. Immunol. **190**, 5329–36.

207 Le, Q., Yao, W., Chen, Y., Yan, B., Liu, C., Yuan, M., Zhou, Y. and Ma, L. (2016) GRK6 regulates ROS response and maintains hematopoietic stem cell self-renewal. Cell Death Dis., Nature Publishing Group **7**, e2478.

208 Nakaya, M., Tajima, M., Kosako, H., Nakaya, T., Hashimoto, A., Watari, K., Nishihara, H., Ohba, M., Komiya, S., Tani, N., et al. (2013) GRK6 deficiency in mice causes autoimmune disease due to impaired apoptotic cell clearance. Nat. Commun. **4**, 1532.

209 Fadok, V. A., Bratton, D. L., Konowal, A., Freed, P. W., Westcott, J. Y. and Henson, P. M. (1998) Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. J. Clin. Invest. **101**, 890–8.

210 Peng, Y., Martin, D. A., Kenkel, J., Zhang, K., Ogden, C. A. and Elkon, K. B. (2007) Innate and adaptive immune response to apoptotic cells. J. Autoimmun. **29**, 303–9.

CHAPTER 2: G-PROTEIN COUPLED RECEPTOR KINASE-2 IS A CRITICAL REGULATOR OF TNFα SIGNALING IN COLON EPITHELIAL CELLS

A major part of this chapter represents a manuscript that was accepted by *Biochemical Journal* on June 1, 2017.

ABSTRACT

G protein-coupled receptor kinase-2 (GRK2) belongs to the GRK family of serine/threonine protein kinases critical in the regulation of GPCRs. Apart from this canonical role, GRK2 is also involved in several signaling pathways via distinct intracellular interactomes. In this study, we examined the role of GRK2 in TNFα signaling in colon epithelial cell-biological processes including wound healing, proliferation, apoptosis, and gene expression. Knockdown of GRK2 in the SW480 human colonic cells significantly enhanced TNFα-induced epithelial cell wound healing without any effect on apoptosis/proliferation. Consistent with wound healing effects, GRK2 knockdown augmented TNFα-induced matrix metalloproteinases (MMP) 7 and 9 as well as urokinase plasminogen activator (uPa) (factors involved in cell migration and wound healing). To assess the mechanism by which GRK2 affects these physiological processes, we examined the role of GRK2 in TNFα-induced MAPK and NFκB pathways. Our results demonstrate that while GRK2 knockdown inhibited TNFα-induced IκBα phosphorylation, activation of ERK was significantly enhanced in GRK2 knockdown cells. Our results further demonstrate that GRK2 inhibits TNFα-induced ERK activation by inhibiting generation of reactive oxygen species (ROS). Using immunoprecipitation of endogenous GRK2 coupled with mass spectrometry, we find that GRK2 interacts with mitochondrial proteins important for ROS generation. Together, these data suggest that GRK2 plays a critical role in TNFα-induced wound healing by modulating MMP7, 9 and uPA levels via ROS-ERK pathway. Consistent with the in vitro findings, GRK2 heterozygous mice exhibited enhanced intestinal wound healing. Together, our results identify a novel role for GRK2 in TNFα signaling in intestinal epithelial cells.

INTRODUCTION

G-protein coupled receptors kinases (GRKs) are serine/threonine kinases that include 7 distinct proteins separated into three subfamilies based on their sequence homology and functional similarities. These include the retinal kinase (GRK1 and GRK7), the GRK2 (GRK2 and GRK3), and the GRK4 (GRK4, 5, and 6) subfamilies [1]. GRKs were initially described for their canonical regulation of G-protein coupled receptor (GPCR) phosphorylation and desensitization. However, it is now clear that their role in cell signaling is not limited to this canonical function. GRK2 can interact with and regulate (or be regulated by) a wide variety of non-GPCRs as well as non-receptor substrates, including IGF-1R, insulin-R, EGFR, ERK, MEK, IκBα, and p38, which demonstrates its capacity to impact a diverse set of cellular functions through both phosphorylation-dependent and -independent mechanisms [2]. These non-canonical roles of GRK2 allow for this kinase to influence basic cellular processes such as inflammatory gene expression, cellular migration, as well as mitochondrial metabolism [3]. The role for GRK2 in these cellular functions has been extensively examined in cardiac, immune and other cell types [2,4,5], but the role of GRK2 in intestinal epithelial cells is not well known.

Mucosal surfaces, including the intestinal tract, are lined by epithelial cells and are critical in defining and maintaining a barrier between the host and external environment. Dysregulation of this barrier integrity leads to inflammatory processes including inflammatory bowel disease (IBD) [6,7][8,9]. Intestinal epithelial cell healing (proliferation, differentiation, migration) and other epithelial dynamics such as permeability become impaired in inflammatory bowel disease [10]. In addition, patients with chronic IBD are also at increased risk for colitis-associated colon cancer. Thus, understanding the signaling mechanisms that regulate colonic

epithelial cell biology is important for long-term drug development strategies for IBD and colitis-associated colon cancer.

IBD is associated with an increase in inflammatory cytokines, such as TNFα, and these cytokines have been shown to alter the ability of epithelial cells to repair damage to the monolayer. These changes are mediated via intracellular signaling through regulation of NFκB and MAPK pathways which affect epithelial cell apoptosis, proliferation and migration from the crypts [11]. In previous studies, we and others showed that GRK2 is an important regulator of TNFα signaling in myeloid cells both *in vitro* and *in vivo* [12–17]. However, the function of GRK2 in intestinal epithelial cells in the context of TNFα signaling processes is not known. Therefore, in this study, we examined the role of GRK2 in colonic epithelial cells in terms of various cell biological responses including cell signaling pathways that are modulated by GRK2. Our studies underscore a critical role of GRK2 in intestinal epithelial cell wound healing *in vitro*. Part of this regulation occurs via the role of GRK2 in TNFα-induced ROS-ERK pathway and subsequent MMP expression. Consistent with *in vitro* findings, we also demonstrate that intestinal wound healing is enhanced in GRK2 heterozygous knockout mice *in vivo*.

MATERIALS AND METHODS

Cell Culture and Knockdown of GRK2

Human colon epithelial cell line SW480 was obtained from ATCC and was maintained in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose and supplemented with 10% (v/v) fetal bovine serum (Life Technologies) along with 5% penicillin/streptomycin (Life Technologies). These cells were grown at 37° C in a humidified 5% CO_2 incubator. One hour prior to stimulation, cells were replenished with culture media without FBS and then stimulated with 20 ng/ml of TNFα (PeproTech) for various time points as indicated. For knockdown of GRK2, 1 million cells were treated with 25 pmoles of "SmartPool siRNA" containing a mixture of four designed siRNAs targeting GRK2 or scrambled siRNA (Dharmacon) and electroporated using an Amaxa V electroporation instrument using the standard protocol provided by the supplier and solution V (Lonza) and program L-024. The cells were then incubated for 48 hours to facilitate knockdown before being used for experiments.

Scratch Wound Healing Assay

SW480 cells were cultured in a 6-well plate and the confluent cells were used for *in vitro* scratch-wound assay [18]. Cells were treated with TNFα (20 ng/ml) or vehicle at time 0 hours. To create the wound, a uniform scratch was created vertically in the culture dish using a 200 µl plastic pipette tip and the plate was then incubated for 48 additional hours in the presence of mitomycin C (1 µg/ml) to inhibit proliferation. The scratch was assessed and photos of the wound were taken at 0, 16, 24, 32, and 48 hours. Images were taken with cellSens Software (Olympus) and the distance of the wound was calculated by measuring the distance between the leading edges of the wound using ImageJ software. Effect of TNFα on wound healing was calculated by comparing the pixel values of TNFα treated cells in control

siRNA and GRK2 siRNA groups to their corresponding untreated cells at each time point and expressed as fold untreated cells.

Proliferation Assay

After siRNA transfection, SW480 cells were cultured in a 96 well plate at a concentration of 50,000/well for 24 or 48 hours and stimulated with TNFα (20 ng/ml) or vehicle for 24 or 48 hours. The amount of proliferation was determined using the CyQuant Cell Proliferation Assay (Thermo Scientific). In short, after TNFα treatment the media was removed and the plate was incubated at -80° C overnight. The following day the plate was thawed and a DNA binding dye in lysis buffer was added to each well and the fluorescence was read on a Tecan M2000 spectrophotometer at 485 nm excitation and 530 nm emission and compared to a standard curve.

Apoptosis Assay

After siRNA transfection, SW480 cells were cultured in 6-well plates at a concentration of 1 million cells/well for 48 hours and were treated with TNFα (20 ng/ml) for 24 or 48 hours. Apoptosis was quantified using Annexin V Apoptosis Detection Kit (eBioscience) according to the manufacturer's protocol. Cells were quantified using LSR II flow cytometer and data was analyzed using FlowJo software. Data was divided into 4 categories: Live (Annexin V “-“, Propidium Iodide (PI) “-“), Early Apoptotic (Annexin V “+”, PI “-“), Apoptotic (Annexin V “+”, PI “+”), and Necrotic/Dead (Annexin V “-“, PI “+”).

ROS Assay

After siRNA transfection, SW480 cells were cultured in 6-well plates at a concentration of 1 million cells/well for 48 hours and stimulated for 10 or 20 minutes with TNFα (20 ng/ml) or vehicle. ROS was measured using CellRox Green Reagent (Thermo Scientific). Briefly, the cells were pretreated with CellRox Green reagent (5 μM) for 30 minutes. The cells were then treated with TNFα and after the treatment

washed 3 times with PBS. The cells were fixed with 3.7% formaldehyde for 15 minutes, washed 3 times with FACS buffer (0.5% BSA, 0.05% Sodium Azide, in 1x PBS) and quantified using LSR II flow cytometer and analyzed using FlowJo software. Data is expressed as mean fluorescent intensity (MFI).

Invasion Assay

After siRNA transfection, SW480 cells were cultured in a 6-well plate to facilitate the siRNA mediated knockdown of GRK2. After 48 hours, the cells were removed from the plate and 20,000 cells were seeded in serum free media in the upper chamber of transwell inserts coated with matrigel (Cell BioLabs Inc) and cellular migration was determined per manufacturer's protocol. In brief, serum-containing media was inserted in the lower chamber and the cells were incubated for 48 hours to allow for migration through the membrane in the presence or absence of TNFα (20ng/ml). After 48 hours the migrated cells were detached using the manufacturer's detachment solution and lysed in lysis buffer. A fluorescent dye was added to the lysate and fluorescence was read on a Tecan M2000 spectrophotometer at 485 nm excitation and 530 nm emission.

RT-PCR

RNA was extracted from SW480 cells using the RNEasy Kit (Qiagen) and treated with DNase (LifeTech) per manufacturer's protocol. A total of 1 μg RNA was used for the template for single-stranded cDNA synthesis. Quantitative real-time PCR was performed for the various genes and the cDNA was amplified using SYBR Green Pro Master Mix and the genes of interest were normalized to the corresponding hypoxanthine-guanine phosphoribosyltransferse (HPRT) controls as described before [19]. All primers used in this set of experiments are listed in **Table 2.1.**

Table 2.1. List of genes measured and primer sequences for RT-PCR

GENE	SEQUENCE
HPRT	F – 5’ GAC CAG TCA ACA GGG GAC AT 3’
	R – 5’ AAC ACT TCG TGG GGT CCT TTT C 3’
IL6	F – 5’ TCG AGC CCA CCG GGA ACG AA 3’
	R – 5’ GCA ACT GGA CCG AAG GCG CT 3’
CXCL8	F – 5’ GTG CAG TTT TGC CAA GGA GT 3’
	R – 5’ CTC TGC ACC CAG TTT TCC T 3’
TNFα	F –5’ TAT CTC TCA GCT CCA CGC CA 3’
	R – 5’ TCT CGA ACC CCG AGT GAC AA 3’
MMP9	F – 5’ GAG TGG CAG GGG GAA GAT GC 3’
	R – 5’ CCT CAG GGC ACT GCA GGA TG 3’
MMP7	F – 5’ GGA GGA GAT GCT CAC TTC GAT 3’
	R – 5’ AGG AAT GTC CCA TAC CCA AAG A 3’
uPA	F – 5’ ATT TGT GAG GCC CAT GGT TG 3’
	R – 5’ AAA CCG CTG CTC CCA CAT T 3’
GRK2	F – 5’ ACC CGC CCA CCC GCC TTT TA 3’
	R – 5’ GCT GGG GCC ACG GGA AAT CA 3’

Western Blot Analysis

Sample preparation and Western blot analysis were done as described before [20]. Briefly, cells were lysed in lysis buffer (20mM Tris-HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl) containing 1% Triton X-100 and protease inhibitors (protease inhibitor cocktail, Roche Diagnostics) and phosphatase inhibitors. Insoluble material was removed by centrifugation and protein concentration was determined by Bradford assay. Proteins in the cellular lysates were separated 10% SDS-PAGE and the gel was electro-blotted against nitrocellulose membrane (Thermo Fisher Scientifics). Following this transfer, the blot was probed with specific primary antibodies for pERK 1/2, total ERK-2, p38, pp38, JNK1/2, pJNK1/2 and tubulin (Santa Cruz) as well as IκBα and pIκBα (Cell Signaling) diluted in LiCor blocking buffer (LiCor) and followed by anti-rabbit or anti-mouse IR dye-conjugated secondary antibodies (LiCor). The blot was scanned and analyzed with an Odyssey LiCor instrument along with its software Odyssey.

Mice

GRK2 heterozygous mice were obtained from Jackson Labs (kindly donated by Dr. Robert Lefkowitz, Duke University). Animals were bred at Michigan State University by breeding GRK2 heterozygous mice with wild-type mice. Note that homozygous knockout of GRK2 are embryonically lethal [21]. All animals were housed in a specific-pathogen-free facility maintained at 22-24 C with a 12H light-dark cycle and were given mouse chow and water *ad libitum*. All animals were performed with age- and sex- matched mice (males) between 8 and 10 weeks of age. All animal procedures were approved by the Michigan State University IACUC and conformed to NIH guidelines.

Statistical Analysis

All values are represented as mean +/- SEM. All data were tested for statistical analysis for statistical significance using unpaired Student's T-test (two sample comparisons) and Analysis of Variance (ANOVA) with Tukey post-hoc test (more than 2 sample comparisons). The analysis was done using Prism GraphPad software. A p-value <0.05 was considered significant.

RESULTS

GRK2 Suppresses TNFα Induced Epithelial Wound Closure

The present studies were designed to investigate the role of GRK2 in TNFα signaling in intestinal epithelial cells. In other studies, TNFα has been shown to significantly modulate the intestinal wound healing response and protect from intestinal epithelial apoptosis [11]. To this end, we performed a scratch wound-healing assay with SW480 cells (human colonic epithelial cell line) to identify the role of GRK2 in TNFα-induced wound healing. For this, cells were transfected with either control siRNA or GRK2 siRNA and plated as described in the methods. GRK2 knockdown was ~80-90% compared to controls **(Figure 2.1.A-B)**. The wound was induced by scratch assay and wound closure was monitored up to 48 hours. Proliferation was inhibited using mitomycin C and therefore any cells in the wound space are the result of cell migration and not due to cell proliferation. The scratch-induced wound size was similar in each experimental group at time 0. The effect of TNFα on wound closing was calculated by normalizing the size of the wound in treated versus untreated cells (i.e. fold untreated) in the respective siRNA groups (Ctrl siRNA and GRK2 siRNA). We observed that the amount of wound closure was similar between untreated and TNFα treated groups in the control siRNA transfected cells, at all the time points tested. However, in the GRK2 knockdown cells, wound size was markedly reduced in response to TNFα **(Figure 2.1.C-D)**. This suggests that GRK2 normally inhibits TNFα-induced wound healing and therefore, its knockdown unmasks the healing potential of TNFα. Importantly, the concentration of TNFα used in these experiments had no effect on SW480 proliferation or apoptosis between the control and the GRK2 knockdown cells **(Figure 2.2.A-B)**. To further understand whether this phenomenon was kinase activity-

Figure 2.1. GRK2 inhibits wound healing in SW480 cells
A) SW480 cells were transfected with either scrambled siRNA (siCtrl) or GRK2 siRNA (siGRK2) and incubated for 48 hours to facilitate knockdown of GRK2. Representative western blot and their quantification showing levels of GRK2 after siCtrl or siGRK2 treatment. Tubulin is shown as loading control. **B)** Gene expression levels of GRK2 in cells treated with either siCtrl or siGRK2 (n=5). **C)** Confluent SW480 cells were wounded through the generation of a linear scratch and the cells were photographed at 0, 16, 24, 40 and 48 hours. At the time of wounding mitomycin C was added to prevent proliferation. The cells were treated with TNFα (20 ng/ml) at the onset of the linear scratch. Differences between amount of wound closed at each time point between untreated and treated cells for both siCtrl and siGRK2 cells (1 = no difference) was calculated through ImageJ Software and by identifying leading edge of the wound. Data expressed as fold untreated cells for each time point. (n=9). Wound closure relative to control at 48 hours is also shown on the right. **D)** Representative image from each group shows the area of wound closure at 0, and 48 hours at 4x magnification. Mean +/- SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

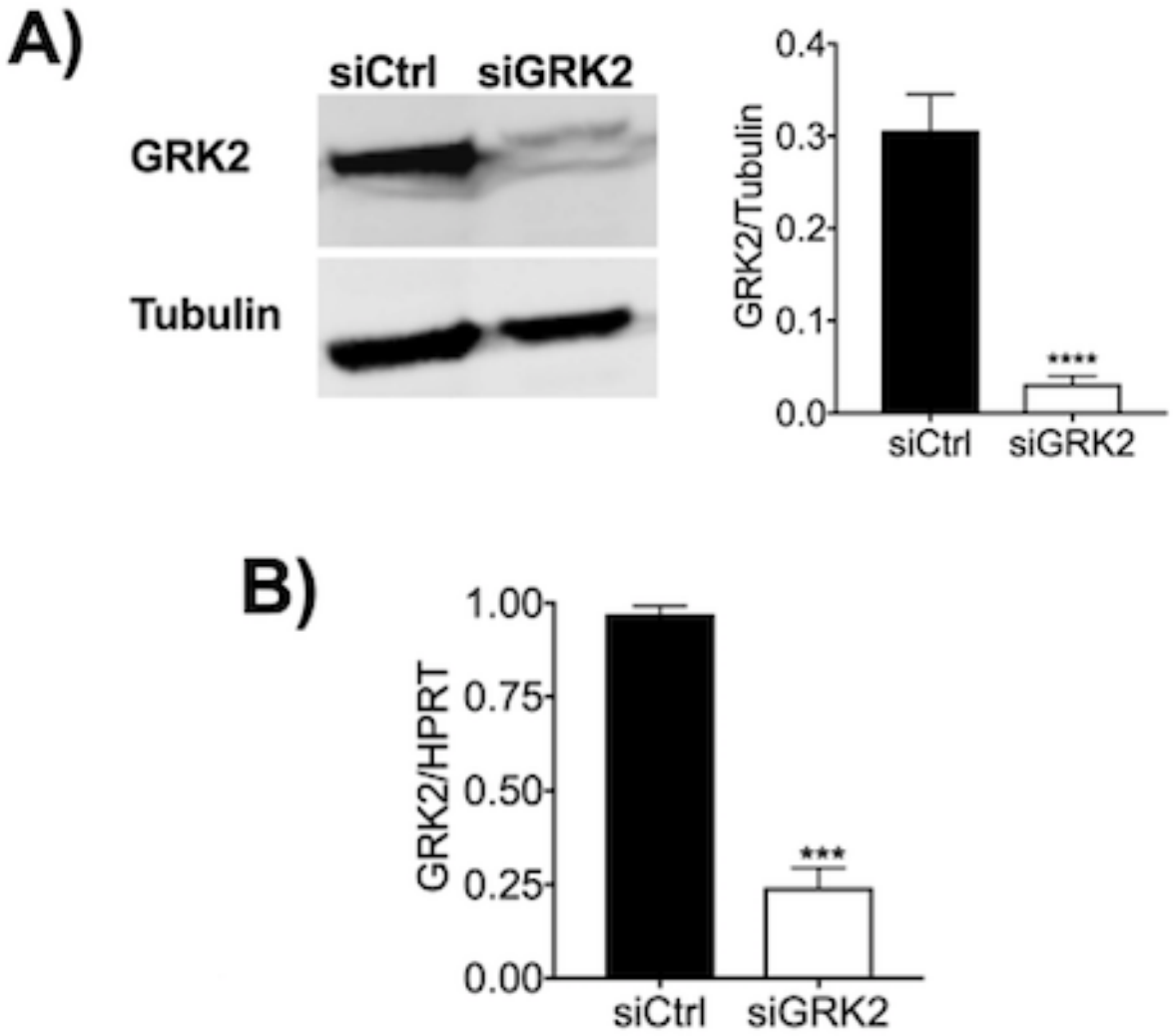


Figure 2.1. (cont'd)

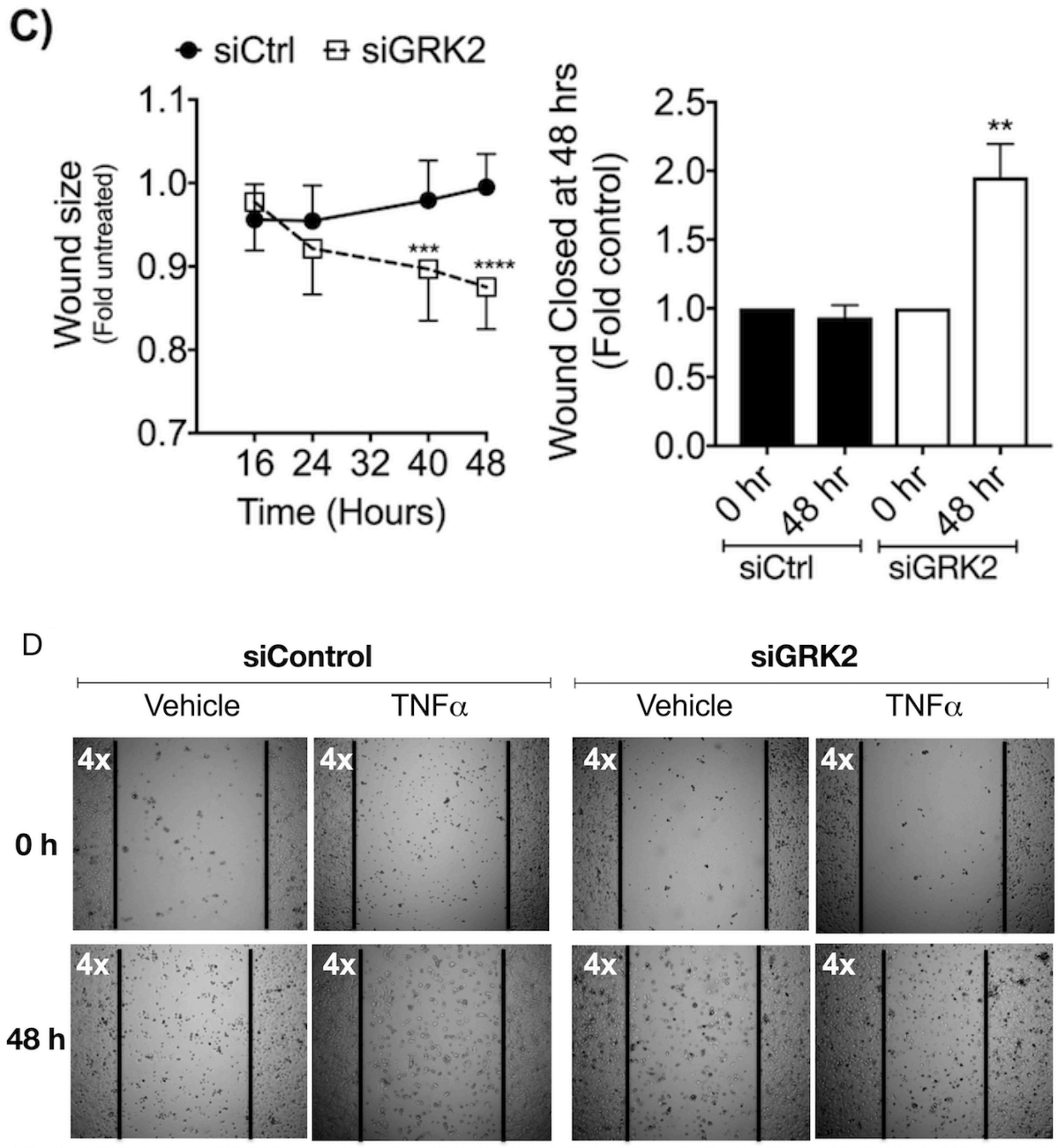
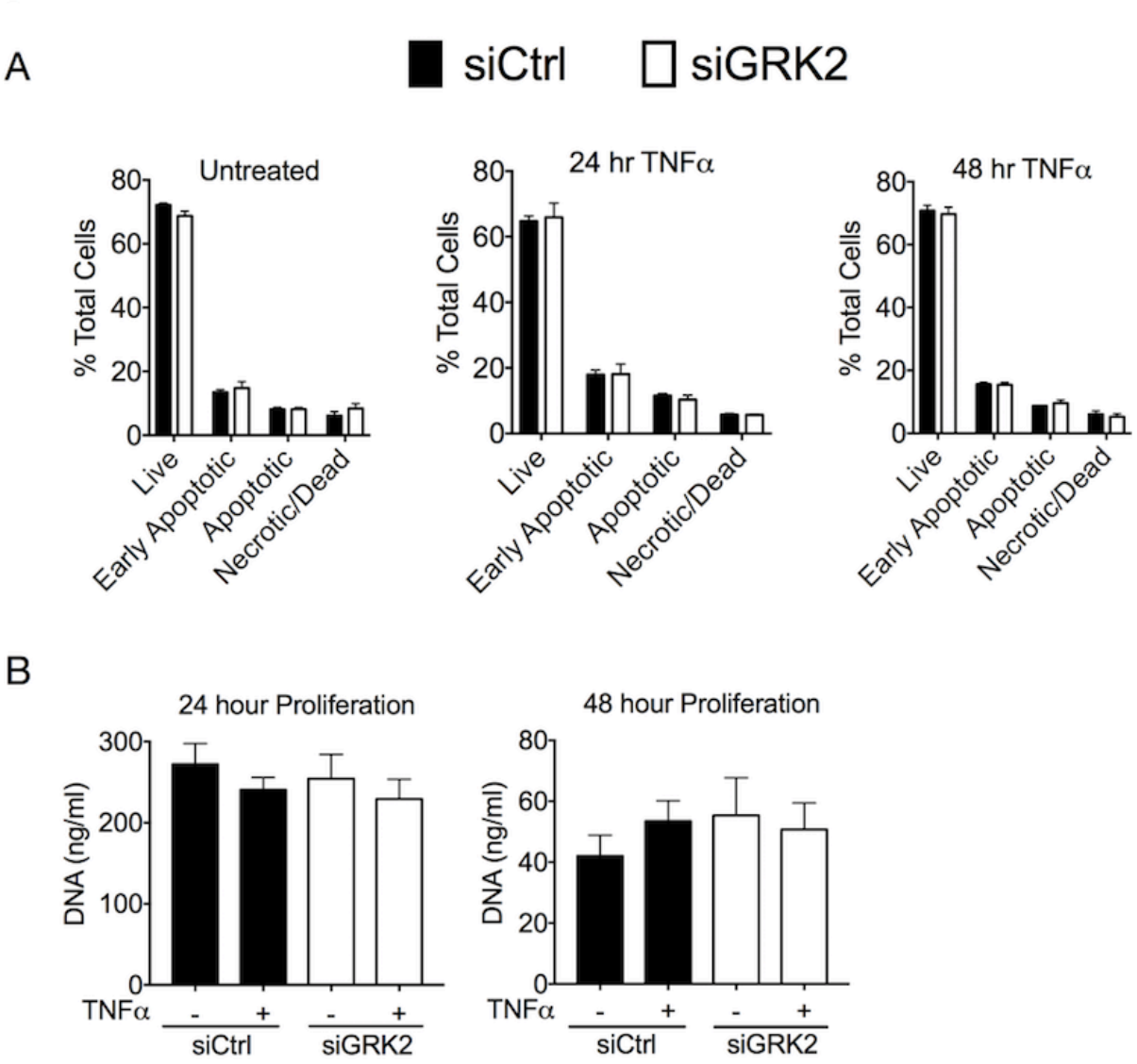


Figure 2.2. GRK2 does not affect TNFα induced apoptosis or proliferation in SW480 cells
A) After siRNA transfection, SW480 cells were cultured in 6-well plates at a concentration of 1 million cells/well and were treated +/-TNFα (20 ng/ml) for 24 and 48 hours in order to induce apoptosis. Data is expressed as % total cells quantified by flow cytometry. (n=3) **B)** Proliferation was assessed by measuring total DNA from either siCtrl or siGRK2 treated cells +/- TNFα (20 ng/ml) for 24 and 48 hours. Equal numbers of cells were plated and DNA concentration was measured after TNFα treatment. (n=6) Mean +/- SEM, * $p < 0.05$.



dependent we performed the wound healing experiments in the presence of a GRK2 kinase inhibitor, paroxetine. Paroxetine binds to the active site of GRK2 inhibiting its kinase function [22]. Pretreatment of SW480 cells with paroxetine (10 μM) did not affect wound healing in the absence or presence of TNFα, suggesting that our observations with GRK2 knockdown are likely kinase independent (data not shown). Together, our results indicate that knockdown of GRK2 in intestinal epithelial cells accelerates TNFα-induced wound healing process.

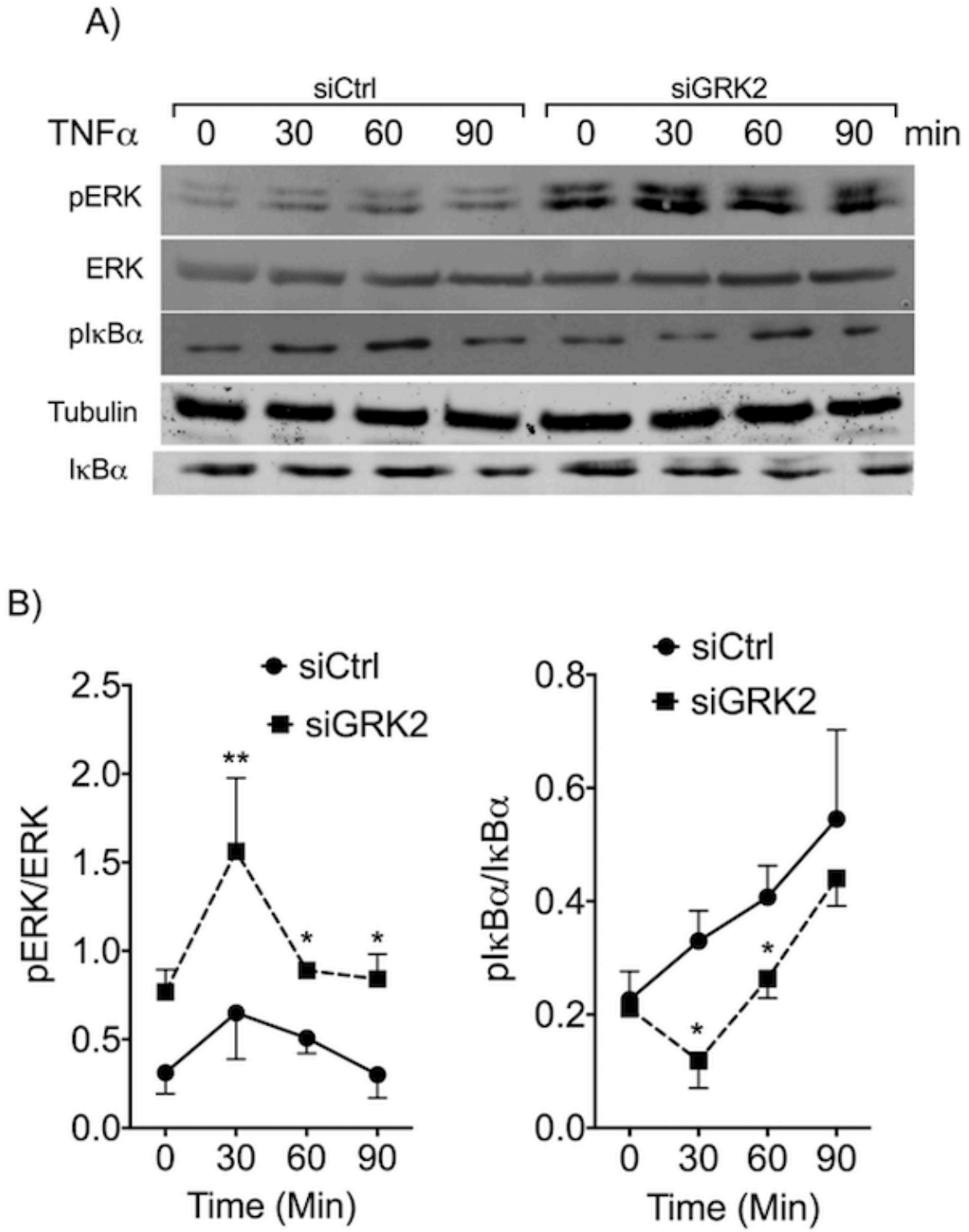
GRK2 Differentially Modulates TNFα Signaling in SW480 Cells

Previous studies have shown that GRK2 can influence TNFα signaling in macrophages [12]. Our results here suggest that GRK2 can significantly impact TNFα-induced wound healing in epithelial cells. To examine the signaling mechanisms by which this occurs, we treated control and GRK2 siRNA transfected SW480 cells with TNFα for various time points (0 to 90 min) and examined the cell signaling pathways. TNFα stimulation led to increases in phosphorylation of IκBα and ERK1/2 in the control siRNA treated cells **(Figure 2.3.A-B)**. Surprisingly, these effects were differentially affected in the GRK2 knockdown cells. TNFα-induced IκBα phosphorylation was significantly inhibited in the GRK2 knockdown cells whereas ERK1/2 phosphorylation was markedly enhanced **(Figure 2.3.A-B)**. These results demonstrate opposing roles of GRK2 in MAPK and NFκB pathways, with GRK2 augmenting the NFκB pathway and simultaneously inhibiting the ERK pathway. Other signaling pathways including JNK and p38 were not consistently affected by GRK2 knockdown (data not shown). Overall, these results suggest that GRK2 is an important regulator of ERK1/2 and IκBα signaling pathways in intestinal epithelial cells.

GRK2 Alters TNFα Induced Gene Expression

Both ERK1/2 and NFκB pathways are potent regulators of gene transcription;

Figure 2.3. GRK2 differentially affects TNFα signaling pathway in SW480 colon epithelial cells
A) Representative blots show protein levels analyzed by Western Blot as well as loading controls. GRK2 knockdown upregulates pERK and downregulates pIκBα. ERK and Tubulin are loading controls. **B)** Quantification of western blots for pERK/ERK and pIκBa/IκBa. Means +/- SEM, * $p < 0.05$. ** $p < 0.01$, compared to the corresponding siCtrl group. (n=5)



therefore we sought to identify which inflammatory and matrix modifying genes are altered by GRK2 knockdown at 6 and 24 hours post-TNFα treatment. TNFα-induced expression of genes that encode extracellular matrix degradation, including matrix metalloproteinase 7 (*MMP7*), matrix metalloproteinase 9 (*MMP9*), as well as urokinase plasminogen activator (*uPA*), were all markedly enhanced in the GRK2 knockdown cells as compared to control cells at 24 hours **(Figure 2.4.A)**. Whereas after 6 hours of stimulation we observed a mixed response in inflammatory gene expression where TNFα-induced expression of *CXCL8* and *TNFα* was markedly enhanced in the GRK2 knockdown cells (compared to control siRNA cells) and *IL6* expression was significantly decreased in GRK2 knockdown compared to control cells **(Figure 2.4.A)**. After 24 hours of TNFα treatment, there essentially was no difference in the expression of these 3 genes between control and GRK2 knockdown groups.

Because MMPs are important in cell migration and MMP9 has been shown to be regulated via the ERK pathway, we investigated whether the TNFα-induced MMP9 in SW480 cells is ERK dependent. To this end, we treated control and GRK2 knockdown cells with MEK1/2 inhibitor U0126 (10 μM, 40-minute pretreatment) prior to TNFα treatment. Inhibition of MEK-ERK pathway abolished TNFα-induced *MMP9* expression, confirming that *MMP9* expression is driven via the ERK pathway in both control and GRK2 knockdown groups **(Figure 2.4.B)**. We also examined the transcripts of *MMP7* and *uPA* but observed that their regulation is not ERK dependent. We attempted to inhibit the IκBα pathway using the IKK inhibitor BMS-345541 but the combination of the inhibition of the pro-survival NFκB and the stimulation with TNFα was detrimental to the cell survival in culture at both 6 and 24 hours (data not shown). To further confirm that an increase in MMP9 activity is important in migration through an extracellular matrix we subjected the SW480

Figure 2.4. GRK2 differentially regulates TNFα-induced gene expression in SW480 cells

A) GRK2 knockdown alters TNFα-induced gene expression in SW480 colon epithelial cells transfected with siCtrl or siGRK2. Gene expression levels were assessed by analyzing mRNA levels using real-time PCR after TNFα stimulation (20ng/ml) for 0 (untreated), 6, and 24 hours and normalized to HPRT then expressed as % maximal expression. (n=5) **B)** Cells were either treated with siCtrl or siGRK2 and stimulated with TNFα (24 hours) in the presence or absence of MEK inhibitor U0126. Gene expression levels of the indicated genes were assessed and normalized to HPRT then expressed as % maximal expression. (n=3) **C)** Following siRNA transfection cells were seeded in the upper chamber of a transwell insert coated with matrigel. Following 48 hours incubation with TNFα, migrated cells were detached and lysed and quantified using a fluorescent dye and expressed as fold control (n=3). Means +/- SEM, $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

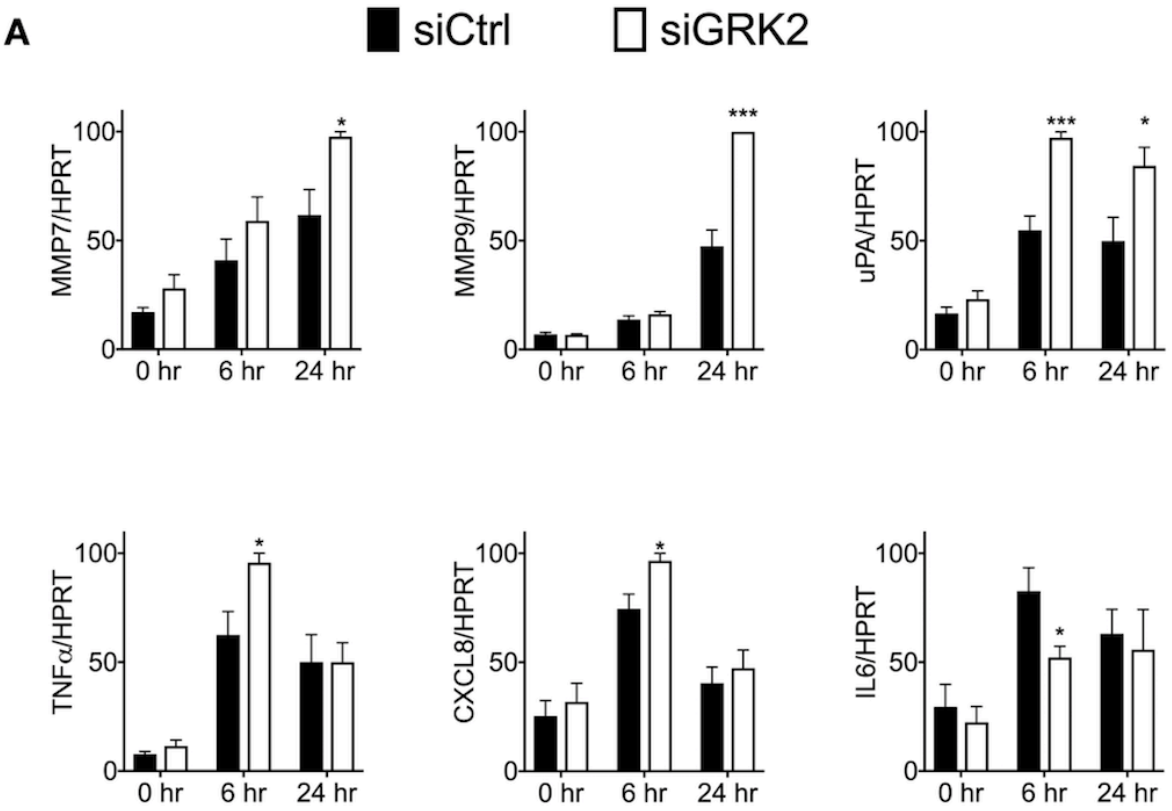
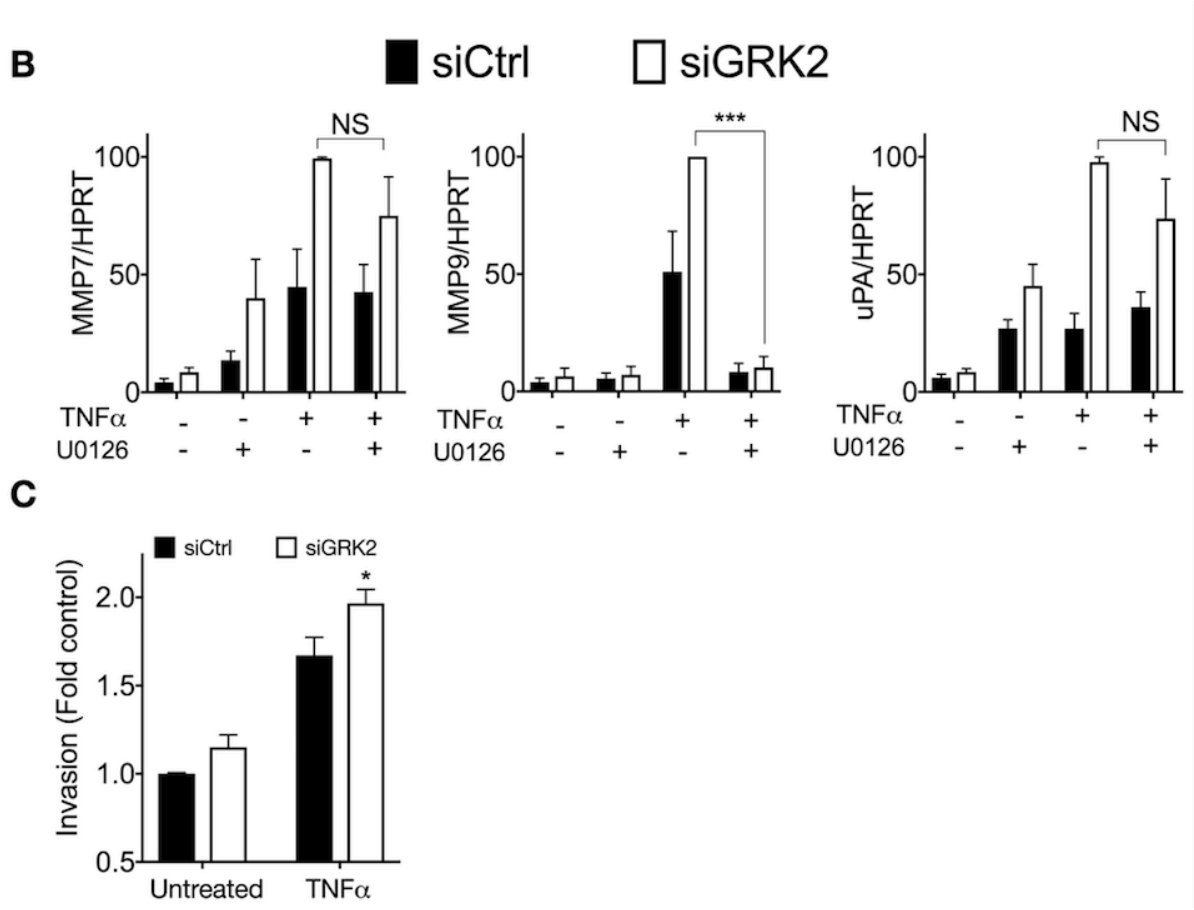


Figure 2.4. (cont'd)



cells to a matrigel invasion assay. Control and GRK2 knockdown SW480 cells were seeded in the upper chamber of a transwell insert coated with matrigel and allowed to migrate towards serum for 48 hours in the presence or absence of TNFα (20ng/ml). As shown, GRK2 knockdown cells treated with TNFα showed enhanced degradation of the matrigel layer and subsequent increase in migration towards the serum stimulus **(Figure 2.4.C)**. Recently, groups have demonstrated that, in other epithelial cell models, MMP9 is critical for the onset and activation of epithelial migration and wound repair [23] suggesting that the effect of GRK2 on ERK1/2 activation and MMP9 expression may be responsible for the increase in TNFα induced wound healing in these cells.

GRK2 Localizes in the Mitochondria and Inhibits ROS Production

In previous studies, we have shown that GRK2 can directly regulate TNFα-induced IκBα phosphorylation [12]. Moreover, because the cellular phenotype (MMP expression and wound healing [23–25]) appears to be regulated via the ERK pathway, we focused on determining the mechanisms by which GRK2 regulates the TNFα-induced ERK pathway. To define how GRK2 knockdown enhanced ERK1/2 phosphorylation, we initially focused on known GRK2 binding partners with respect to the ERK pathway (mainly MEK1/2). However, our immunoprecipitation approach did not identify MEK1/2 as a binding partner for GRK2 in these cells (data not shown). Therefore, we pursued an unbiased mass-spectrometry approach to identify the various partners of GRK2 in the SW480 cells. For this we immunoprecipitated GRK2 from the lysates of untreated cells and subjected the eluate to gel electrophoresis and reverse-phase chromatography, followed by ionization and mass spectrometry. After fragmentation through high-energy collision-induced dissociation, data analysis on the fragments was performed primarily through Proteome Discoverer, with a secondary analysis performed using Scaffold. Note that the

controls were immunoprecipitated using a species- and isotype- matched non-specific IgG. This approach provided 27 unique proteins that co-immunoprecipated with GRK2 in the SW480 cells **(Figure 2.5.A-C)**. Several of these proteins are localized/associated with mitochondria or mitochondrial functions suggesting that GRK2 may be playing a critical role in mitochondrial behavior **(Figure 2.5.B and Table 2.1.)**.

To further determine if GRK2 can localize to the mitochondria, we isolated mitochondria from untreated and TNFα treated SW480 cells and subjected them to SDS-PAGE followed by Western blotting for GRK2. We found that GRK2 is detectable in the mitochondria even under basal conditions as well as at 10 minutes and 3 hours after TNFα stimulation, indicating that GRK2 is not only present in the mitochondria but remains there even under inflammatory conditions **(Figure 2.6.A)**. To further assess the functional consequences of GRK2 being present in the mitochondria we measured generation of reactive oxygen species (ROS) in control and GRK2 knockdown cells. We focused on ROS because previous studies have shown that ROS generation can lead to ERK activation [26]. Therefore, we treated control and GRK2 siRNA transfected SW480 cells with TNFα and examined ROS production using flow cytometry. There was a modest increase in TNFα-induced ROS production in control cells over the time points tested; however, in the GRK2 knockdown cells, ROS production was significantly enhanced and this was more evident at 10 min following TNFα treatment **(Figure 2.6.B-C)**.

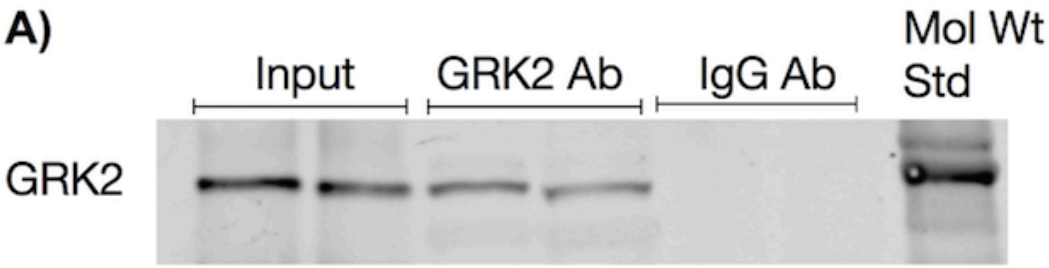
Increase in ROS is Responsible for Increase in ERK1/2 Phosphorylation

ROS is produced by a variety of cell types but is normally viewed as harmful to both cells and tissues. Over the last few decades, the functional capabilities of ROS have been expanding and ROS has been shown to play alternative roles other than causing harmful effects. ROS generation in non-phagocytotic cells (at low

Figure 2.5. GRK2 interacts with a number of intracellular proteins in SW480 cells

Endogenous GRK2 was immunoprecipitated from untreated SW480 cells and the eluates subjected to SDS-PAGE. After confirmation of efficient and specific IP of GRK2 **(A)**, the bands from the gels were extracted and subjected to digestion, extraction and Mass spectrometry as described in the methods. **B)** The list of mitochondrial proteins identified proteins. All other proteins identified are provided in the supplementary table. Note that the list includes proteins that were exclusively co-immunoprecipitated by GRK2 IgG (n=3). **C)** Representative readout of identification process for proteins. Shows entire amino acid sequence for protein selected and shows minimum peptide threshold for identification.

A)



B)

Protein	p Value
HSP70-protein 4	p<0.016
Succinate Dehydrogenase	p<0.016
Elongation Factor Tu	p<0.018
Stress Protein 70	p<0.034
Mitochondrial Ribosome-associated GTPase 2	p<0.046
Serine Hydroxymethyltransferase	p<0.046

C)

SDHA_HUMAN (100%), 72,691.8 Da
Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial OS=Homo sapiens GN=SDHA PE=1 SV=2
1 exclusive unique peptides, 1 exclusive unique spectra, 1 total spectra, 14/664 amino acids (2% coverage)

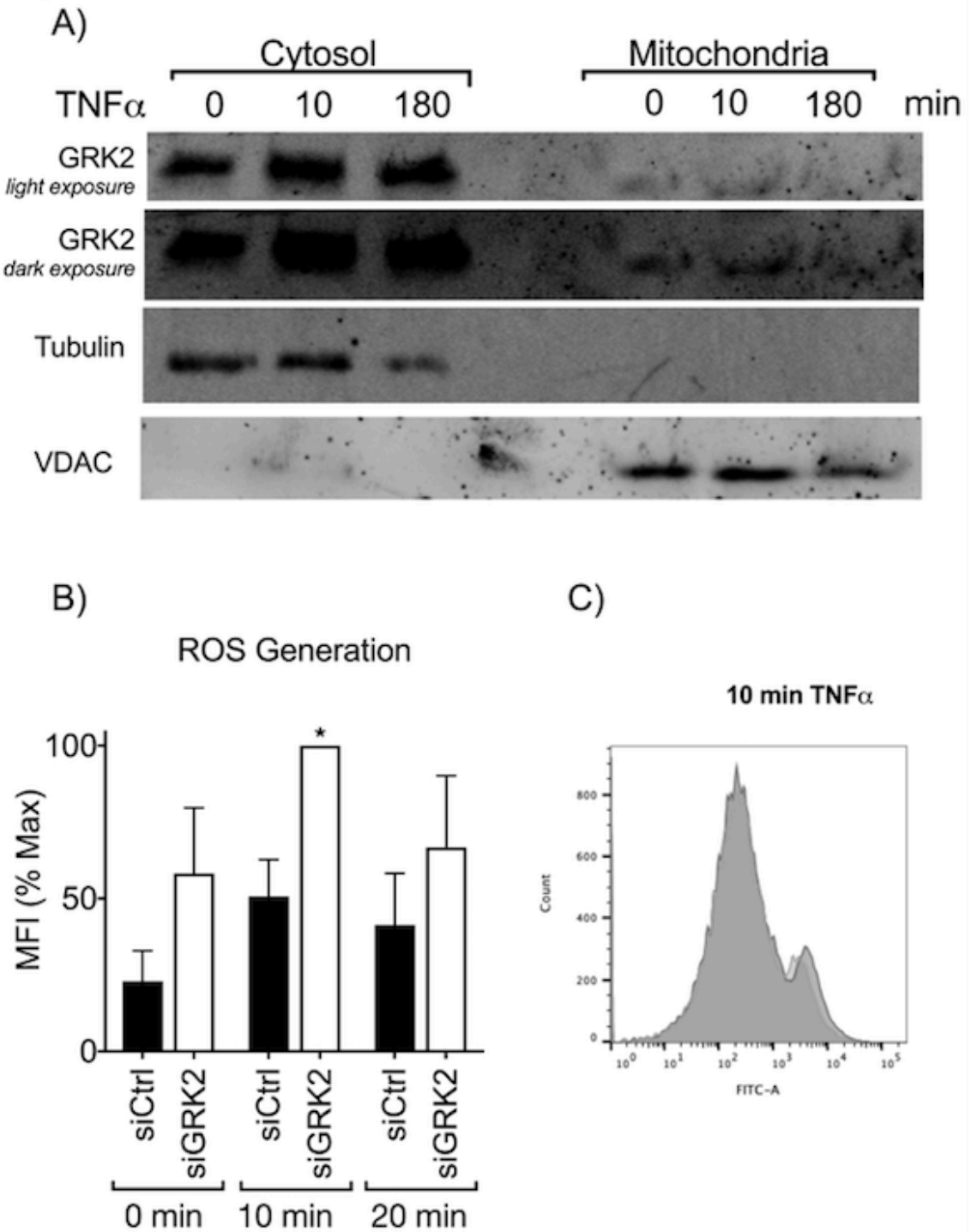
MSGVRGLSRL LSARRLALAK AWPTVLQTGT RGFHFTVDGN KRASAKVSDS ISAQYPVVDH
EFDAVVVGAG GAGLRAAFGL SEAGFNTACV TKLFPTRSHT VAAQGGINAA LGNMEEDNWR
WHFYDTVKGS DWLGDQDAIH YMTEQAPAAV VELENYGMPF SRTEDGKIYQ RAFGGQSLKF
GKGGQAHRCC CVADRTGHSL LHTLYGRSLR YDTSYFVEYF ALDLLMENGE CRGVIALCIE
DGSIHRIRAK NTVVATGGYG RTYFSCTSAH TSTGDGTAMI TRAGLPCQDL EFVQFHPTGI
YGAGCLITEG CRGEGGILIN SQGERFMERY APVAKDLASR DVVSRSMTLE IREGRGCGPE
KDHVYLQLHH LPPEQLATRL PGISETAMIF AGVDVTKEPI PVLPTVHYNM GGIPTNYKGQ
VLRHVNGQDQ IVPGLYACGE AACASVHGAN RLGANSLLDL VVFGRACALS IEESCRPGDK
VPPIKPNAGE ESVMNLDKLR FADGSIRTSE LRLSMQKSMQ NHAAVFRVGS VLQEGCGKIS
KLYGDLKHLK TFDRGMVWNT DLVETLELQN LMLCALQTIY GAEARKESRG AHAREDYKVR
IDEYDYSKPI QGQQKKPFEE HWRKHTLSYV DVGTGKVTLE YRPVIDKTLN EADCATVPPA
IRSY

Table 2.2. Unique proteins co-immunoprecipitated with GRK2
SW480 cells were lysed and 2 mg of total lysate was immunoprecipitated with the anti-GRK2 antibody on Protein A agarose beads. After isolation, bound proteins were analyzed and identified using mass spectrometry. All significant proteins bound to GRK2 identified are listed in the above table in order of significance. (n=3)

Protein	p Value
Beta-adrenergic receptor kinase 1	0.00048
E3 ubiquitin-protein ligase TRIM21	0.00056
60S ribosomal protein L10a	0.0058
40S ribosomal protein S24	0.0075
78 kDa glucose-regulated protein	0.0094
Acetyl-CoA carboxylase 1	0.016
Arginine--tRNA ligase, cytoplasmic	0.016
F-box only protein 22	0.016
Heat shock 70 kDa protein 4	0.016
Heterogeneous nuclear ribonucleoprotein H3	0.016
Maleylacetoacetate isomerase	0.016
Probable ATP-dependent RNA helicase DDX41	0.016
Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	0.016
Elongation factor Tu, mitochondrial	0.024
Heterogeneous nuclear ribonucleoprotein M	0.026
Stress-70 protein, mitochondrial	0.034
Nucleolin	0.035
60S ribosomal protein L23	0.038
60S ribosomal protein L38	0.038
T-complex protein 1 subunit theta	0.039
Nucleophosmin	0.04
Heterogeneous nuclear ribonucleoprotein U-like protein 1	0.041
Mitochondrial ribosome-associated GTPase 2	0.046
D-3-phosphoglycerate dehydrogenase	0.047
Heat shock cognate 71 kDa protein	0.047
Platelet-activating factor acetylhydrolase IB subunit gamma	0.047
Serine hydroxymethyltransferase, mitochondrial	0.047

Figure 2.6. GRK2 is present in the mitochondria in SW480 cells and regulates TNFα-induced ROS generation

Isolated Mitochondria shows GRK2 localization in mitochondria in the absence and presence of TNFα (20 ng/ml). **A)** Representative western blot identifies location of GRK2 in different cellular fractions. To see specificity of fractionation, tubulin is used for cytoplasmic fraction control and VDAC is used for mitochondrial fraction. (n=3) **B)** GRK2 knockdown enhances ROS Expression. SW480 cells were treated with either siCtrl or siGRK2 and after 0, 10, or 20 minutes of TNFα stimulation; ROS production was measured and analyzed using flow cytometry. Data expressed as % maximal expression (n=3). **C)** Representative histogram of ROS expression after 10 minutes of TNFα expression. Lighter color is siCtrl and darker is siGRK2. (n=3). Means +/- SEM, * $p < 0.05$.



concentrations) is recognized as ubiquitous intracellular messengers that can activate mitogen-associated signal transduction pathways, including ERK1/2 [27,28]. To determine if the observed increase in ROS generation was causative for the increases in phosphorylated ERK1/2 in SW480 cells, we treated these cells with a superoxide inhibitor, N-acetylcysteine (NAC), to inhibit the production of ROS and demonstrate the link between ROS and ERK1/2 activation. Control and GRK2 knockdown SW480 cells were pretreated with NAC (100 μM) for 30 minutes and then stimulated with TNFα for 30 minutes. In the groups untreated with NAC we observed that knocking down GRK2 caused a significant increase in the pERK1/2 (as expected), but in the presence of NAC, ERK activation was suppressed especially in the GRK2 knockdown cells **(Figure 2.7.A)**. This data demonstrates that the enhanced ERK activation observed in the GRK2 knock down cells following TNFα stimulation is due to increase in ROS generation. To further link ROS generation to wound healing response, we repeated the wound healing assay in control and GRK2 knockdown cells pretreated with NAC and untreated or treated with TNFα for 48 hours. As shown NAC significantly inhibited the effect of GRK2 knockdown on TNFα-induced wound closure **(Figure 2.7.B)**. Together this data supports the role that GRK2 inhibits TNFα induced wound healing in these cells via the ROS pathway.

GRK2 Heterozygous Mice are protected in Intestinal Wound Healing Model

Our *in vitro* wound-healing assay indicated that knocking down GRK2 in intestinal epithelial SW480 cells improved their ability to close the wound in the presence of TNFα. A common characteristic of dextran sodium sulfate (DSS) induced colitis in mice is gross epithelial damage and high levels of TNFα in the colon [29,30]. To determine if our *in vitro* results would correlate to an *in vivo* model we used mice that were heterozygous for GRK2 and subjected them to a 2.5% DSS

Figure 2.7. ROS scavenging prevents effect of GRK2 on ERK activation and wound closure

A) Treatment with N-acetylcysteine (NAC) prevents TNFα-induced increase in pERK in siGRK2 cells. Cells were untreated or treated with TNFα and NAC as shown. TNFα treatment was performed for 30 minutes. pERK/ERK levels were assayed as described in the methods. Representative blot is shown in the top and quantitation in the bottom. Expressed as fold siCtrl for either no treatment or NAC treatment. (n=3). Means +/- SEM, * $p < 0.05$, ** $p < 0.01$. **B)** Treatment with NAC prevents TNFα-induced wound closure in siGRK2 cells. Wound healing experiments were done as described in the methods. siControl and siGRK2 cells were untreated or pretreated with NAC followed by treatment (or not with TNFα) for 48 hours as shown. (N=3). ***p<0.01.

A)

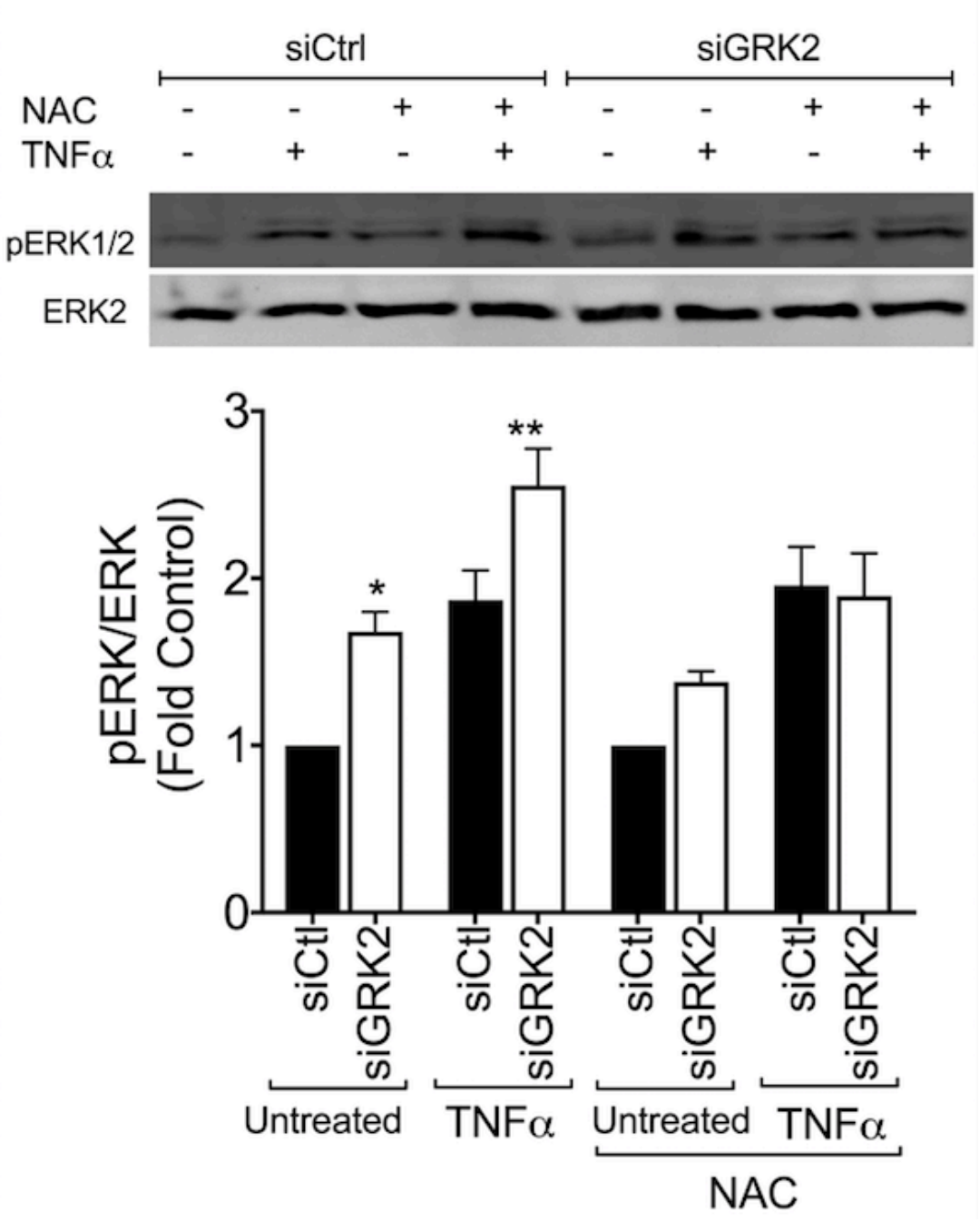
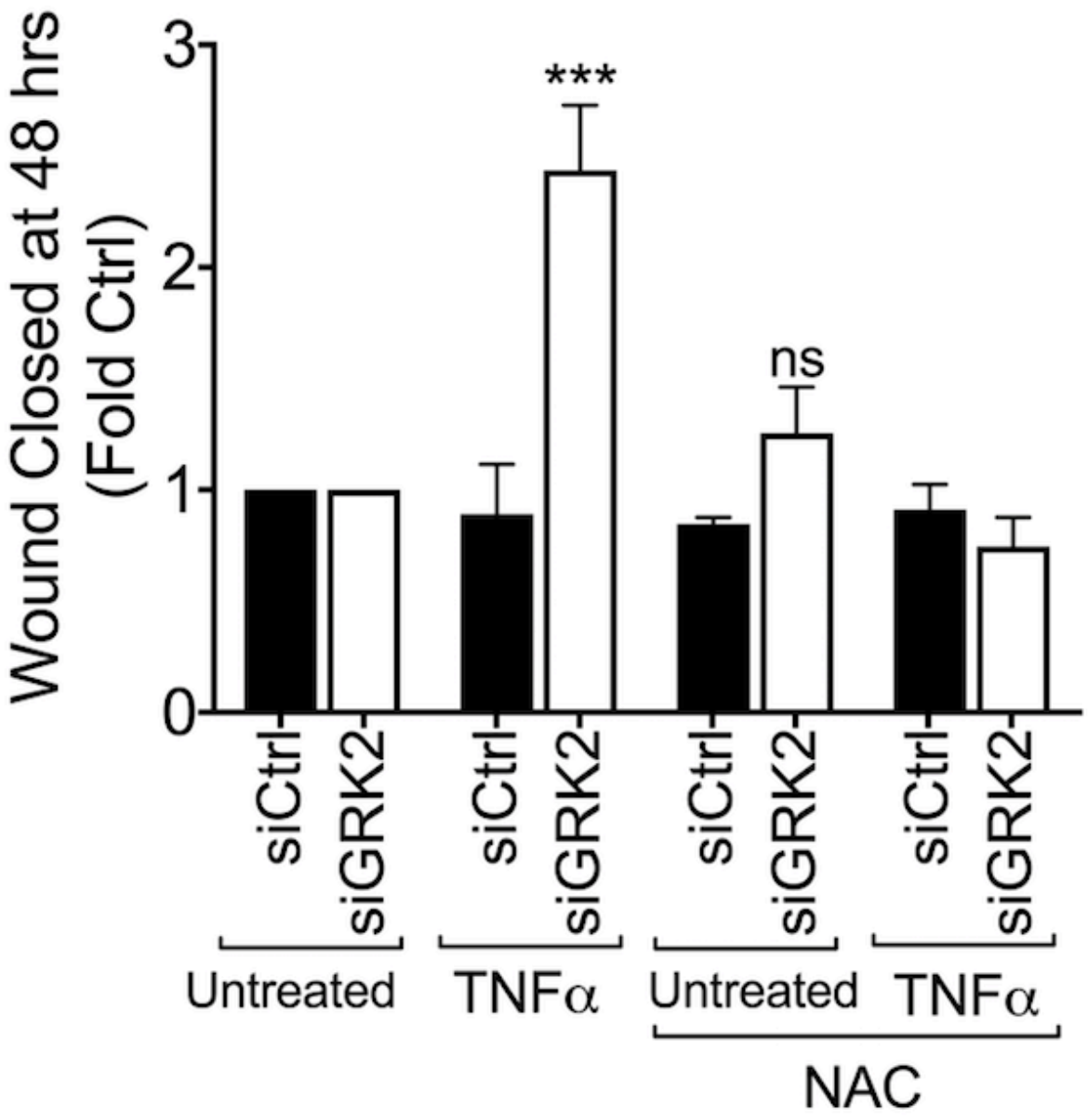


Figure 2.7. (cont'd)

B)



colitis. Both wild-type (WT) and heterozygous GRK2 mice ($GRK2^{+/-}$) were administered 2.5% DSS in drinking water. Mice were scored daily for weight loss and external signs of disease measured as Disease Activity Score (DAI, indicated by weight loss, loose and bloody stools, hunched posture, crusty eyes, and ruffled hair coat) [31]. After 7 days of DSS, the mice were given clean water and allowed to heal from intestinal inflammation for an additional 5 days. After DSS treatment, DAI increased after day 7 but recovered after day 12 and were back to normal by day 13 in the wild type mice **(Figure 2.8.A)**. Interestingly, the DAI was significantly decreased in the $GRK2^{+/-}$ group. This was also evident in the histopathology where GRK2 heterozygous knockout mice showed marked protection **(Figure 2.8.B)**. GRK2, therefore, is protective in *in vivo* wound healing, perhaps through alterations of the epithelial response to TNFα and its influence on wound healing.

Figure 2.8. Intestinal wound healing is enhanced in GRK2 heterozygous mice Wild type and GRK2 heterozygous mice were subjected to 2.5% DSS for the indicated times. **A)** Disease activity index was determined as described in the text (n=6-8). **B)** Histology of the colon from mice subjected to intestinal inflammation at day 13 (n=6-8). Means +/- SEM, * $p < 0.05$.

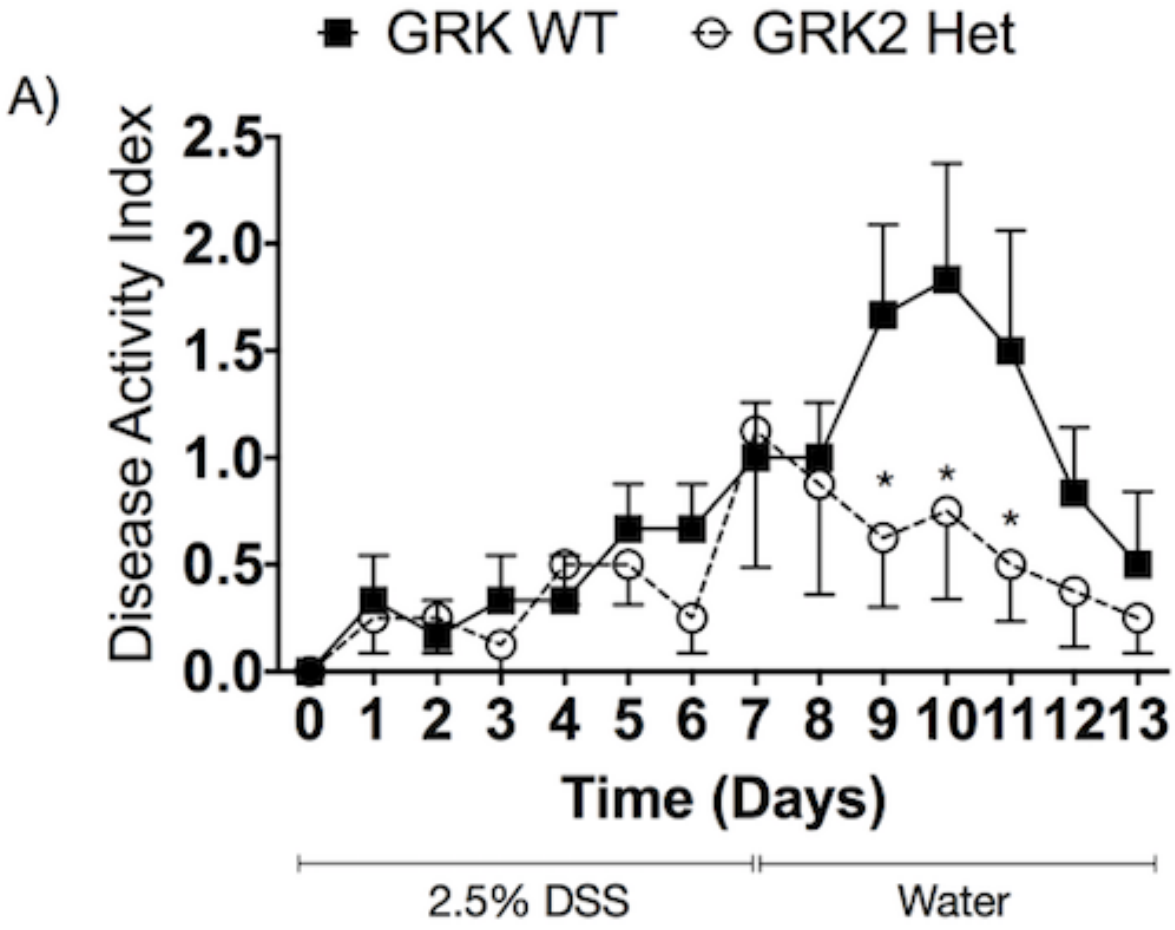
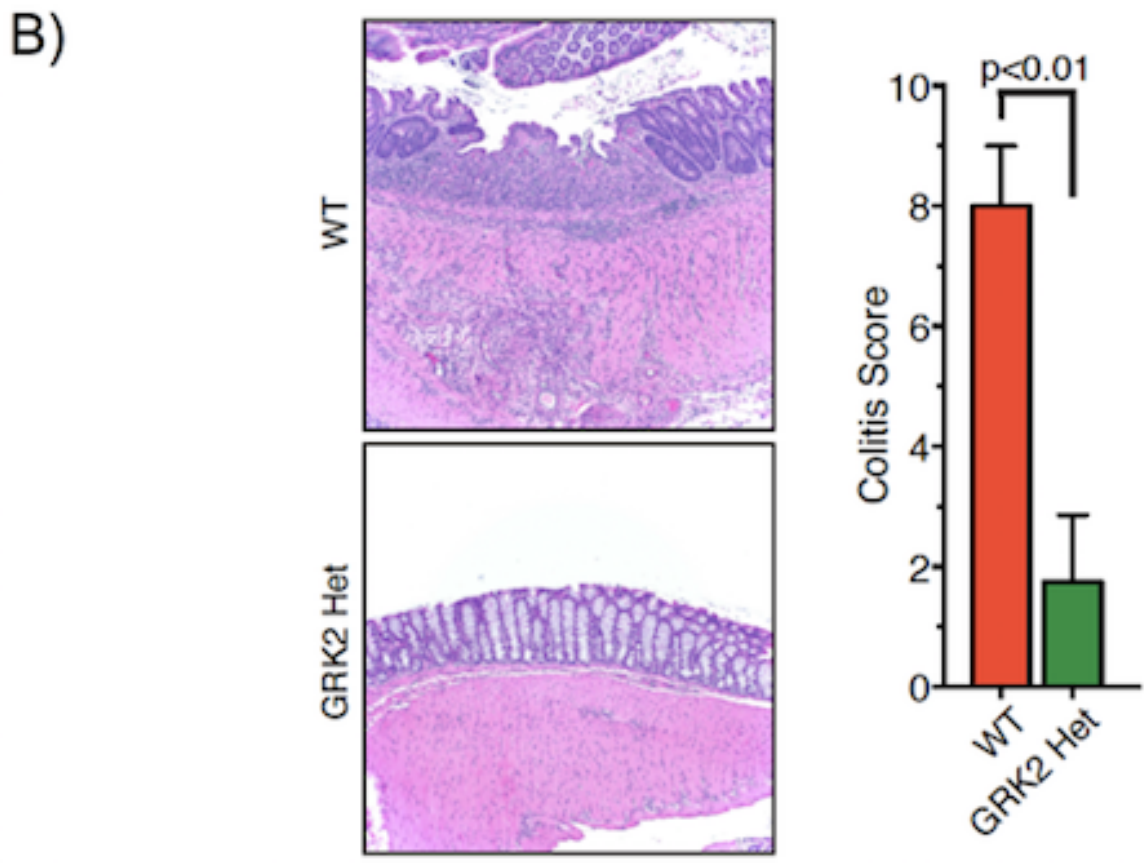


Figure 2.8. (cont'd)



DISCUSSION

Since the discovery of GRK2, it has been shown as a key regulator of G-protein coupled receptor phosphorylation and desensitization. As it continued to be studied, its role has expanded and it has been shown to have many different functions both dependent and independent of its catalytic activity [3]. Furthermore, GRK2 has been shown to regulate non-GPCRs as well as a variety of intracellular targets leading to a number of cellular roles for this kinase. In this study, we investigated these non-receptor regulatory capabilities of GRK2 in intestinal epithelial cells in response to TNFα stimulation.

In previous studies, GRK2 has been demonstrated to play a negative role in wound healing that is also associated with increased levels of TNFα. Measuring levels of inflammatory cytokines in the hearts of mice lacking β1-adrengergic receptor (B1KO) and expressing βARKct (a GRK2 inhibitor) there was an increase in TNFα, IL-6 and IL-1β 24 hours after undergoing myocardial infarction which is indicative of increased healing associated inflammation [32]. Alternatively GRK2 was shown to positively mediate sphingosine-1 phosphate (S1P)-induced ERK1/2 activation and wound closing in HEK293 cells as well as mouse embryonic fibroblasts [33]. In our studies with colonic epithelial cells, GRK2 inhibited TNFα-induced ERK1/2 phosphorylation as well as wound closure, and thus the role of GRK2 in wound healing per se is different between the two studies. In the above study GRK2 regulates ERK signaling through modifications of the scaffolding ability of GIT1 through direct interactions. This modification alters the Rac/PAK/MEK/ERK pathway leading to eventual changes in migration. While these differences in how GRK2 regulates the ERK pathway could be attributed to different cell types and ligands, the role of GRK2 in the SW480 cells offers an alternative pathway of ERK regulation through modification of TNFα-induced ROS production that acts a

mitogenic signaling factor to stimulate ERK. Thus, the critical factor between both of these studies is the activation of the ERK pathway. The role of the ERK pathway in wound healing is becoming more and more apparent through studies in other epithelial cells types and several studies have now shown ERK to be a critical regulator of wound healing and cellular migration [23,24]. Importantly, activation of the ERK pathway has been linked to improved wound healing responses in a variety of different cell types, including colonic epithelial cells [34]. Thus, understanding the mechanisms by which the ERK pathway is regulated in colonic epithelial cells could lead to identification of better therapeutic targets for intestinal inflammation. In this context, our results using GRK2 suggest that this may be one additional mechanism by which ERK pathway and its consequent effect on wound healing is regulated. Thus, we show for the first time that GRK2 inhibits the TNFα induced wound healing response in colonic epithelial cells.

The ERK1/2 pathway has a diverse set of roles within the cell including regulation of cell growth, survival, as well as the regulation of cell motility [35]. A necessary mechanism for cellular migration and wound healing is the capacity for matrix degradation through the localized expression of matrix metalloproteinases. One suggested mechanism for the ability of ERK1/2 to alter migration and wound healing is the upregulation of MMPs for extracellular matrix remodeling [36]. We provide evidence that MMP7, MMP9, and urokinase plasminogen activator (uPA) (another ECM remodeler) are all elevated in GRK2 knockdown cells and that the increase in MMP9 expression especially is ablated by inhibition of the ERK pathway. Other studies demonstrate that MMP9 is critical to the onset and activation of epithelial migration and wound repair in both SW480 cells as well as alternative cell types such as HEK293 cells [23–25]. Consistent with those studies, our data has shown that GRK2 knockdown cells have increased invasive capabilities through a

matrigel layer. Taken together, our studies suggest that GRK2 regulation of the ERK pathway in SW480 cells modulates MMP9 and consequent epithelial cell migration.

It is possible that MMP7 and uPA (as well as the inflammatory cytokines, CXCL8, IL6, and TNFα) are regulated through the changes observed in NFκB signaling or other unidentified pathways. Our lab has previously confirmed that GRK2 regulates the expression of CXCL8 and IL6 through alterations of NFκB in murine macrophages [17]. In this study, we focused on mechanisms and consequences by which GRK2 regulates the ERK pathway. Thus, regulation of MMP9 through pERK1/2 and the importance of this MMP in published studies indicate that it may be sufficient to drive the phenotype we observe in our system.

Previously we showed that GRK2 could directly influence TNFα signaling through binding to IκBα [12]. Additionally, GRK2 has been shown to interact with other signaling proteins such as PI3K [37], AKT [38], GIT [39], and MEK1 [40]. Based on these previous studies we hypothesized that GRK2 would interact with an unknown component of the TNFα signaling pathway to influence regulation of ERK1/2 in SW480 cells. Interestingly, when we performed an immunoprecipitation assay for GRK2 in SW480 cells the most abundantly co-purified peptides were not from the canonical TNFα signaling proteins but rather from mitochondrial proteins. Indeed, when we isolated the mitochondrial fraction from these cells we observed that GRK2 is present in the mitochondria both basally as well as after TNFα stimulation. This ability for GRK2 to be present in the mitochondria has been shown by other groups in both fibroblasts [41] as well as macrophages [42] and cardiac myocytes [43] but has never been shown in colonic epithelial cells. Previous work investigating the role of GRK2 in the mitochondria showed that knocking down GRK2 increases the mitochondrial generation of ROS in response to lipopolysaccharide in macrophages [42]. Our results on ROS generation in SW480 cells, following TNFα

stimulation, are consistent with these findings. It should be noted that one of the co-immunoprecipitated proteins with GRK2 in SW480 cells was succinate dehydrogenase. Although we do not yet know whether GRK2 directly binds to succinate dehydrogenase to influence its function, its identification in the immunoprecipitation experiment is intriguing. Previous studies have shown that succinate dehydrogenase is important in the generation of ROS [44,45] and therefore in future studies we will examine the link between GRK2, succinate dehydrogenase and ROS generation. Our results on ROS generation and ERK activation are consistent with previous studies demonstrating a strong link between ROS and ERK pathway [26,46]. In line with this, treatment with superoxide inhibitor completely abolished the enhanced ERK activation observed in GRK2 knockdown cells. Together these results indicate that GRK2 is present both in the cytoplasm as well as in the mitochondria under normal and inflammatory conditions in the colonic epithelial cells and this presence regulates ROS generation and subsequent modulation of ERK-mediated wound healing response.

Inflammatory bowel disease is a chronic inflammatory state characterized by persistent damage to the epithelial barrier in the colon. A common characteristic of dextran sodium sulfate (DSS) induced colitis model in mice is gross epithelial damage and high levels of TNFα in the colon. To determine if our *in vitro* results with SW480 cells can be correlated to *in vivo* pathophysiology, we used mice that were heterozygous for GRK2 and subjected them to a wound healing DSS model. Our data indicates that knocking down GRK2 does, in fact, protect against intestinal inflammation, consistent with the *in vitro* data. It should however, be noted that in vivo intestinal inflammation involves several different cell types. Even though the injury is initiated by DSS at the level of the intestinal epithelial cells, pathogenesis of inflammation involves participation of many different cell types including immune

cells. Since GRK2 is expressed in many different cell types, it is possible that the effect observed in the heterozygous mice is not restricted to its role in intestinal epithelial cells. These certainly are important questions for future studies.

Although very little is known about the role of GRK2 in the colon or in colitis, GRK2 has been implicated in several diseases including multiple sclerosis [14], Alzheimer's disease [47], and rheumatoid arthritis as well as sepsis and endotoxemia [15]. In addition, others have examined the role of other GRKs, including GRK6, in DSS-induced colitis model and shown that mice deficient in GRK6 have enhanced immune cell infiltration and enhanced severity of colitis [48]. These results suggest that the role of the various GRKs in colitis models could be different and warrant further detailed investigation. Altogether these studies show a regulatory capacity for GRKs in the colon and a vital role of GRK2 in inflammation, this merits future work on the mechanism of protection and the role of GRK2 in colitis.

In conclusion, our studies unravel a critical role for GRK2 in TNFα signaling in colonic epithelial cells and potential role for GRK2 in intestinal inflammation. Through regulation of ROS-generated ERK1/2 phosphorylation GRK2 knockdown enhances the ability of SW480 cells to close a wound possibly through increased MMP9 expression. These studies suggest GRK2 as a possible therapeutic target in intestinal inflammation.

ACKNOWLEDGEMENTS

We gratefully acknowledge the support from NIH (grants HL095637, AI099404 and AR056680 to N.P.). We thank the University lab animal resources for taking excellent care of our animals, and the Histopathology laboratory for their excellent service.

REFERENCES

REFERENCES

1 Gurevich, E. V., Tesmer, J. J. G., Mushegian, A. and Gurevich, V. V. (2012) G protein-coupled receptor kinases: more than just kinases and not only for GPCRs. Pharmacol. Ther., Elsevier Inc. **133**, 40–69.

2 Packiriswamy, N. and Parameswaran, N. (2015) G-protein-coupled receptor kinases in inflammation and disease. Genes Immun. **16**, 367–77.

3 Evron, T., Daigle, T. L. and Caron, M. G. (2012) GRK2: Multiple roles beyond G protein-coupled receptor desensitization. Trends Pharmacol. Sci., Elsevier Ltd **33**, 154–164.

4 Penela, P., Murga, C., Ribas, C., Lafarga, V. and Mayor, F. (2010) The complex G protein-coupled receptor kinase 2 (GRK2) interactome unveils new physiopathological targets. Br. J. Pharmacol. **160**, 821–832.

5 Woodall, M. C., Ciccarelli, M., Woodall, B. P. and Koch, W. J. (2014) G protein-coupled receptor kinase 2: A link between myocardial contractile function and cardiac metabolism. Circ. Res.

6 Sansonetti, P. J. (2004) War and Peace at Mucosal Surfaces. Nat. Rev. Immunol. **4**, 953–964.

7 Kagnoff, M. F. and Eckmann, L. (1997) Epithelial cells as Sensors for Microbial Infection. J. Clin. Invest. **100**, 6–10.

8 Xavier, R. J. and Podolsky, D. K. (2007) Unravelling the pathogenesis of inflammatory bowel disease. Nature **448**, 427–434.

9 Gribar, S. C., Richardson, W. M., Sodhi, C. P. and Hackam, D. J. (2008) No Longer an Innocent Bystander: Epithelial Toll-Like Receptor Signaling in the Development of Mucosal Inflammation. Mol. Med. **14**, 645–659.

10 Okamoto, R. and Watanabe, M. (2005) Cellular and Molecular Mechanisms of the Epithelial Repair in IBD. Dig. Dis. Sci. **50**, 34–38.

11 Leppkes, M., Roulis, M., Neurath, M. F., Kollias, G. and Becker, C. (2014) Pleiotropic functions of TNF-α in the regulation of the intestinal epithelial response to inflammation. Int. Immunol. **26**, 509–515.

12 Patial, S., Luo, J., Porter, K. J., Benovic, J. L. and Parameswaran, N. (2011) G-protein coupled receptor kinases mediate TNFa induced NFkB signaling via direct interaction with and phosphorylation of IkBa. Biochem. J. **425**, 169–178.

13 Patial, S. and Parameswaran, N. (2010) Tumor Necrosis Factor alpha Signaling in Macrophages. Crit. Rev. Eukaryot. Gene Expr. **20**, 87–103.

14 Vroon, A., Kavelaars, A., Limmroth, V., Lombardi, M. S., Goebel, M. U., Van Dam, A.-M., Caron, M. G., Schedlowski, M. and Heijnen, C. J. (2005) G protein-coupled receptor kinase 2 in multiple sclerosis and experimental autoimmune encephalomyelitis. J. Immunol. **174**, 4400–6.

15 Parvataneni, S., Gonipeta, B., Packiriswamy, N., Lee, T., Durairaj, H. and Parameswaran, N. (2011) Role of myeloid-specific G-protein coupled receptor kinase-2 in sepsis. Int. J. Clin. Exp. Med. **4**, 320–30.

16 Durairaj, H., Steury, M. D. and Parameswaran, N. (2015) Paroxetine differentially modulates LPS-induced TNFα and IL-6 production in mouse macrophages. Int. Immunopharmacol., Elsevier B.V. **25**, 485–492.

17 Patial, S., Saini, Y., Parvataneni, S., Appledorn, D. M., Dorn, G. W., Lapres, J. J., Amalfitano, A., Senagore, P. and Parameswaran, N. (2011) Myeloid-specific GPCR kinase-2 negatively regulates NFkB1p105-ERK pathway and limits endotoxemic shock in mice. J. cell Physiol. **226**, 627–637.

18 Gao, C., Negash, S., Guo, H. T., Ledee, D., Wang, H. and Zelenka, P. (2002) CDK5 Regulates Cell Adhesion and Migration in Corneal Epithelial Cells. Mol. Cancer Res. **1**, 12–24.

19 Loniewski, K., Shi, Y., Pestka, J. and Parameswaran, N. (2008) Toll-like receptors differentially regulate GPCR kinases and arrestins in primary macrophages. Mol. Immunol. **45**, 2312–2322.

20 Parameswaran, N., Pao, C. S., Leonhard, K. S., Kang, D. S., Kratz, M., Ley, S. C. and Benovic, J. L. (2006) Arrestin-2 and G protein-coupled receptor kinase 5 interact with NFkappaB1 p105 and negatively regulate lipopolysaccharide-stimulated ERK1/2 activation in macrophages. J. Biol. Chem. **281**, 34159–70.

21 Jaber, M., Koch, W. J., Rockman, H., Smith, B., Bond, R. A., Sulik, K. K., Ross Jr., J., Lefkowitz, R. J., Caron, M. G. and Giros, B. (1996) Essential role of β-adrenergic receptor kinase 1 in cardiac development and function. Proc. Natl. Acad. Sci. U. S. A. **93**.

22 Thal, D. M., Homan, K. T., Chen, J., Wu, E. K., Hinkle, P. M., Huang, Z. M., Chuprun, J. K., Song, J., Gao, E., Cheung, J. Y., et al. (2012) Paroxetine is a direct inhibitor of g protein-coupled receptor kinase 2 and increases myocardial contractility. ACS Chem. Biol. **7**, 1830–9.

23 Bove, P. F., Hristova, M., Wesley, U. V., Olson, N., Lounsbury, K. M. and van der Vliet, A. (2008) Inflammatory levels of nitric oxide inhibit airway epithelial cell migration by inhibition of the kinase ERK1/2 and activation of hypoxia-inducible factor-1 alpha. J. Biol. Chem. **283**, 17919–28.

24 Kim, H. C., Kim, Y. S., Oh, H., Kim, K., Oh, S.-S., Kim, J., Kim, B. Y., Lee, S.-J., Choe, Y., Kim, D.-H., et al. (2014) Collagen Triple Helix Repeat Containing 1 (CTHRC1) acts via ERK-dependent induction of MMP9 to promote invasion of colorectal cancer cells. Oncotarget **5**, 519–529.

25 Luo, Y., Liang, F. and Zhang, Z. Y. (2009) PRL1 Promotes Cell Migration and Invasion by Increasing MMP2 and MMP9 Expression through Src and ERK1/2 Pathways. Biochemistry **48**, 1838–1846.

26 Iovine, B., Iannella, M. L., Nocella, F., Pricolo, M. R. and Bevilacqua, M. A. (2012) Carnosine inhibits KRAS-mediated HCT116 proliferation by affecting ATP and ROS production. Cancer Lett. **315**, 122–128.

27 Zhang, J., Jin, N., Liu, Y. and Rhoades, R. A. (1998) Hydrogen Peroxide Stimulates Extracellular Signal-regulated Protein Kinases in Pulmonary Arterial Smooth Muscle Cells. Am. J. Respir. Cell Mol.Biol. **19**, 324–332.

28 Chen, K. C., Zhou, Y., Xing, K., Krysan, K. and Lou, M. F. (2004) Platelet derived growth factor (PDGF)-induced reactive oxygen species in the lens epithelial cells: the redox signaling. Exp. Eye Res. **78**, 1057–1067.

29 Neurath, M. F. (2014) New targets for mucosal healing and therapy in inflammatory bowel diseases. Mucosal Immunol. **7**, 6–19.

30 Iizuka, M. and Konno, S. (2011) Wound healing of intestinal epithelial cells. World J. Gastroenterol.

31 Sharma, D., Malik, A., Steury, M. D., Lucas, P. C. and Parameswaran, N. (2015) Protective Role of β-arrestin2 in Colitis Through Modulation of T-cell Activation. Inflamm. Bowel Dis. **12**, 2766–2777.

32 Salazar, N. C., Vallejos, X., Siryk, A., Rengo, G., Cannavo, A., Liccardo, D., De Lucia, C., Gao, E., Leosco, D., Koch, W. J., et al. (2013) GRK2 blockade with βARKct is essential for cardiac β2-adrenergic receptor signaling towards increased contractility. Cell Commun. Signal. **11**, 64.

33 Penela, P., Ribas, C., Aymerich, I., Eijkelkamp, N., Barreiro, O., Heijnen, C. J., Kavelaars, A., Sánchez-Madrid, F. and Mayor, F. (2008) G protein-coupled receptor kinase 2 positively regulates epithelial cell migration. EMBO J. **27**, 1206–18.

34 Murray, D., Morrin, M. and McDonnell, S. (2004) Increased Invasion and Expression of MMP-9 in Human Colorectal Cell Lines by a CD44-dependent Mechanism. Anticancer Res. **24**, 489–494.

35 Vial, E. and Pouyssegur, J. (2004) Regulation of Tumor Cell Motility by ERK Mitogen-Activated Protein Kinases. Ann. NY Acad. Sci **1030**, 208–218.

36 Mccawley, L. J., Li, S., Wattenberg, E. V and Hudson, L. G. (1999) Sustained Activation of the Mitogen-activated Protein Kinase Pathway. J. Biol. Chem. **274**, 4347–4353.

37 Naga Prasad, S. V, Barak, L. S., Rapacciuolo, A., Caron, M. G. and Rockman, H. A. (2001) Agonist-dependent Recruitment of Phosphoinositide 3-Kinase to the Membrane by Beta-Adrenergic Receptor Kinase 1. J. Biol. Chem. **276**, 18953–18959.

38 Liu, S., Premont, R. T., Kontos, C. D., Zhu, S. and Rockey, D. C. (2005) A crucial role for GRK2 in regulation of endothelial cell nitric oxide synthase function in portal hypertension. Nat. Med. **11**, 952–958.

39 Premont, R. T., Claing, A., Vitale, N., Freeman, J. L. R., Pitcher, J. A., Patton, W., Moss, J., Vaughan, M. and Lefkowitz, R. J. (1998) Beta2-Adrenergic receptor regulation by GIT1, a G protein-coupled receptor kinase-associated ADP ribosylation factor GTPase-activating protein. Proc. Natl. Acad. Sci. **95**, 14082–14087.

40 Jiménez-Sainz, M. C., Murga, C., Kavelaars, A., Jurado-Pueyo, M., Krakstad, B. F., Heijnen, C. J., Mayor, F. and Aragay, A. M. (2006) G protein-coupled receptor kinase 2 negatively regulates chemokine signaling at a level downstream from G protein subunits. Mol. Biol. Cell **17**, 25–31.

41 Fusco, A., Santulli, G., Sorriento, D., Cipolletta, E., Garbi, C., Dorn, G. W., Trimarco, B., Feliciello, A. and Iaccarino, G. (2012) Mitochondrial localization unveils a novel role for GRK2 in organelle biogenesis. Cell. Signal. **24**, 468–475.

42 Sorriento, D., Fusco, A., Ciccarelli, M., Rungi, A., Anastasio, A., Carillo, A., Dorn, G. W., Trimarco, B. and Iaccarino, G. (2013) Mitochondrial G protein coupled receptor kinase 2 regulates proinflammatory responses in macrophages. FEBS Lett., Federation of European Biochemical Societies **587**, 3487–94.

43 Chen, M., Sato, P. Y., Chuprun, J. K., Peroutka, R. J., Otis, N. J., Ibetti, J., Pan, S., Sheu, S., Gao, E. and Koch, W. J. (2013) Prodeath Signaling of G Protein–Coupled Receptor Kinase 2 in Cardiac Myocytes After Ischemic Stress Occurs Via Extracellular Signal–Regulated Kinase-Dependent Heat Shock Protein 90-Mediated Mitochondrial Targeting. Circ. Res. **112**, 1121–1134.

44 Mills, E. L., Kelly, B., Logan, A., Costa, A. S. H., Varma, M., Bryant, C. E., Tourlomousis, P., Däbritz, J. H. M., Gottlieb, E., Latorre, I., et al. (2016) Succinate Dehydrogenase Supports Metabolic Repurposing of Mitochondria to Drive Inflammatory Macrophages. Cell **167**, 1–14.

45 Yankovskaya, V., Horsefield, R., Tornroth, S., Luna-Chavez, C., Miyoshi, H., Leger, C., Byrne, B., Cecchini, G. and Iwata, S. (2003) Architecture of Succinate Dehydrogenase and Reactive Oxygen Species Generation. Science (80-.). **299**, 700–704.

46 Samavati, L., Monick, M. M., Sanlioglu, S., Buettner, G. R., Oberley, L. W. and Hunninghake, G. W. (2002) Mitochondrial K ATP channel openers activate the ERK kinase by an oxidant-dependent mechanism. Am J Physiol Cell Physiol **283**, 273–281.

47 Obrenovich, M. E., Palacios, H. H., Gasimov, E., Leszek, J. and Aliev, G. (2009) The GRK2 Overexpression Is a Primary Hallmark of Mitochondrial Lesions during Early Alzheimer Disease. Cardiovasc. Psychiatry Neurol. **2009**, 327360.

48 Eijkelkamp, N., Heijnen, C. J., Lucas, A., Premont, R. T., Elsenbruch, S., Schedlowski, M. and Kavelaars, A. (2007) G protein-coupled receptor kinase 6 controls chronicity and severity of dextran sodium sulphate-induced colitis in mice. Gut **56**, 847–54.

CHAPTER 3: GRK2 DEFICIENT MICE ARE PROTECTED FROM DEXTRAN SODIUM SULFATE-INDUCED ACUTE COLITIS

ABSTRACT

G-protein coupled receptor kinase 2 (GRK2) levels have been shown to be elevated in inflammatory diseases such as sepsis. In addition, GRK2 has been shown to modulate inflammatory response in vitro and in vivo via canonical (GPCR phosphorylation-dependent) and non-canonical (GPCR phosphorylation-independent) functions. Therefore, we hypothesized that ablation of GRK2 would significantly modulate the pathogenesis of dextran sodium sulfate (DSS) induced acute colitis in mice. To test this, we administered DSS to C56BL/6 wild-type (WT) and GRK2 heterozygous ($GRK^{+/-}$) mice in their drinking water for 7 days. $GRK2^{+/-}$ mice were protected from DSS induced colitis as demonstrated by decreased weight loss ($GRK^{+/-}$ end weight at 89% of initial vs WT 80%), lower disease activity index scores ($GRK^{+/-}$ 4.1, WT 9.1), and increased colon lengths ($GRK^{+/-}$ 5.3 cm vs WT 4.7 cm) as compared to WT mice. To examine the cellular mechanism by which GRK2 deficient mice are protected from colitis, we investigated the role for GRK2 in a myeloid specific knockout. Our results demonstrate that GRK2 deficiency in the myeloid cells is sufficient for the protection observed in the heterozygous mice. In both models we observed no major differences between WT and KO in immune populations in the colonic lamina propria or the mesenteric lymph nodes. Interestingly, we observed reduced inflammatory cytokines IL17, GMCSF, as well as the IL6/IL10 ratio in the colon of GRK2 knockout compared to the WT mice. Together our results indicate that the protective effects on colitis observed in both the GRK2 myeloid and heterozygous knock out may be regulated by a non-canonical, non-GPCR related function of GRK2 in inflammation.

INTRODUCTION

The intestinal tract, one of the body's largest mucosal lined surfaces, is essential in creating and maintaining the barrier between the luminal environment and the host. Alterations and damage to the integrity of this barrier can lead to various inflammatory processes and pathologies, one of which is inflammatory bowel disease (IBD) [1–4]. IBD itself presents as a multifactorial disease that results from dysregulation of the immune response and the epithelial cells lining the intestinal barrier. Regulating the response of the immune system to foreign antigens is particularly important in controlling the levels of inflammatory mediators and maintaining gut homeostasis. Modern therapies for the treatment of IBD include both anti-inflammatory as well as immunosuppressive therapies but given its diverse etiology these therapies are not effective for a significant proportion of patients or decrease in their efficacy over time [5]. Therefore it is critical to further our understanding of both the progression of IBD and the factors that regulate its pathogenesis. Importantly, we need to identify novel targets and methods for treating IBD.

G-protein coupled receptor kinases (GRKs) were originally identified in their ability to regulate G-protein coupled receptors through phosphorylation of the intracellular domains and recruiting β-arrestins for the subsequent desensitization of those receptors. Despite this vital biological role, our knowledge on the behavior of various GRKs, specifically GRK2, has been expanding to include non-receptor substrates including members of the NFκB pathways, MAP kinase pathways, and other non-GPCR receptors including EGFR and IGF-1R. This diverse set of binding partners allows for GRK2 to regulate cellular processes such as inflammatory gene expression and cellular migration [6]. This role of this kinase for these cellular processes has been

extensively examined in cardiac, immune, and other cell types [7–9] and recently work in our lab has shown a critical role for GRK2 in intestinal epithelial wound healing [10]. Therefore in this study we examined the role of GRK2 in the pathogenesis of acute DSS induced colitis using GRK2 deficient mice. Our studies highlight a critical role for GRK2 in the onset and regulation of acute colitis as seen by improved disease scores and weight loss in the heterozygous knockout mice than seen in wild type mice. Our results further demonstrate that the myeloid-specific GRK2 is sufficient for this protective response. This protection is in part due to alterations in inflammatory cytokine production in the mucosal tissue and not likely due to differences in immune cell chemotaxis.

MATERIALS AND METHODS

Mice

GRK2 heterozygous mice (C57BL6 background) were obtained from Jackson Labs (kindly donated by Dr. Robert Lefkowitz, Duke University) and were bred at Michigan State University by breeding GRK2 heterozygous mice with wild-type mice. It is important to note that homozygous knockout of GRK2 mice are embryonically lethal [11]. Myeloid-specific GRK2 deficient mice were generated as previously described [12]. In short, $GRK2^{fl/fl}$ mice where exons 3-6 of GRK2 are flanked by LoxP sites (kindly donated by Dr. Gerald Dorn II, Washington University School of Medicine, St. Louis). These mice were backcrossed to C57BL6 background for more than 12 generations. These mice were crossed with LysMCre mice (C57BL6 background from Jackson Labs) resulting in $GRK2^{fl/fl+LysMcre}$ mice. These mice were bred by mating $GRK2^{fl/fl}$ mice with $GRK2^{fl/fl+LysMCre}$. For experiments $GRK2^{fl/fl+LysMCre}$ were used in experiments and compared to littermate $GRK2^{fl/fl}$ controls. The mice were bred and housed in a specific-pathogen-free facility and were maintained at 22-24°C with 50% humidity a 12 hour light dark cycle. Mouse chow and water were provided *ad libitum*. All experiments (with exception of the initial female cohort) were performed with age- and sex- matched mice between 8 and 12 weeks of age. All animal procedures were approved by the Michigan State University IACUC and conformed to NIH guidelines.

DSS-Induced Colitis Model

Mice were administered either 3.5 to 4.25% dextran sodium sulfate (wt/vol) in their drinking water for the indicated times (generally 7 days) and then provided water for an additional day before euthanasia. During this treatment, mice were examined daily and scored for disease activity index under the following criteria: stool consistency

(1-loose), blood present in stool (1-mild, 2-gross), ruffled hair coat (0 or 1), crusty eyes (0 or 1), and hunched posture (0 or 1) and weight loss (1-0-5%, 2-5-10%, 3-10-15%). At the time of harvest splenic weight, colon length, and colon weight were additionally measured.

Sample Preparation

At the termination of DSS treatment, mice were euthanized through CO_2 asphyxiation. Spleen, mesenteric lymph nodes (MLN) and bone marrow were collected and processed as previously described [13]. In short, spleen and MLN were homogenized, treated with red blood cell lysis buffer, filtered through 40 um nylon mesh and counted for stimulation or flow cytometry analysis. Bone marrow cells were analyzed as previously described [14]. At harvest, after removal of fecal content, colon lengths and weights were noted and 5 mm segments of colon were flash frozen for mRNA and protein isolation or prepared for histology. The remaining colon was processed as previously described for leukocyte isolation [15]. In short, the colon was cut into 5 mm segments and incubated in epithelial dissociation buffer at 25°C with gentle agitation for 30 minutes. These segments were further cut into 1 mm segments and incubated for one hour in 0.5 mg/ml collagenase D. Finally, the pieces were strained through a 100 micron filter and loaded onto 80:40 percoll gradients. Cells were ultimately collected from the interface and used as leukocyte fraction after a wash in phosphate-buffered saline.

Flow Cytometry

Processed cells were incubated with fcγR blocking antibody to block non-specific binding and were then surface stained with antibody cocktail and washed with staining buffer (phosphate-buffered saline with sodium azide and bovine calf serum). When

required, intracellular staining was performed by fixing the cells using fixation buffer (eBioscience) and permeabilized and washed with perm buffer (phosphate-buffered saline with sodium azide and saponin, eBioscience). The antibodies used against cell surface markers were CD11b, F4/80, Ly6G, CD3, CD4, CD8; intracellular cytokines were IL-10 and TNFα. All antibodies were obtained from eBiosciences and used as per the manufacturer's instructions. All cells were run on LSR II and the data was analyzed using FlowJo software.

Cytokine/Chemokine Measurements

Cytokines were measured from plasma and ex vivo culture supernatants using enzyme-linked immunosorbent assay kits from eBiosciences Inc as per manufacturer's protocol.

RT-PCR

For determination of relative levels of RNA transcripts, RNA was isolated from snap frozen tissue using Qiagen RNeasy mini kit following the manufacturer's protocol. Reverse transcription was performed on 1 μg of RNA for single-stranded cDNA synthesis. Quantitative real-time PCR was performed for various genes of interest and the cDNA was amplified using SYBR Green Pro Master Mix with hypoxanthine-guanine phosphoribosyltransferase (HPRT) as the normalization control as described before [16].

Permeability (Ussing, In Vivo FITC)

In vivo permeability was determined by measuring paracellular permeability of 4 kDa fluorescein isothiocyanate (FITC)-dextran as previously described [17,18]. In short, mice were orally gavaged 300 mg/kg FITC-dextran in a total volume of 150 μL. 4 hours after administration serum was obtained by cardiac puncture after CO_2 asphyxiation and

fluorescence intensity was read on a Tecan M2000 spectrophotometer at 485 nm excitation and 530 nm emission and compared to a standard curve. *Ex vivo* permeability was determined as previously described [19] by removing proximal sections of colon and mounting them in Lucite chambers that were then placed in Ussing chambers (Physiologic Instruments) that exposed mucosal and serosal surfaces to oxygenated (95% O_2, 5% CO_2) Krebs bicarbonate ringer buffer (Sigma). Intestinal sections were not stripped of underlying muscle. The buffer was heated to 37 C by a heated water jacket and the samples were allowed to equilibrate to this temperature for 30 min. For measuring flux across the tissue, 4 kDa FITC-dextran (2.2 mg/ml final concentration) was added to the mucosal chamber; 10 kDa rhodamine B isothiocyanate (RITC)-dextran (0.55 mg/ml final concentration) was also added to the mucosal chamber and used as a control for tissue integrity. Serosal chamber samples were collected at 0 and 60 minutes and fluorescence was read as before (FITC 485 nm excitation, 530 nm emission; RITC 595 nm excitation, 615 nm emission).

Histopathology

Colon tissue was harvested, swiss rolled, and fixed overnight in 10% formalin followed by 70% ethanol. The tissue was then embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) and was analyzed in a blinded fashion by a board certified pathologist (P.C.L).

Statistical Analysis

All values are represented as mean +/- SEM. All data were tested for statistical significance using unpaired Student's T-test (two sample comparisons) and Analysis of Variance (ANOVA) with Tukey post-hoc test (more than 2 sample comparisons). All

analysis was done using Prism GraphPad software. A p-value of < 0.05 was considered significant.

RESULTS

GRK2 Heterozygous Mice are protected from DSS-Induced Colitis

This study was designed to investigate the role of GRK2 in the onset and progression of colitis. Other members of the GRK family have been implicated in playing crucial roles in colitis [20] as well as various β-arrestin proteins [21], which are closely associated with the classical role of GRKs in desensitization. Given the influential roles of these proteins and recent work from our lab demonstrating a critical role for GRK2 in intestinal epithelial wound healing we hypothesized that GRK2 would play an important regulatory role in the onset and progression of colitis. To ascertain the role of GRK2 in basal conditions, we investigated intestinal permeability of both, the colon using an Ussing chamber as well as the entire intestine through *in vivo* FITC-dextran leak from GI lumen to blood in wild type mice as well as GRK2 heterozygous mice (GRK2 het) **(Figure 3.1.A-B)**. For these initial experiments we focused on male mice. Our results indicated modest decreases in in vivo permeability in the GRK2 het mice compared to the wild types. Although this was not statistically significant (P=0.08), these results pointed towards potentially tighter junctions and protection from diseases such as IBD that rely in part on translocation and dissemination of luminal contents across the epithelial barrier. To directly test the role for GRK2 in colitis we treated male GRK2 het and wild-type mice with 4.25% DSS in their drinking water in order to induce colitis. After DSS treatment, wild-type mice lost significantly more body weight beginning at day 4 and displayed more severe signs of disease as measured by Disease Activity Score **(Figure 3.2.A-B)** (indicated by loose and bloody stools, hunched posture, crusty eyes, ruffled hair, etc, see methods for complete description). Compared to the wild type, GRK2 het mice were significantly protected from clinical signs of colitis.

Figure 3.1. GRK2 influences permeability in mice under basal conditions
A) Wild-type mice or GRK2$^{+/-}$ mice were cohoused and untreated. At baseline levels GRK2$^{+/-}$ mice have lower permeability across the colon *ex vivo* as assessed by the rate of FITC dextran transport measured by Ussing chamber. **B)** GRK2$^{+/-}$ mice showed decreased permeability across the length of the intestine *in vivo* as measured by the administration of FITC dextran by oral gavage (300 mg/kg) and measured in the serum after 4 hours. n =5 mice per genotype +/- SEM.

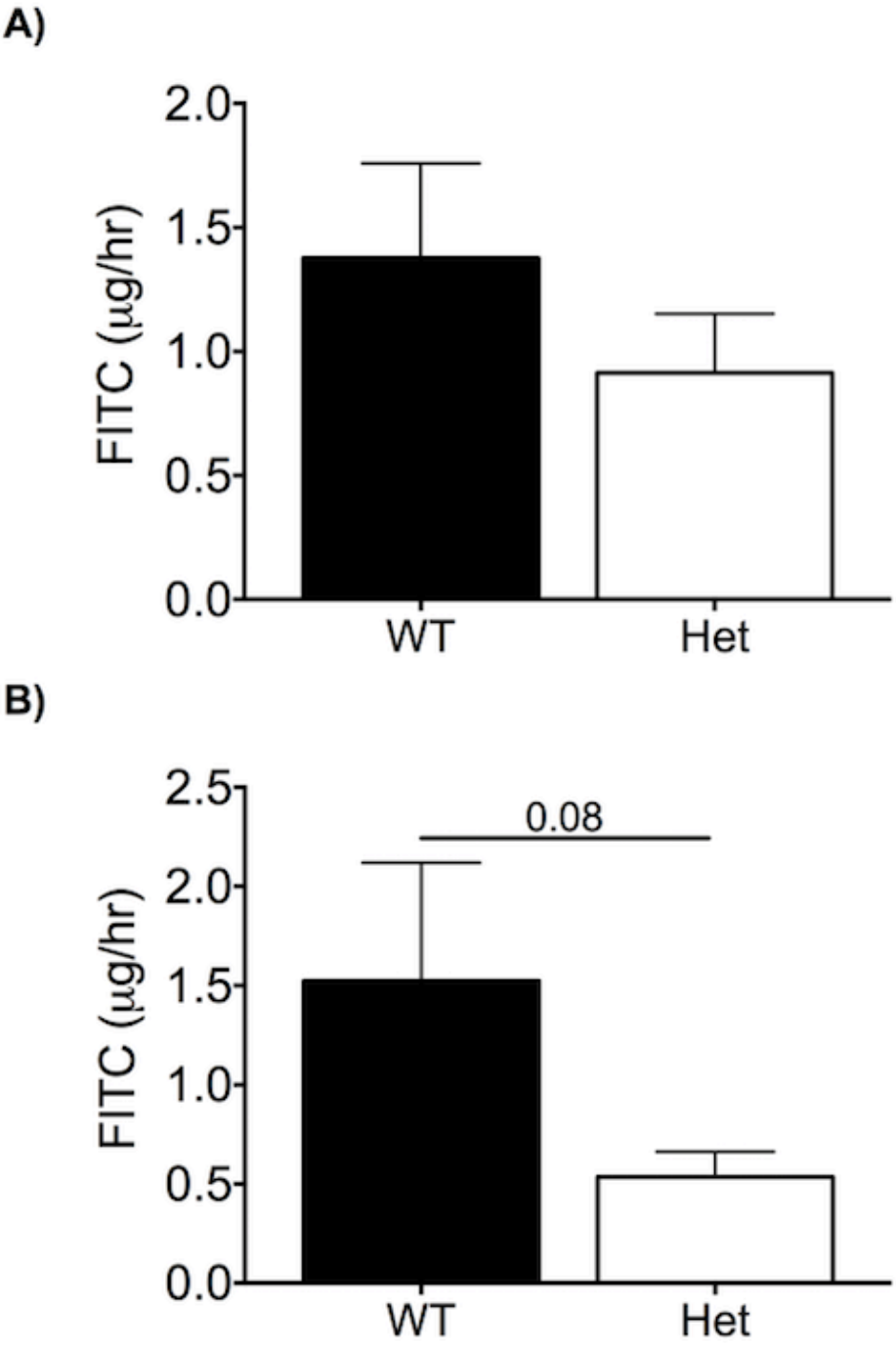
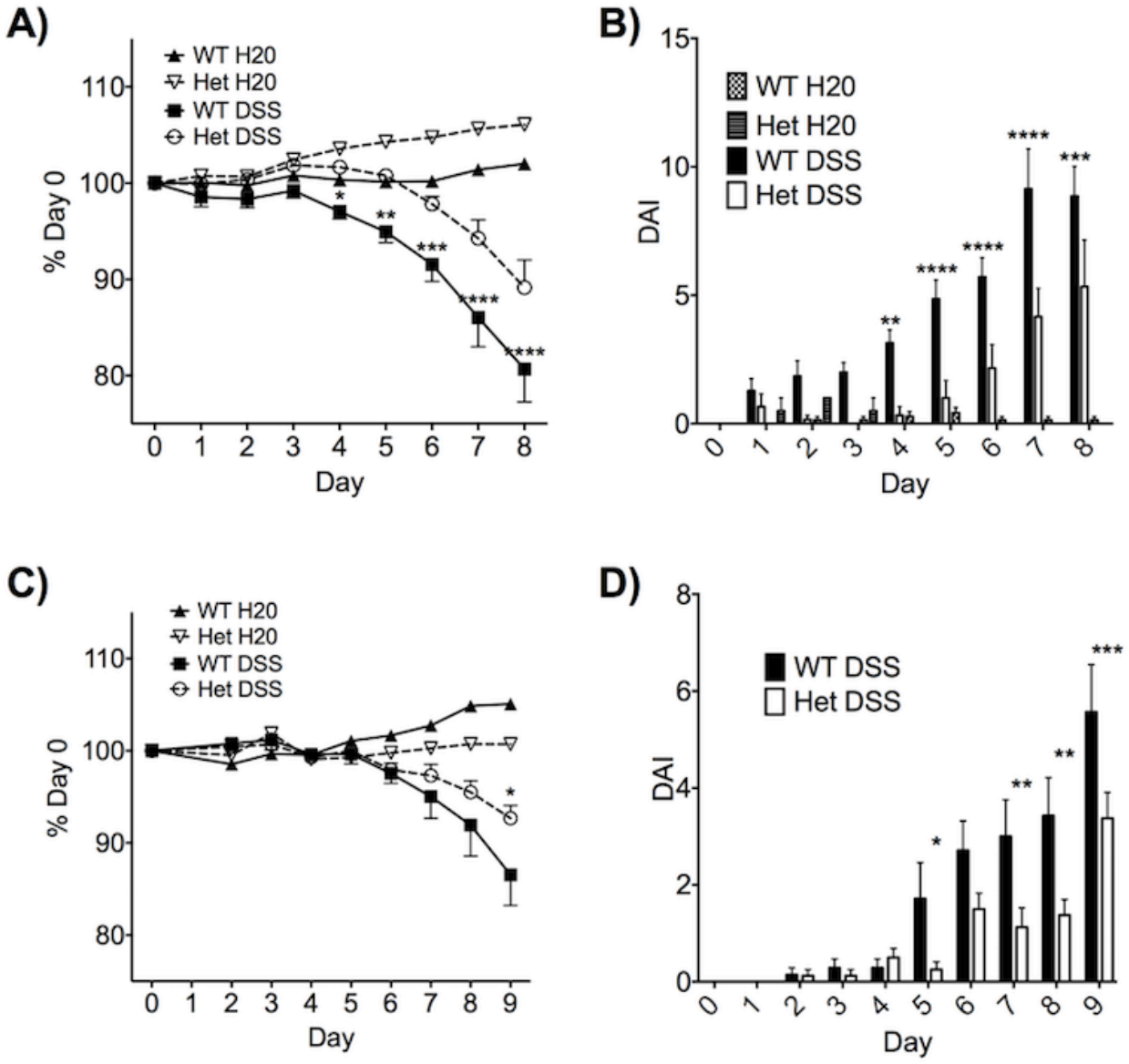


Figure 3.2. GRK2$^{+/-}$ mice are protected from acute DSS induced colitis in male and female mice

WT and GRK2$^{+/-}$ mice were administered either water or 4.25% DSS in their drinking water *ad libitum* for 6 days to induce colitis, switched to water on day 7 and euthanized on day 8. **A)** Percentage of body weight in male mice over the course of the experiment expressed as % starting weight. **B)** Disease activity index in male mice observed over the course of the experiment. **C)** Percentage of body weight in female mice. **D)** Disease activity index measured in female mice over the course of the experiment. n= 6-7, +/- SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.



To determine if there are any sex differences in the role of GRK2 in colitis pathogenesis, we administered DSS to female WT and GRK2 het mice. Interestingly, and as noted in previous studies [22], female mice showed delayed onset of colitis in WT mice and GRK2 het mice were protected from the clinical signs of colitis **(Figure 3.2.C-D)**. These results suggest that while there may be differences in the kinetics of colitis pathogenesis in males and females, with regard to the role of GRK2, it may be sex-hormone independent. Because the phenotypic differences were striking in the male mice, we focused our studies here on out in male mice.

Similar to the clinical signs, male WT mice following DSS displayed significantly shortened colon **(Figure 3.3.A-B)**, a morphometric measurement of the degree of inflammation in these animals. This was significantly attenuated in GRK2 het mice (longer colons compared to WT). Interestingly, the GRK2 heterozygous mice had enlarged spleens at the time of harvest despite their overall protection **(Figure 3.3.C)**. Finally, despite the changes in DAI and gross morphology, the colon tissue itself did not display any differences in histological damage between the WT and $GRK2^{+/-}$ mice **(Figure 3.3.D)**. Together, these results suggest that GRK2 has a negative role in the onset and progression of DSS induced colitis in mice.

Protection from DSS-Induced Colitis in $GRK2^{+/-}$ Mice is Independent of Immune Infiltration

Upon observing that the GRK2 heterozygous mice are protected from DSS induced colitis we further examined levels of cellular infiltration into the colonic lamina propria, mesenteric lymph nodes as well as the populations of innate and adaptive leukocytes in the bone marrow. Interestingly, we observed no differences in the numbers of macrophages, dendritic cells, and granulocytes as well as CD3+, CD4+ and

Figure 3.3. Colon length, spleen weight and histology at harvest
WT mice have significantly shortened colons in comparison to GRK2$^{+/-}$ mice. **A)** Quantification of colon lengths at time of harvest and **B)** Representative images of colons at time of harvest. **C)** Spleen weight taken at time of harvest. **D)** Histological images of proximal colon and colitis score as determined by a board certified pathologist. N=6-7, +/- SEM, * $p < 0.05$.

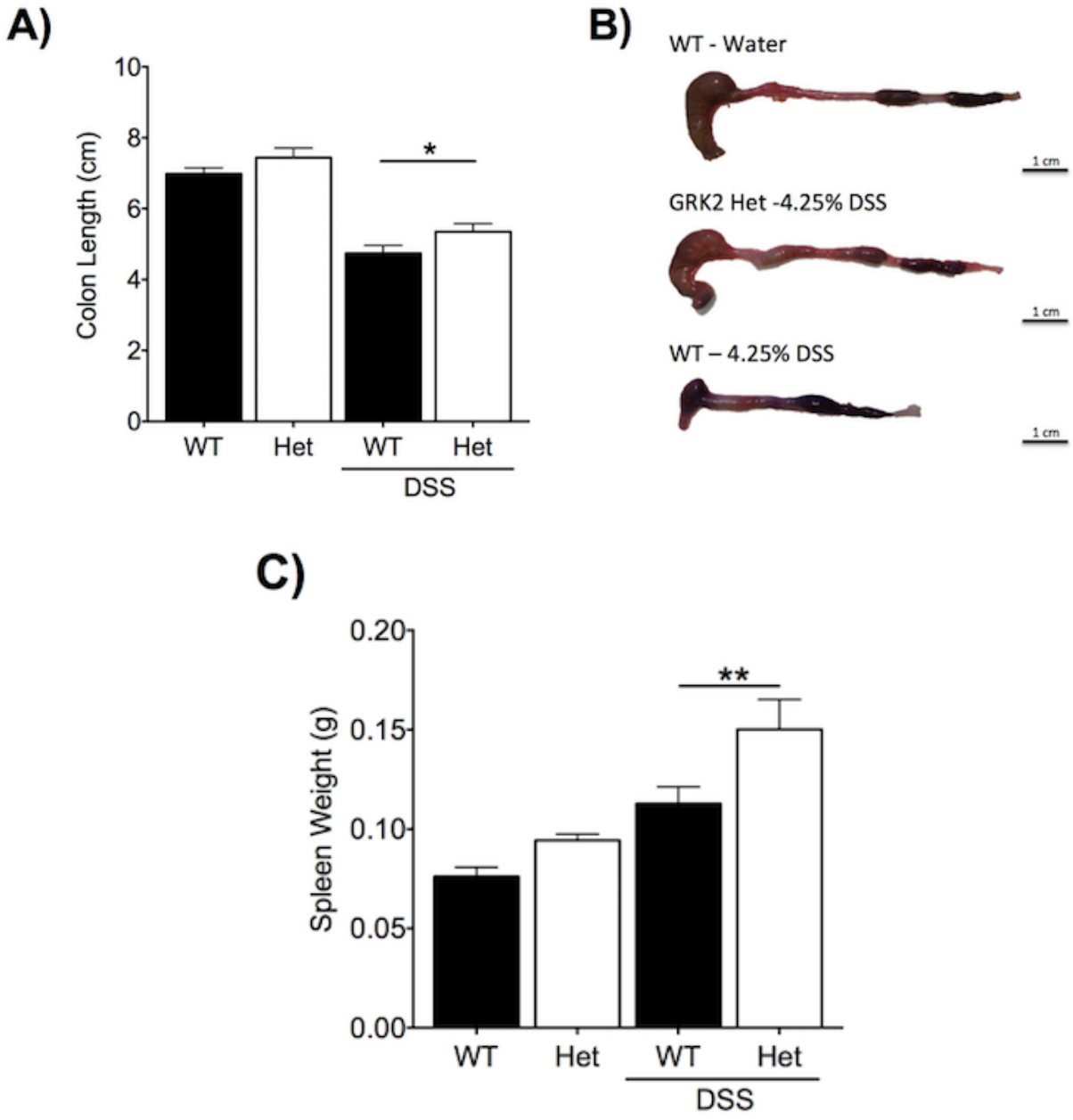
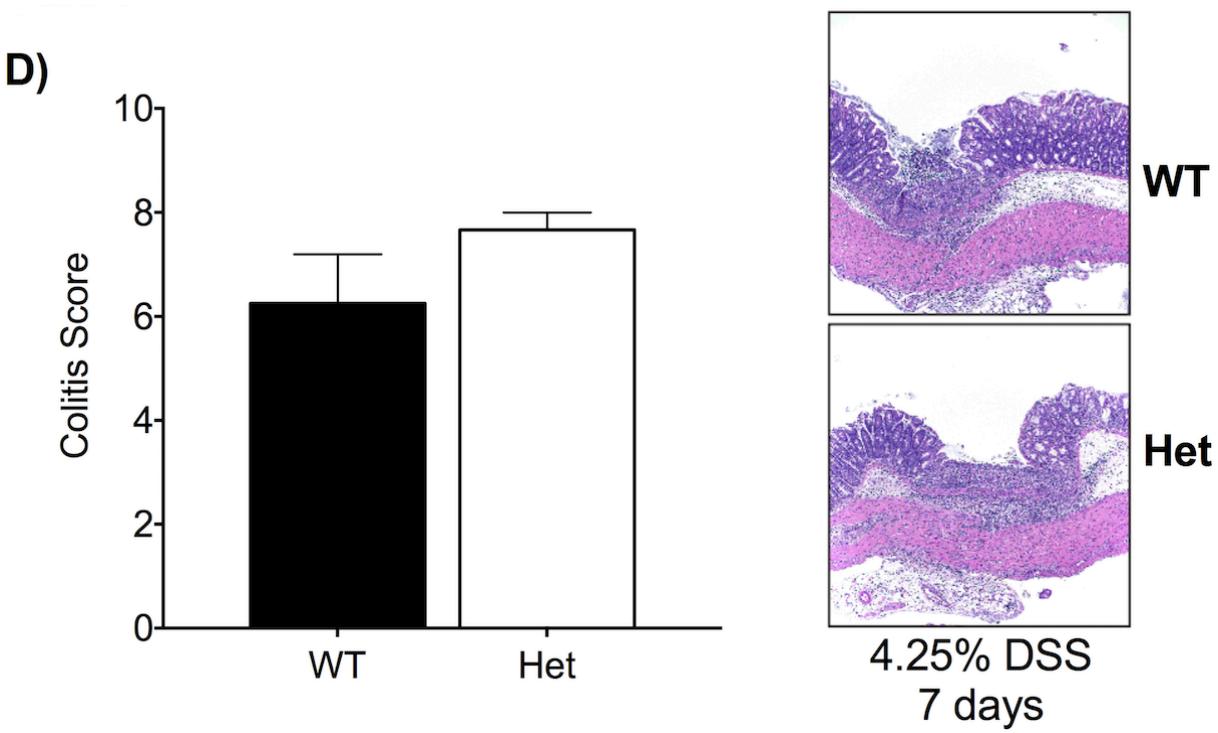


Figure 3.3. (cont'd)



CD8+ T cells in the GRK2 heterozygous mice compared to the wild-type mice in both the colonic lamina propria **(Figure 3.4.)** and mesenteric lymph nodes **(Figure 3.5.)**. When analyzing the populations of the bone marrow in these animals, again there were no differences seen in the lymphocyte population or in the macrophages, or dendritic cell population. There was, however, a significant increase in the number of granulocytes detected in the WT mice verses the $GRK2^{+/-}$ mice **(Figure 3.6.)**.

Reviewing the onset of colitis in these animals, we observed differences between the two groups as early as day 4 of DSS treatment. Therefore, we hypothesized that there were alterations in immune infiltration earlier in the time-course that may have resolved by the time of harvest. To that end, we administered DSS to both WT and $GRK2^{+/-}$ mice for 4 days followed by euthanasia, in an attempt to identify the mechanism responsible for the early differences observed over the time-course. As expected, WT mice began to significantly lose more weight by day 4 than their $GRK2^{+/-}$ counterparts and their overall higher DAI score reflected this as well **(Figure 3.7.A-B)**. Despite their higher disease score and weight loss, at day 4 the WT and $GRK2^{+/-}$ mice do not show any difference in colon length or spleen weight at this time point **(Figure 3.7.C-D)**. Furthermore, once again there were no detectable differences in the number of innate or adaptive leukocytes in the colonic lamina propria **(Figure 3.8.)**, mesenteric lymph node **(Figure 3.9.)** and the bone marrow **(Figure 3.10.)**. Together these data suggest that the protection from DSS induced colitis in $GRK2^{+/-}$ mice is independent of immune infiltration per se into the colonic lamina propria and the mesenteric lymph nodes and likely independent of changes in bone marrow immune populations.

Figure 3.4. DSS does not influence immune infiltration in the colonic lamina propria at day 8

A) Cellular populations in the colonic lamina propria of WT and GRK2$^{+/-}$ mice expressed as fold WT H20. N= 6-7 per genotype. Data pooled from 2 independent experiments.

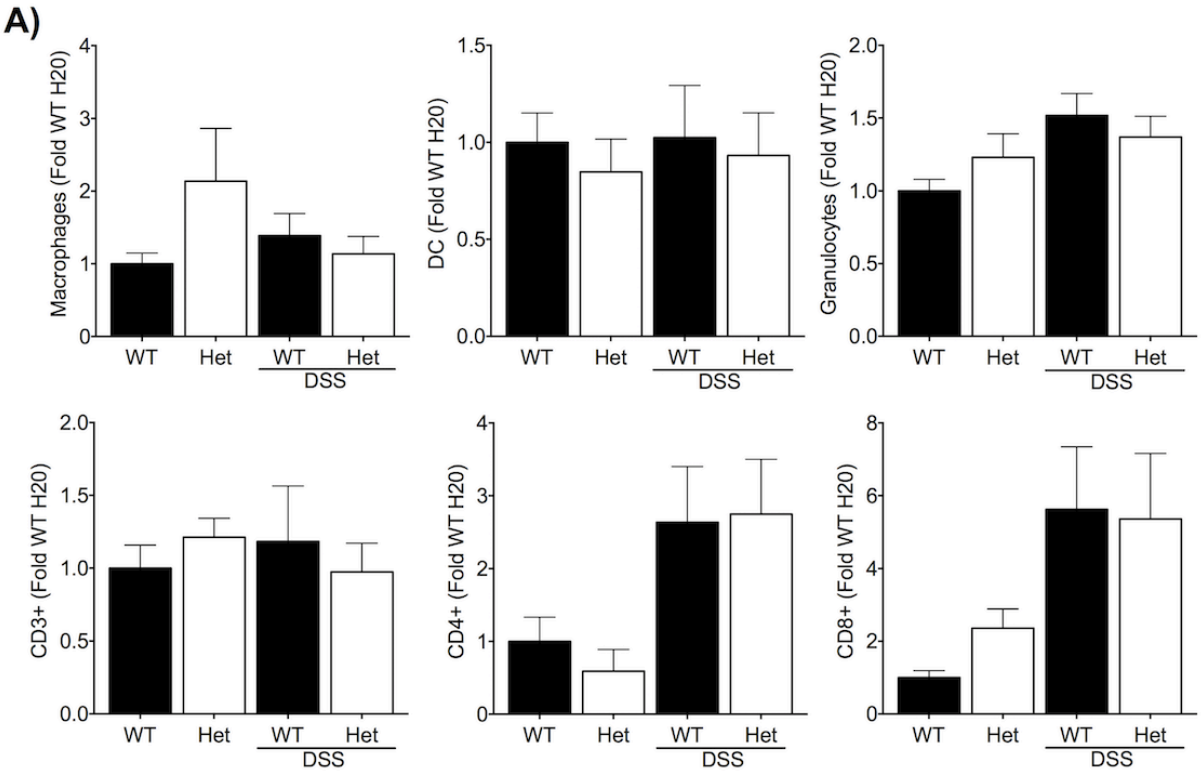


Figure 3.5. DSS does not influence immune infiltration in the mesenteric lymph node at day 8

A) Cellular populations in the mesenteric lymph nodes of WT and GRK2$^{+/-}$ mice expressed as fold WT H20. N= 6-7 per genotype. Data pooled from 2 independent experiments.

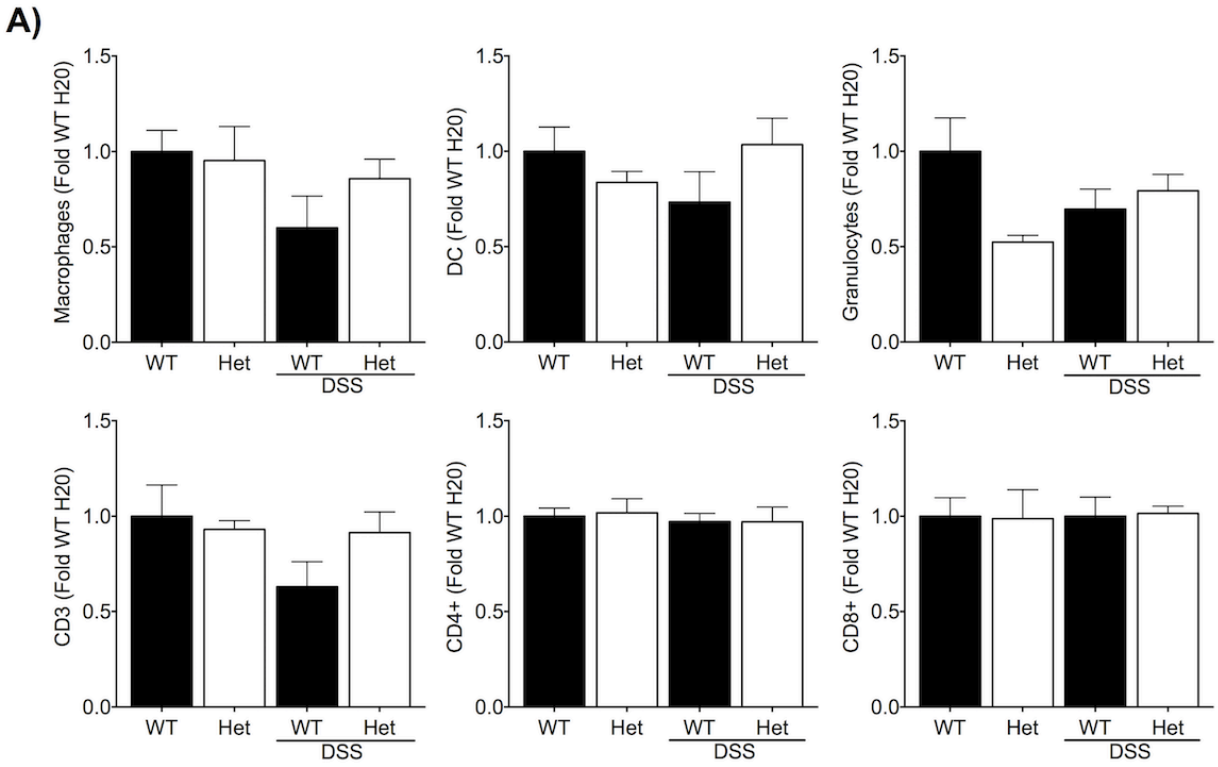


Figure 3.6. DSS increases granulocytes numbers in WT mice in the bone marrow
A) Cellular populations in the bone marrow of WT and GRK2$^{+/-}$ mice expressed as fold WT H20. N= 6-7 per genotype. Data pooled from 2 independent experiments. * $p < 0.05$

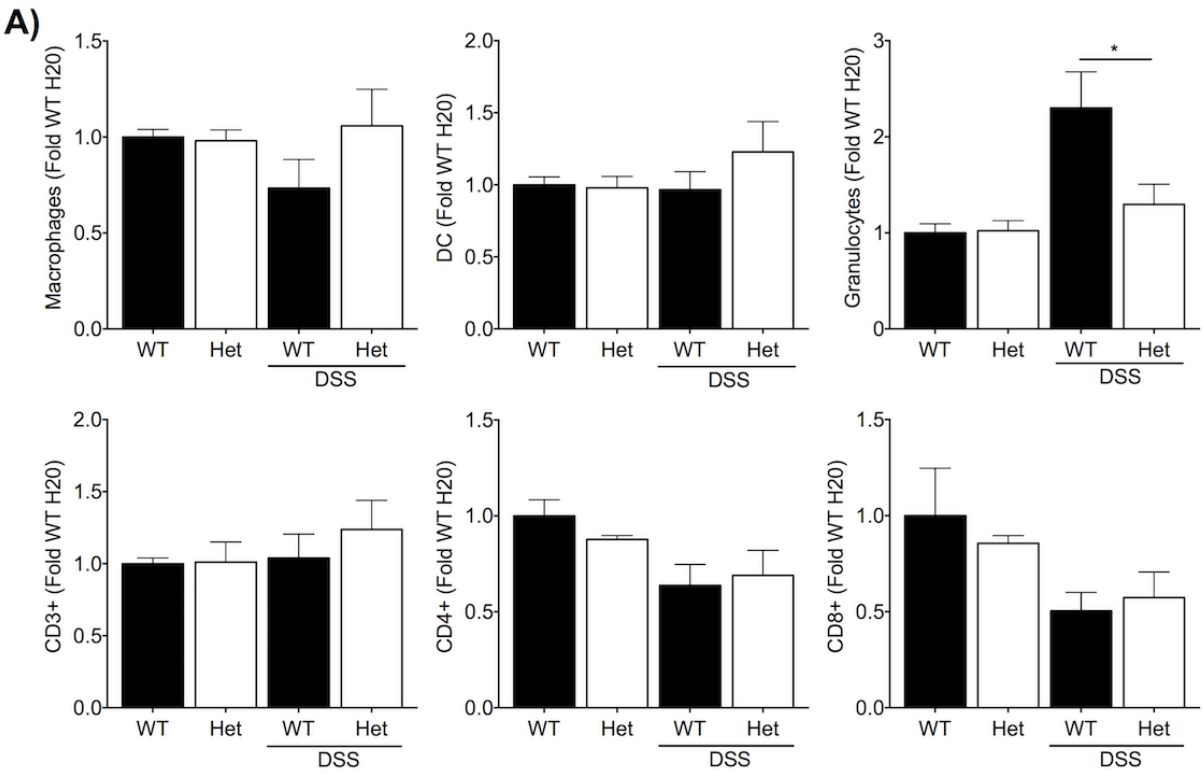


Figure 3.7. GRK2$^{+/-}$ mice are protected from acute DSS induced colitis at day 4
WT and GRK2$^{+/-}$ mice were administered either water or 4.25% DSS in their drinking water *ad libitum* for 4 days to induce colitis and euthanized on day 4. **A)** Percentage of body weight in male mice over the course of the experiment expressed as % starting weight. **B)** Disease activity index in mice observed over the course of the experiment. **C)** Quantification of the colon lengths and **D)** spleen weight taken at time of harvest. N = 3-4, +/- SEM. ** $p < 0.01$, *** $p < 0.001$.

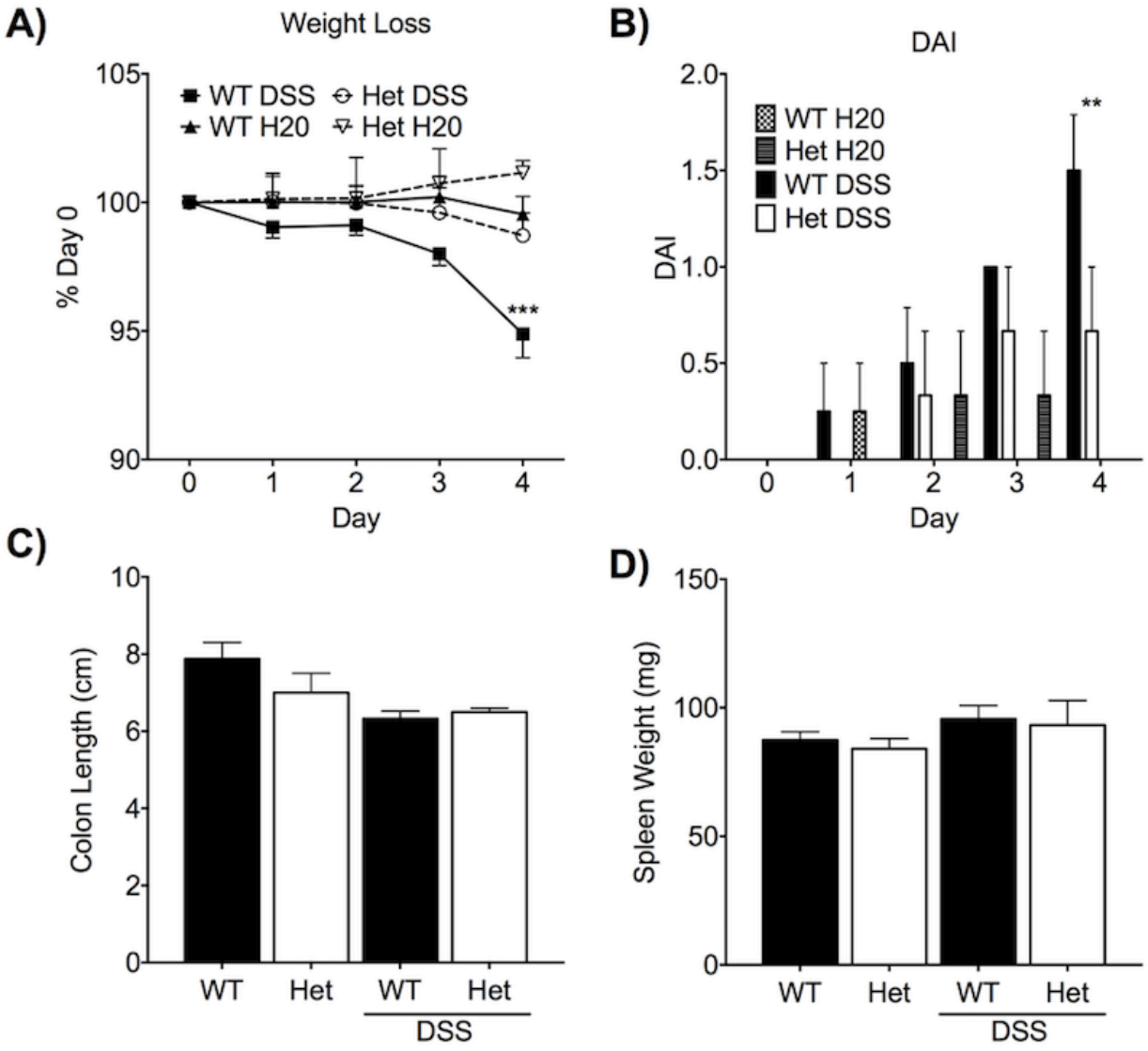


Figure 3.8. DSS does not influence immune infiltration in the colonic lamina propria at day 4

A) Cellular populations in the colonic lamina propria of WT and GRK2$^{+/-}$ mice expressed as fold WT H20. N= 3-4 per genotype.

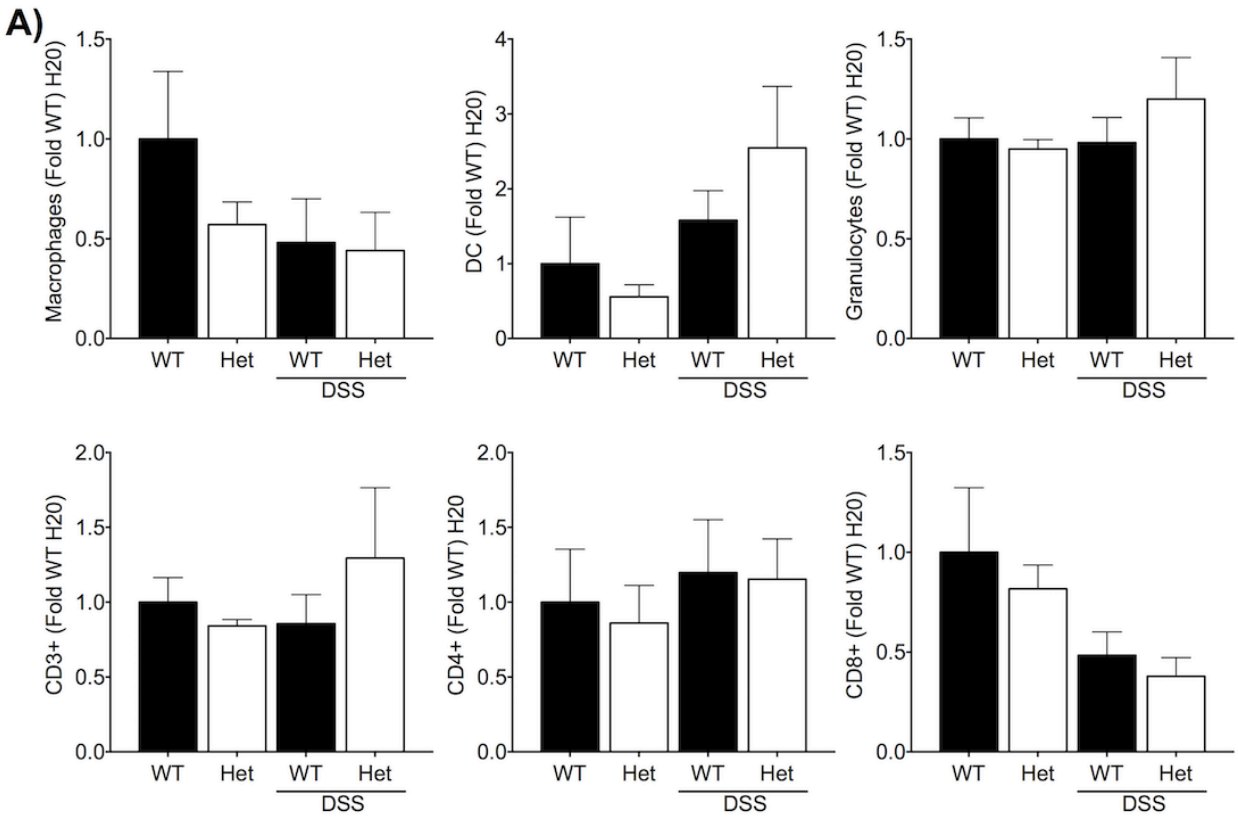


Figure 3.9. DSS does not influence immune infiltration in the mesenteric lymph node at day 4
A) Cellular populations in the mesenteric lymph nodes of WT and GRK2$^{+/-}$ mice expressed as fold WT H20. N= 3-4 per genotype.

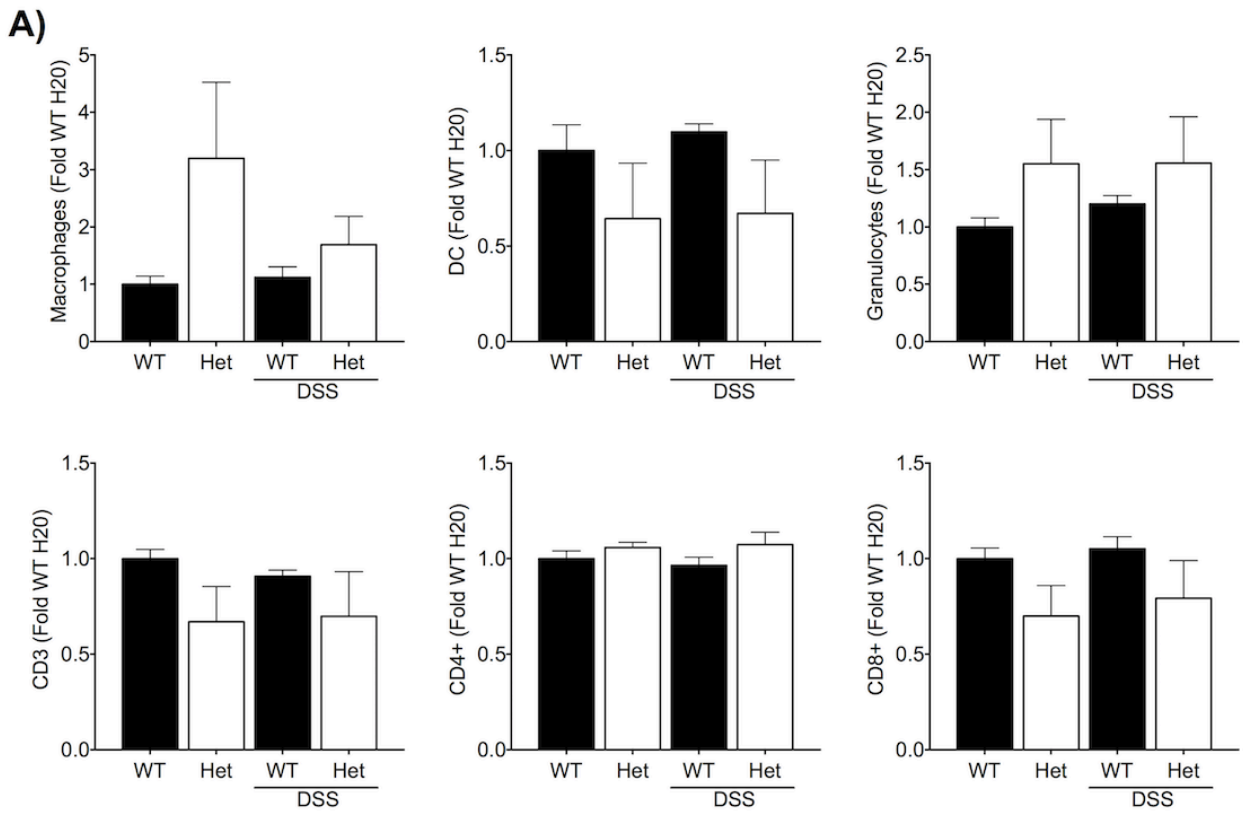
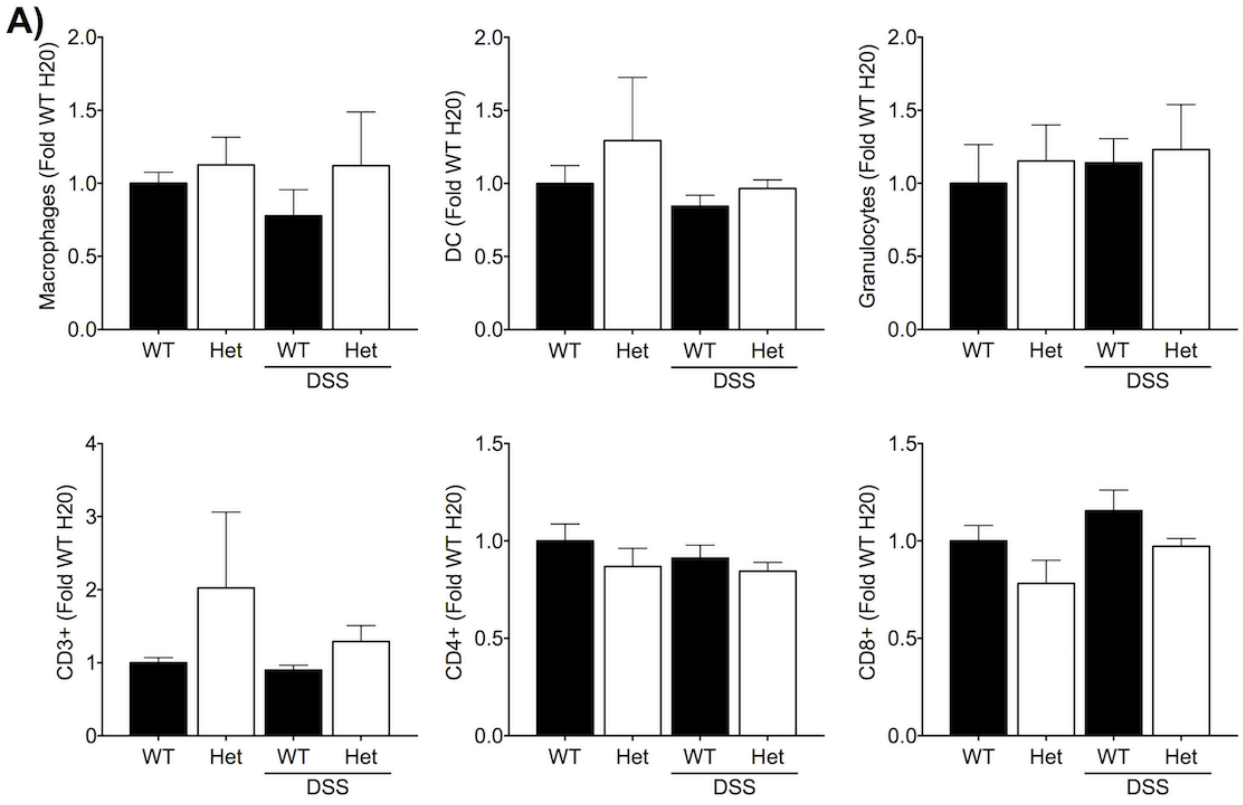


Figure 3.10. DSS does not influence immune infiltration in the bone marrow at day 4

A) Cellular populations in the bone marrow of WT and GRK2$^{+/-}$ mice expressed as fold WT H20. N= 3-4 per genotype.



GRK2 Knockout in Myeloid Cells is able to protect against DSS-Induced Colitis

To begin to dissect the mechanisms by which GRK2 regulates DSS-induced colitis, we resorted to using targeted GRK2 knockout mice. Even though there were no major differences in immune cell infiltration, we reasoned that given the role of GRK2 in inflammatory signaling pathways in immune cells, knocking out GRK2 in one of the immune populations may shed light on the role of GRK2 in colitis. For this we examined the effect of DSS in myeloid-specific GRK2 knockout. Previously our lab has demonstrated that GRK2 levels in these animals are significantly reduced in the macrophages and neutrophils but remains present at equivalent levels to wild type in other organ tissues [12]. Upon administration of DSS to these animals, we observed that the myeloid specific GRK2 knockout animals phenocopied the whole body GRK2 heterozygous mice in both weight loss **(Figure 3.11.A)** and DAI score **(Figure 3.11.B)** when compared to wild-type animals. This protection from DSS induced colitis was further substantiated by significantly longer colon in the myeloid knockout animals compared to wild type mice; similar to previous experiments, spleens from myeloid KOs were larger than their WT counterparts **(Figure 3.11.C-D)**. Interestingly, the histopathology of the myeloid animals showed a significantly improved colitis score and protection from damage to colonic tissue, compared to the WT animals **(Figure 3.12.A-B)**.

We again measured immune infiltration into the colonic lamina propria, mesenteric lymph nodes and populations in the bone marrow to see the effect that GRK2 is having in this mouse model in response to DSS-induced colitis. Similar to earlier studies, we saw minimal changes in these populations in the bone marrow **(Figure 3.13.)** although there were slight increases in lamina propria CD8+

Figure 3.11. GRK2^{LysM} mice are protected from acute DSS induced colitis
WT and GRK2^{LysM} mice were administered either water or 3.25% DSS in their drinking water *ad libitum* for 7 days to induce colitis and euthanized on day 7. **A)** Percentage of body weight in male mice over the course of the experiment expressed as % starting weight. **B)** Disease activity index in mice observed over the course of the experiment. **C)** Representative images of colons taken at time of harvest and quantification of the colon lengths and **D)** spleen weight taken at time of harvest. n= 7-12 Data pooled from 2 independent experiments. +/- SEM. * $p < 0.05$ ** $p < 0.01$, *** $p < 0.001$.

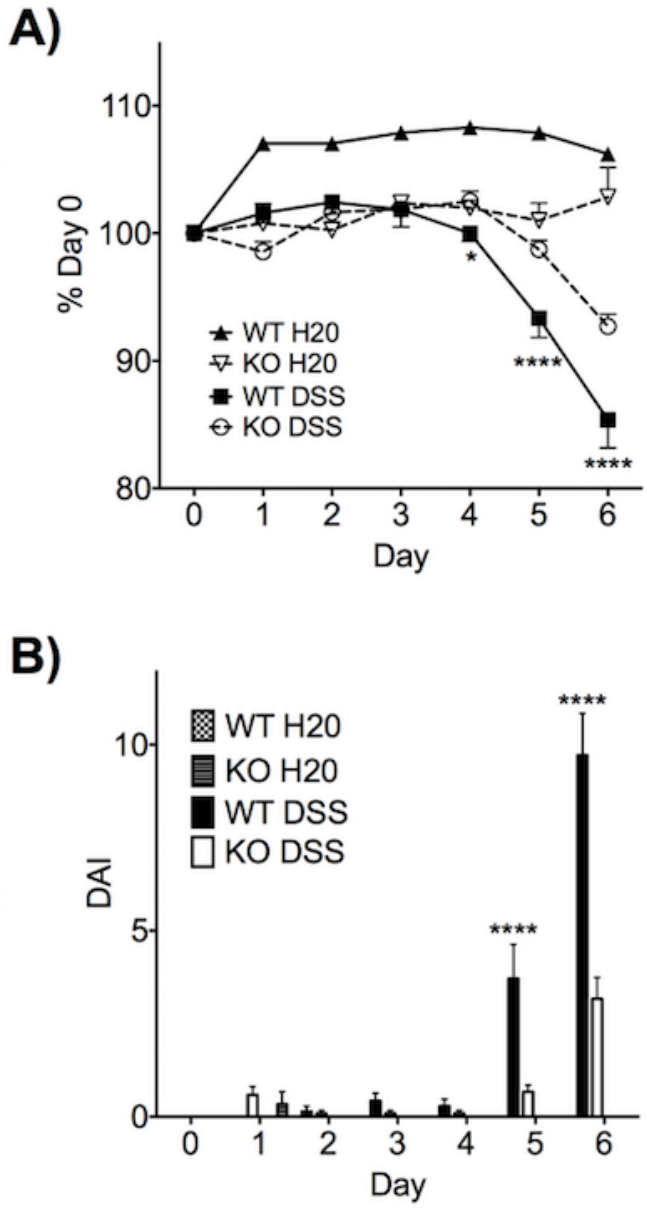


Figure 3.11. (cont'd)

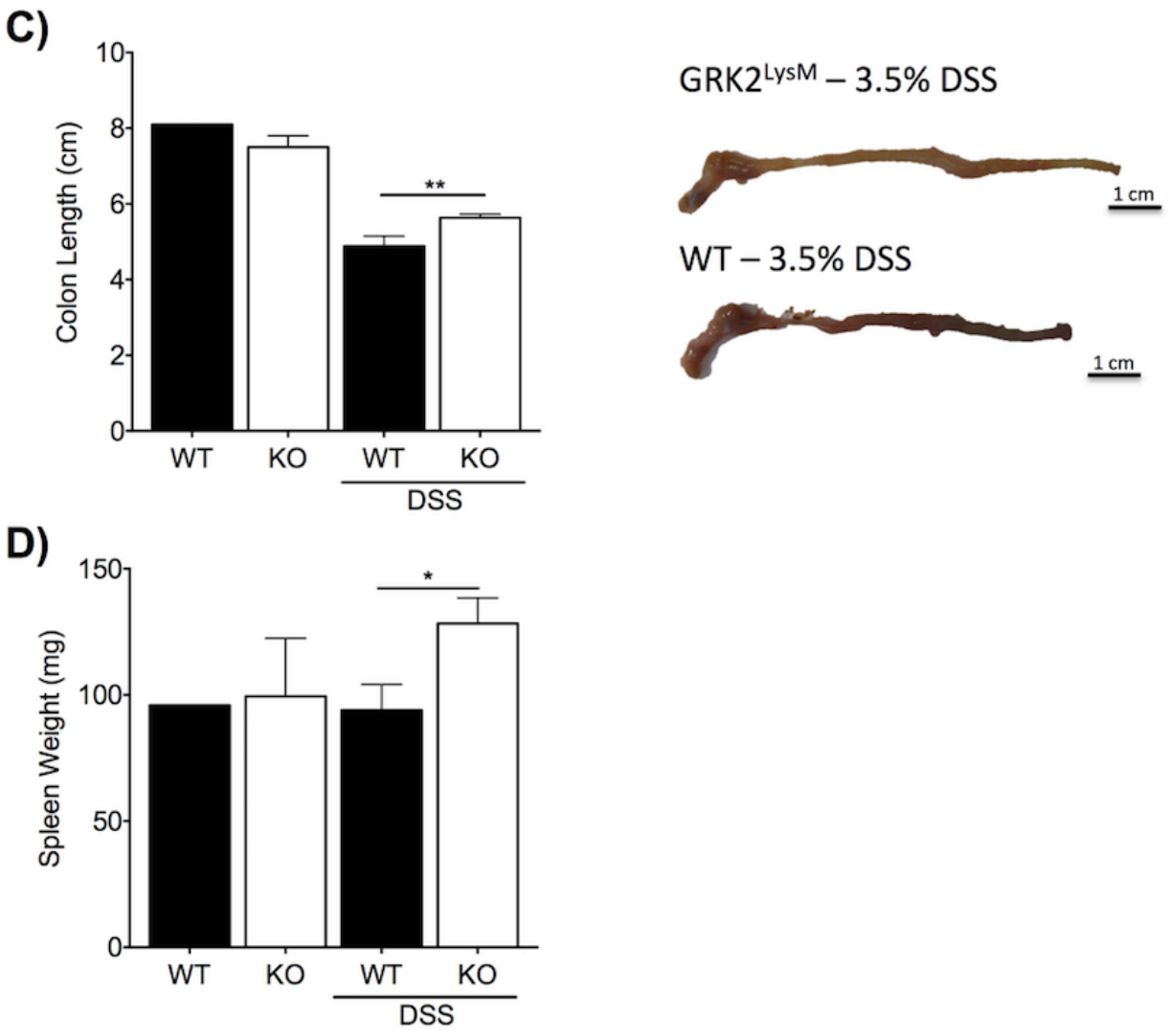


Figure 3.12. WT mice display more severe histological damage than GRK2^{LysM} mice
A) Representative images of colon histology taken at time of harvest and **B)** disease pathology score for WT and GRK2^{LysM} mice. Mice given water had scores of 0 and are not included in graph. n= 7-12 Data pooled from 2 independent experiments. +/- SEM. * $p < 0.05$

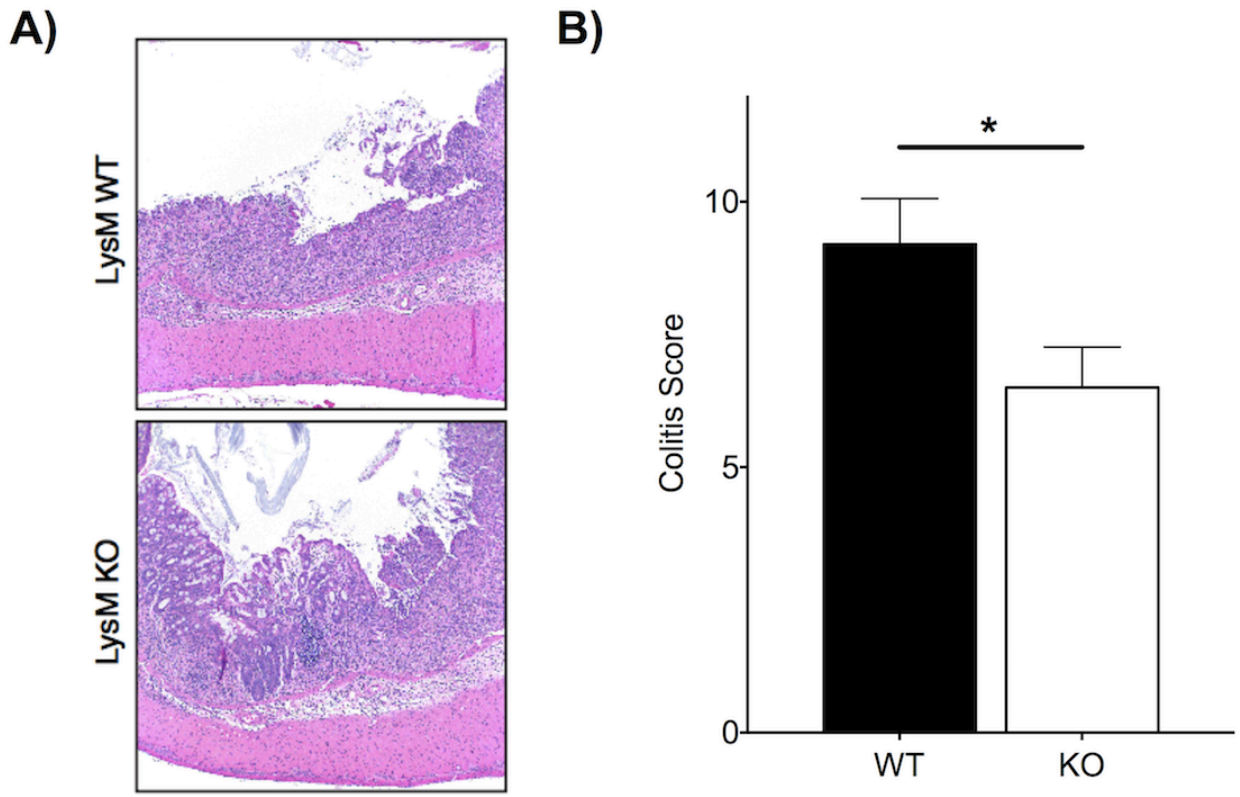
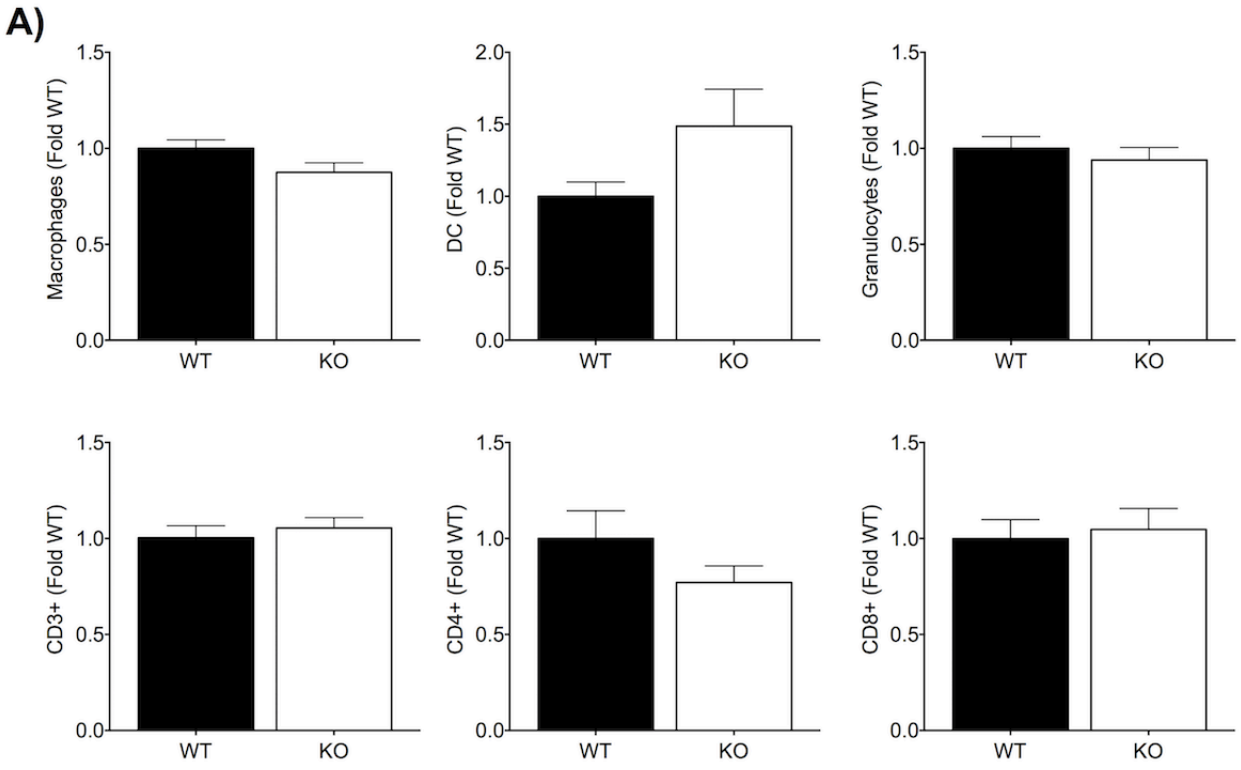


Figure 3.13. DSS does not influence immune infiltration in the bone marrow in GRK2^{LysM} mice

A) Cellular populations in the bone marrow of DSS treated WT and GRK2^{LysM} mice expressed as fold WT DSS. N= 7-12 per genotype, +/- SEM. Data pooled from 2 independent experiments.



(Figure 3.14.) cells in the wild-type mice as well as T-cell alterations in the mesenteric lymph nodes with decreased CD3+, CD4+ and dendritic cells in the WT and decreased CD8+ in the GRK2 LysM mice **(Figure 3.15.)**. Together, these data reveal that knockout of GRK2 in myeloid cells is sufficient for protection against DSS-induced colitis.

Decreased Levels of GRK2 inhibit Mucosal and Splenic Inflammation in DSS-Induced Colitis

GRK2 has been shown to be an important regulator of GPCR receptor phosphorylation and desensitization but GRK2 also has a myriad of functions independent of GPCRs and can regulate signaling for receptors and agonists in both a kinase-dependent and –independent fashion [23]. Due to the fact that we see very little changes in immune populations in the various tissue groups, we hypothesized that GRK2 may be regulating other functional aspect of these cells. To test this, we examined expression of various cytokines and chemokines in the colon from WT and GRK2 het mice subjected to DSS-induced colitis. We found significant differences in the expression of inflammatory mediators including components of innate cytokines, chemokines, and T-cell effectors that were significantly lower in the GRK2 het compared to the wild type mice **(Table 3.1)**. In particular, we observed IL-6 to IL-10 ratio, granulocyte macrophage colony stimulating factor, IL-12p40 and MIP2 in the GRK2 het mice were significantly lower compared to the WT mice. Wild-type mice also had higher expression of T-cell cytokines including IL-17A as well as GATA3 indicating that there may be an increase in the Th17 and Th2 responses in these mice that might be in response to increased disease effects in these mice. Due to the importance and known roles for IL-6 and IL-10 [24–26] we examined the expression levels of these cytokines in the $GRK2^{LysM}$ mice and again saw a marked increase in the IL-6:IL-10 ratio

Figure 3.14. DSS does not influence immune infiltration in the colonic lamina propria in GRK2^{LysM} mice

A) Cellular populations in the colonic lamina propria of DSS treated WT and GRK2^{LysM} mice expressed as fold WT DSS. N= 7-12 per genotype, +/- SEM. Data pooled from 2 independent experiments. * $p < 0.05$

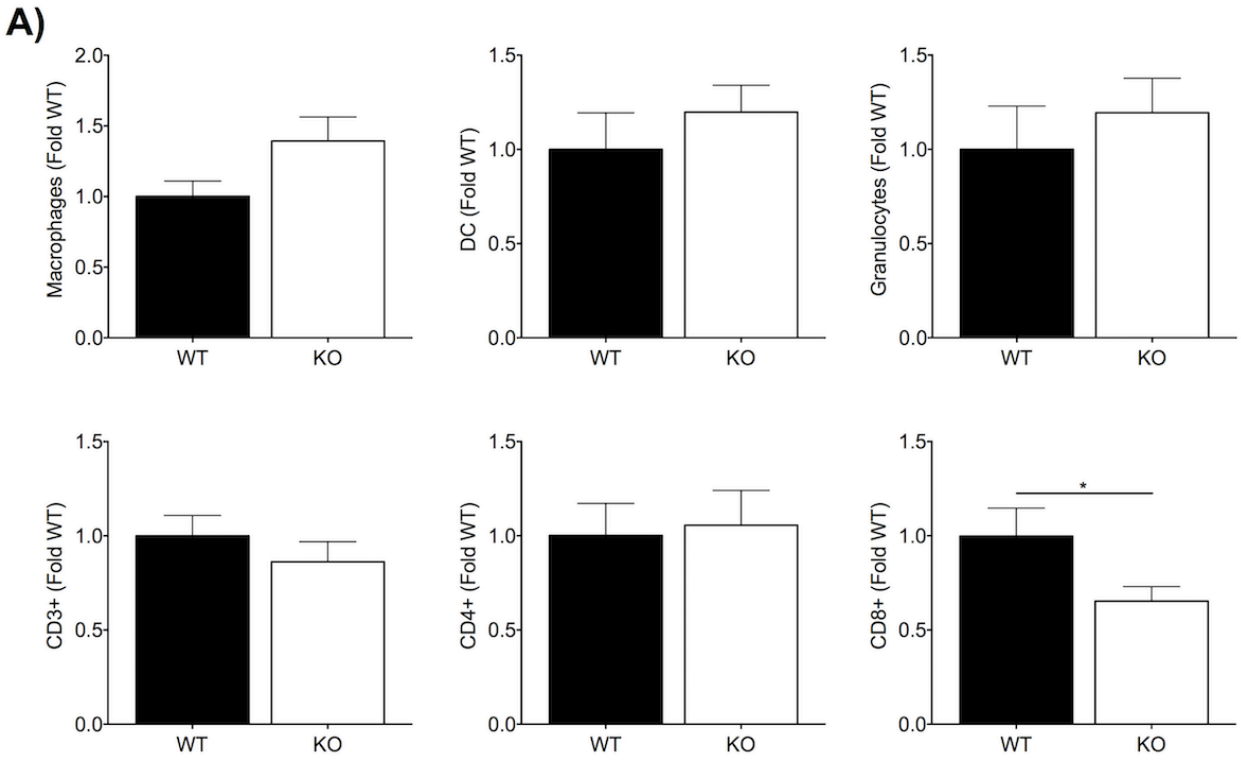


Figure 3.15. DSS does not influence immune infiltration in the mesenteric lymph node in GRK2^{LysM} mice
A) Cellular populations in the mesenteric lymph node of DSS treated WT and GRK2$^{+/-}$ mice expressed as fold WT DSS. N= 7-12 per genotype, +/- SEM. Data pooled from 2 independent experiments. * $p < 0.05$, ** $p < 0.01$

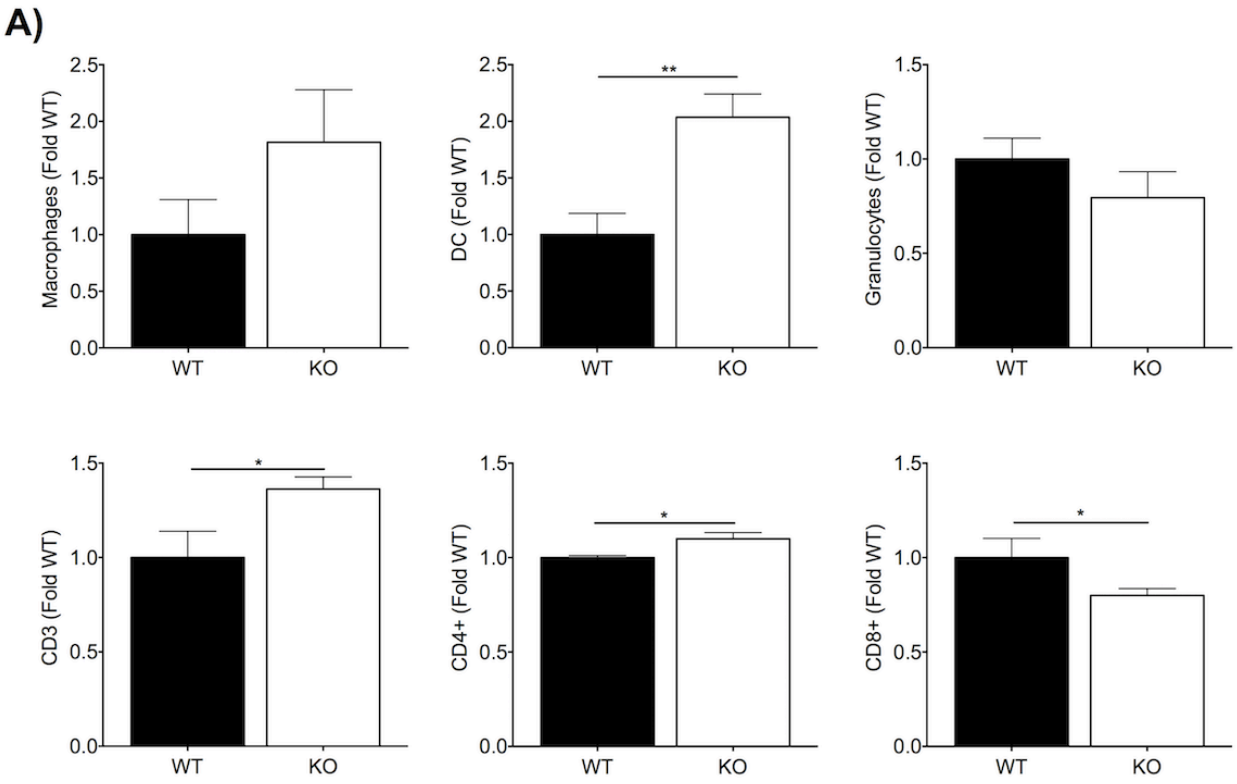


Table 3.1. Inflammatory Mediator Expression in Colon at Day 8
RNA was extracted from proximal colon segments of WT and GRK2$^{+/-}$ mice after DSS treatment, subjected to complementary DNA synthesis and q/RT PCR to quantify the expression of genes using primers noted in Supplemental Table. Data are expressed as % maximum expression. Genes statistically significant from WT are marked in bold.

	WT	GRK2$^{+/-}$
Innate Cytokines		
IL-6/IL10	**53.9 ± 7.90**	**29.14 ± 7.94**
TNF-α	52.80 ± 10.17	58.28 ± 16.48
Granulocyte macrophage-colony stimulating factor	**71.79 ± 9.53**	**42.57 ± 14.53**
IL-12p40	**56.74 ± 15.16**	**24.4 ± 6.51**
Chemokines		
IP-10	46.84 ± 12.54	54.06 ± 16.61
CXCL9	45.6 ± 14.02	45.1 ± 14.23
CXCL11	44.89 ± 9.84	67.26 ± 15.61
CCL20	75.25 ± 32.07	49.55 ± 3.78
CX3CL1	42.24 ± 4.92	37.85 ± 6.3
KC	57.52 ± 14.18	35.33 ± 12.13
MIP2	**59.28 ± 14.4**	**31.32 ± 6.34**
T-cell cytokines/differentiation markers		
IFN-γ	40.11 ± 17.51	43.07 ± 15.83
IL-17A	**62.53 ± 13.53**	**25.48 ± 7.79**
IL-22	50.04 ± 13.85	50.72 ± 16.76
RORγT	53.59 ± 10.64	39.35 ± 6.93
GATA3	**72.09 ± 13.27**	**35.43 ± 6.3**

in the colon tissue of WT mice compared to the KO (data not shown). Together these data suggests that GRK2 is important in regulating inflammatory gene expression in the colon and this regulation is independent of total immune infiltration into this tissue and may influence the onset and progression of colitis.

DISCUSSION

In this study, we demonstrate that mice lacking GRK2 (one allele) displayed lower colonic inflammation following the disruption of the intestinal barrier by DSS and were protected from colitis pathogenesis. Further, this protection was seen despite no gross changes in immune infiltration in the early time points (4 day) or later time points of DSS induced colitis. These results suggest that the likely effect of GRK2 in colitis is independent of immune infiltration into the colon. However, it is possible that the time points we examined are insufficient to make this conclusion. Attempting to look at specific cell groups to ascertain mechanism for this protection we investigated the myeloid population and again saw that GRK2 KO protected against DSS induced colitis. This was again in the absence of gross changes in immune infiltration. These data together clearly demonstrates that GRK2 is detrimental to the onset and progression of colitis and its role in the myeloid population is a key component in the colitogenic potential of this kinase.

Immune infiltration is one of the hallmarks of colitis and the hyper-activation and prolonged duration of immune activation is one of the primary causes of damage to the intestinal lining [27–29]. With the role that GRKs play in receptor desensitization and the influence they can have on immune chemotaxis this is a common place to begin when identifying the role of a GRK in IBD. Indeed, other GRKs and β-arrestins have been implicated in colitis through regulation of chemotaxis. Our lab has shown that β-arrestin2 is protective in DSS-induced colitis through its regulation in the infiltration and activation status of CD4+ T-cells into the colonic lamina propria [21]. Other work on GRK6 shows a similar result in that GRK6 expression is protective against DSS-induced colitis through regulation of CD4+ T-cell and granulocyte infiltration accompanied by

increases in inflammatory mediators [20]. However, as the role for these proteins continue to expand and the number of non-receptor substrates increase it is becoming more and more clear that these proteins can influence the pathogenesis of diseases independently of their effect on immune infiltration. In fact, β-arrestin1 (another protein involved in desensitization of GPCRs) has been shown to be protective in DSS and TNBS induced colitis [30] and it is its role in the non-hematopoietic cells that confers the protection from colitis [31] as well as sepsis [32]. Additionally, GRK5 has been shown in our lab to regulate inflammation and the pathogenesis of polymicrobial sepsis independently of cellular infiltration in mice [33] and we have recently shown a role for GRK2 in the intestinal epithelial cells which can modulate the TNFα driven inflammatory response and wound healing in those cells aiding in the restoration of the epithelial barrier *in vivo* [10].

While we didn't see alterations in immune infiltration while investigating the role of GRK2 in this system, we did observe significant fluctuations in key cytokines known to be associated with the onset and severity of colitis including IL-6 [24–26], IL12p40 and MIP2. IL-6 in particular has been shown in IBD to be dependent on macrophage and epithelial cells [34] supporting the results seen in our experiments. Interestingly, several genes differentially regulated in the GRK2 hets compared to the wild-type mice are associated with T-cell differentiation. One of the genes that was significantly increased in the wild-type mice (compared to GRK2 het mice) was GATA3. Expression of GATA3 is known as a Th2 lineage commitment transcription factor [35] and recent work has shown that increased levels of GATA3 can accelerate acute DSS-induced colitis [36]. Another T-cell cytokine significantly increased in the wild-type animals (compared to het) was IL17A. Produced by a unique subset of memory T cells, IL-17

has a broad impact on the immune system and has been shown to stimulate fibroblasts, endothelial cells, macrophages and epithelial cells stimulating the secretion of pro-inflammatory mediators including IL-1, IL-6, TNFα, MMPs and many others [37]. Recent work on IL-17 has shown that this cytokine can be both pathogenic or protective depending on the source of the cytokine. IL-23 stimulated production of IL-17A results in T-helper cells producing the cytokine and causing influx and activation of immune cells but new work has shown that IL-23 independent release of IL-17 from the γδ T-cells is critical in maintaining and protecting the epithelial barrier [18]. The source of IL17 and whether it is contributing to the increased inflammation and damage or trying to restore the epithelial barrier in our mice remains unknown at this time. While our study investigated the CD3+, CD4+ and CD8+ T-cells the specific lineages and differentiated cell types was not investigated in this study and the production of inflammatory cytokines seen in the non-hematopoietic and myeloid population driving this differentiation may in part be responsible for the outcomes observed.

In our studies, we observe that knockout of GRK2 in the myeloid population was sufficient to confer the protection from acute DSS-induced colitis in our animal model. Work done in our lab and others have begun to elucidate GRK2's role in the regulation of this population of macrophages, neutrophils and other myeloid cells. Although GRK2 has been implicated in other inflammatory systems it has never been investigated in the context of colitis. Work done in models such as hyperalgesia demonstrate that the effect of GRK2 is through alterations in myeloid IL-10 production [38]. Other models including sepsis [39] and endotoxemia [12] have shown that knocking down GRK2 in the myeloid cells generally exacerbates inflammation potentially via negative regulation of NFκB leading to increases in IL6:IL10 ratios at least early on in the onset of inflammation. This

is in contrast to the gene expression seen in the current colitis model where the IL6:IL10 ratio was significantly decreased in the KO mice compared to the wild type mice. These contrasting results with previous studies highlight a diverse role of GRK2 in different models of inflammation and demonstrate that the response seen in these mice may be differentially regulated depending on the disease context and tissue microenvironment.

Understanding how proteins respond in different disease models is important for the development and implementation of novel therapeutic options. IBD is a complex disease where the etiology and specific causation largely remains unknown but, as shown in this paper and others, it results from an imbalance in pro-inflammatory mediators and dysregulated recruitment of leukocytes to the sites of inflammation [5]. IBD has conventionally been treated with nonspecific immune suppression and more recently targeted therapies against specific inflammatory cytokines or pathways such as TNFα. However, because of the diversity of this disease a large number of patients often do not respond to certain types of therapies or they eventually develop tolerance to treatments due to the chronic administration of these therapies [40]. Therefore, identifying new therapies is of great importance to the treatment of this disease and as a way to overcome the limitations to the currently approved treatments. GRK2 offers a new targetable option for the management of IBD and production of inhibitors of this kinase is currently being pursued in part for the role of GRK2 in heart failure. One promising inhibitor is the SSRI paroxetine (Paxil) which is already approved by the Food and Drug Administration and been shown to have potent inhibition of GRK2 both *in vitro* and *in vivo* [41]. In addition to paroxetine, there are a considerable number of GRK2 inhibitors currently in development that may prove to be an even more effective inhibitor

of GRK2 (reviewed in [42]) indicating a realistic possibility that therapeutics could target pathways regulated by GRK2 therefore influencing its effect in IBD in the future.

In summary, this study demonstrates an important negative role for GRK2 in regulating mucosal inflammation under colitic conditions. This result is determined likely not due to dysregulation in immune infiltration but rather through alterations in inflammatory mediator production, possibly in the myeloid population. Further work would determine the mechanism that GRK2 influences these responses and provide insight into the specific cells involved in the onset and pathogenesis of DSS-induced colitis.

ACKNOWLEDGEMENTS

We gratefully acknowledge the support from NIH (grants HL095637, AI099404 and AR056680 to N.P). We thank the University lab animal resources for taking excellent care of our animals.

REFERENCES

REFERENCES

1 Sansonetti, P. J. (2004) War and Peace at Mucosal Surfaces. Nat. Rev. Immunol. **4**, 953–964.

2 Kagnoff, M. F. and Eckmann, L. (1997) Epithelial cells as Sensors for Microbial Infection. J. Clin. Invest. **100**, 6–10.

3 Xavier, R. J. and Podolsky, D. K. (2007) Unravelling the pathogenesis of inflammatory bowel disease. Nature **448**, 427–434.

4 Gribar, S. C., Richardson, W. M., Sodhi, C. P. and Hackam, D. J. (2008) No Longer an Innocent Bystander: Epithelial Toll-Like Receptor Signaling in the Development of Mucosal Inflammation. Mol. Med. **14**, 645–659.

5 D'Haens, G. R., Sartor, R. B., Silverberg, M. S., Petersson, J. and Rutgeerts, P. (2014) Future directions in inflammatory bowel disease management. J. Crohns. Colitis, European Crohn's and Colitis Organisation **8**, 726–34.

6 Evron, T., Daigle, T. L. and Caron, M. G. (2012) GRK2: Multiple roles beyond G protein-coupled receptor desensitization. Trends Pharmacol. Sci., Elsevier Ltd **33**, 154–164.

7 Packiriswamy, N. and Parameswaran, N. (2015) G-protein-coupled receptor kinases in inflammation and disease. Genes Immun. **16**, 367–77.

8 Penela, P., Murga, C., Ribas, C., Lafarga, V. and Mayor, F. (2010) The complex G protein-coupled receptor kinase 2 (GRK2) interactome unveils new physiopathological targets. Br. J. Pharmacol. **160**, 821–832.

9 Woodall, M. C., Ciccarelli, M., Woodall, B. P. and Koch, W. J. (2014) G protein-coupled receptor kinase 2: A link between myocardial contractile function and cardiac metabolism. Circ. Res.

10 Steury, M. D., Lucas, P. C., McCabe, L. R. and Parameswaran, N. (2017) G-Protein Coupled Receptor Kinase-2 is a Critical Regulator of TNFα Signaling in Colon Epithelial Cells. Biochem. J. **Accepted Manuscript**.

11 Jaber, M., Koch, W. J., Rockman, H., Smith, B., Bond, R. a, Sulik, K. K., Ross, J., Lefkowitz, R. J., Caron, M. G. and Giros, B. (1996) Essential role of beta-adrenergic receptor kinase 1 in cardiac development and function. Proc. Natl. Acad. Sci. U. S. A. **93**, 12974–9.

12 Patial, S., Saini, Y., Parvataneni, S., Appledorn, D. M., Dorn, G. W., Lapres, J. J., Amalfitano, A., Senagore, P. and Parameswaran, N. (2011) Myeloid-specific GPCR kinase-2 negatively regulates NFkB1p105-ERK pathway and limits endotoxemic shock in mice. J. cell Physiol. **226**, 627–637.

13 Sharma, D., Malik, A., Lee, E., Britton, R. a. and Parameswaran, N. (2013) Gene dosage-dependent negative regulatory role of β-arrestin-2 in polymicrobial infection-induced inflammation. Infect. Immun. **81**, 3035–44.

14 Patial, S., Shahi, S., Saini, Y., Lee, T., Packiriswamy, N., Appledorn, D. M., Lapres, J. J., Amalfitano, A. and Parameswaran, N. (2011) G-protein coupled receptor kinase 5 mediates lipopolysaccharide-induced NFκB activation in primary macrophages and modulates inflammation in vivo in mice. J. Cell. Physiol. **226**, 1323–33.

15 Malik, A., Sharma, D., St Charles, J., Dybas, L. A. and Mansfield, L. S. (2014) Contrasting immune responses mediate Campylobacter jejuni-induced colitis and autoimmunity. Mucosal Immunol., Nature Publishing Group **7**, 802–17.

16 Loniewski, K., Shi, Y., Pestka, J. and Parameswaran, N. (2008) Toll-like receptors differentially regulate GPCR kinases and arrestins in primary macrophages. Mol. Immunol. **45**, 2312–2322.

17 Laukoetter, M. G., Nava, P., Lee, W. Y., Severson, E. a, Capaldo, C. T., Babbin, B. a, Williams, I. R., Koval, M., Peatman, E., Campbell, J. a, et al. (2007) JAM-A regulates permeability and inflammation in the intestine in vivo. J. Exp. Med. **204**, 3067–76.

18 Lee, J. S., Tato, C. M., Joyce-Shaikh, B., Gulen, M. F., Gulan, F., Cayatte, C., Chen, Y., Blumenschein, W. M., Judo, M., Ayanoglu, G., et al. (2015) Interleukin-23-Independent IL-17 Production Regulates Intestinal Epithelial Permeability. Immunity, Elsevier Inc. **43**, 727–38.

19 Collins, F. L., Rios-Arce, N. D., Atkinson, S., Bierhalter, H., Schoenherr, D., Bazil, J. N., McCabe, L. R. and Parameswaran, N. (2017) Temporal and regional intestinal changes in permeability, tight junction, and cytokine gene expression following ovariectomy-induced estrogen deficiency. Physiol. Rep. **5**, e13263.

20 Eijkelkamp, N., Heijnen, C. J., Lucas, A., Premont, R. T., Elsenbruch, S., Schedlowski, M. and Kavelaars, A. (2007) G protein-coupled receptor kinase 6 controls chronicity and severity of dextran sodium sulphate-induced colitis in mice. Gut **56**, 847–54.

21 Sharma, D., Malik, A., Steury, M. D., Lucas, P. C. and Parameswaran, N. (2015) Protective Role of β-arrestin2 in Colitis Through Modulation of T-cell Activation. Inflamm. Bowel Dis. **12**, 2766–2777.

22 Bábíčková, J., Tóthová, Ľ., Lengyelová, E., Bartoňová, A., Hodosy, J., Gardlík, R. and Celec, P. (2015) Sex Differences in Experimentally Induced Colitis in Mice: a Role for Estrogens. Inflammation **38**, 1996–2006.

23 Evron, T., Daigle, T. L. and Caron, M. G. (2012) GRK2: multiple roles beyond G protein-coupled receptor desensitization. Trends Pharmacol. Sci. **33**, 154–64.

24 Yamamoto, M., Yoshizaki, K., Kishimoto, T. and Ito, H. (2000) IL-6 is required for the development of Th1 cell-mediated murine colitis. J. Immunol. **164**, 4878–82.

25 Atreya, R., Mudter, J., Finotto, S., Müllberg, J., Jostock, T., Wirtz, S., Schütz, M., Bartsch, B., Holtmann, M., Becker, C., et al. (2000) Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in crohn disease and experimental colitis in vivo. Nat. Med. **6**, 583–8.

26 Sander, L. E., Obermeier, F., Dierssen, U., Kroy, D. C., Singh, A. K., Seidler, U., Streetz, K. L., Lutz, H. H., Müller, W., Tacke, F., et al. (2008) Gp130 signaling promotes development of acute experimental colitis by facilitating early neutrophil/macrophage recruitment and activation. J. Immunol. **181**, 3586–94.

27 Trottier, M. D., Irwin, R., Li, Y., McCabe, L. R. and Fraker, P. J. (2012) Enhanced production of early lineages of monocytic and granulocytic cells in mice with colitis. Proc. Natl. Acad. Sci. **109**, 16594–16599.

28 Kuhl, A. A., Erben, U., Kredel, L. I. and Siegmund, B. (2015) Diversity of intestinal macrophages in inflammatory bowel diseases. Front. Immunol. **6**, 1–7.

29 Sartor, R. B. (2006) Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. Nat. Clin. Pract. Gastroenterol. Hepatol. **3**, 390–407.

30 Lee, T., Lee, E., Irwin, R., Lucas, P. C., McCabe, L. R. and Parameswaran, N. (2013) β-Arrestin-1 deficiency protects mice from experimental colitis. Am. J. Pathol. **182**, 1114–23.

31 Lee, T., Lee, E., Arrollo, D., Lucas, P. C. and Parameswaran, N. (2016) Non-Hematopoietic β-Arrestin1 Confers Protection Against Experimental Colitis. J. Cell. Physiol. **231**, 992–1000.

32 Sharma, D., Packiriswamy, N., Malik, A., Lucas, P. C. and Parameswaran, N. (2014) Nonhematopoietic β-Arrestin-1 inhibits inflammation in a murine model of polymicrobial sepsis. Am. J. Pathol. **184**, 2297–309.

33 Packiriswamy, N., Lee, T., Raghavendra, P. B., Durairaj, H., Wang, H. and Parameswaran, N. (2013) G-protein-coupled receptor kinase-5 mediates inflammation but does not regulate cellular infiltration or bacterial load in a polymicrobial sepsis model in mice. J. Innate Immun. **5**, 401–13.

34 Kusugami, K., Fukatsu, A., Tanimoto, M., Shinoda, M., Haruta, J., Kuroiwa, A., Ina, K., Kanayama, K., Ando, T. and Matsuura, T. (1995) Elevation of interleukin-6 in inflammatory bowel disease is macrophage- and epithelial cell-dependent. Dig. Dis. Sci. **40**, 949–59.

35 Zhu, J., Yamane, H., Cote-Sierra, J., Guo, L. and Paul, W. E. (2006) GATA-3 promotes Th2 responses through three different mechanisms: induction of Th2 cytokine production, selective growth of Th2 cells and inhibition of Th1 cell-specific factors. Cell Res. **16**, 3–10.

36 Okamura, M., Yoh, K., Ojima, M. and Morito, N. (2014) Overexpression of GATA-3 in T Cells Accelerates Dextran Sulfate Sodium-Induced Colitis. Exp. Anim. **63**, 133–140.

37 Eyerich, K., Dimartino, V. and Cavani, A. (2017) IL-17 and IL-22 in immunity: Driving protection and pathology. Eur. J. Immunol. **47**, 607–614.

38 Willemen, H. L. D. M., Eijkelkamp, N., Garza Carbajal, A., Wang, H., Mack, M., Zijlstra, J., Heijnen, C. J. and Kavelaars, A. (2014) Monocytes/Macrophages control resolution of transient inflammatory pain. J. Pain **15**, 496–506.

39 Parvataneni, S., Gonipeta, B., Packiriswamy, N., Lee, T., Durairaj, H. and Parameswaran, N. (2011) Role of myeloid-specific G-protein coupled receptor kinase-2 in sepsis. Int. J. Clin. Exp. Med. **4**, 320–30.

40 Melmed, G. Y. and Targan, S. R. (2010) Future biologic targets for IBD: potentials and pitfalls. Nat. Rev. Gastroenterol. Hepatol., Nature Publishing Group **7**, 110–117.

41 Thal, D. M., Homan, K. T., Chen, J., Wu, E. K., Hinkle, P. M., Huang, Z. M., Chuprun, J. K., Song, J., Gao, E., Cheung, J. Y., et al. (2012) Paroxetine is a direct inhibitor of g protein-coupled receptor kinase 2 and increases myocardial contractility. ACS Chem. Biol. **7**, 1830–9.

42 Guccione, M., Ettari, R., Taliani, S., Da Settimo, F., Zappalà, M. and Grasso, S. (2016) G-Protein-Coupled Receptor Kinase 2 (GRK2) Inhibitors: Current Trends and Future Perspectives. J. Med. Chem. **59**, 9277–9294.

CHAPTER 4: SUMMARY AND CONCLUSIONS

SPECIFIC AIMS AND RESULTS OF THE STUDY

The overall aim of this study was to investigate and identify the role for GRK2 in intestinal mucosal inflammation, using both in vitro and in vivo models. It was hypothesized that GRK2 would influence both receptor and non-receptor substrates and critically regulate the inflammatory response towards colonic insults. Therefore, this study would elucidate a molecular mechanism behind intestinal inflammation in both a cell specific and disease model generating targetable proteins for novel therapeutic options. A summary of the research objectives and the obtained results are shown below:

Objective 1 and Results

To characterize the role of GRK2 *in vitro* in human colon epithelial cells and to determine its regulatory capabilities on TNFα induced inflammation and cellular wound healing. Results from this objective include:

1. GRK2 suppresses TNFα induced epithelial wound closure.
2. GRK2 differentially modulates TNFα signaling through IκBα and ERK1/2 in SW480 cells.
3. GRK2 alters TNFα induced expression of inflammatory and matrix remodeling genes.
4. Specific genes modulated by GRK2 are regulated via the ERK pathway.
5. GRK2 localizes in the mitochondria and inhibits ROS production.
6. The increase in ROS production in the GRK2 knockdown cells is responsible for the observed alterations in ERK1/2 signaling.
7. GRK2 heterozygous mice are protected in an *in vivo* intestinal wound-healing model.

Objective 2 and Results

To identify the role of GRK2 in DSS-induced colitis and determine the mechanism behind influence of GRK2 in the resultant pathophysiology. Results from this objective include:

1. GRK2 heterozygous mice are protected from acute DSS-induced colitis.
2. Protection from DSS-induced colitis is independent of immune infiltration.
3. GRK2 knockout in the myeloid cell population protects against DSS-induced colitis.
4. Decreased levels of GRK2 attenuate intestinal inflammation in DSS possibly through similar mechanisms in the myeloid and GRK2 heterozygous models.

LIMITATIONS OF THE STUDY

Although many positives can be taken from this study, there are some limitations that must be considered:

1. *In vitro* work was done using a colon carcinoma cell line that has been immortalized for scientific research. These cells do not fully characterize and/or represent human intestinal epithelial cells.
2. While an advantage of using mass spectrometry allows for an unbiased search for GRK2 binding partners in the SW480 cells, it is possible that we are simply seeing the most highly expressed proteins that can interact with GRK2 and not all possible outcomes. Additionally, this experiment was done in unstimulated cells; it is possible that TNFα treatment can change the cohort of proteins capable of interacting with GRK2.
3. The *in vivo* components of this study are limited by the availability of only GRK2 heterozygous mice (GRK2 homozygous mice are lethal). While we still see phenotypic effects with the removal of one allele it is possible that aspects of GRK2s role in these cells are hidden by the incomplete ablation of this protein. To overcome this, work was done using myeloid specific knockout mice but this may be ignoring critical components of the epithelial regulation as seen in Objective 1.

STUDY OUTCOME

This dissertation investigated the role that GRK2 plays in the intestinal epithelium in response to inflammation. Two individual studies were designed and carried out to answer these questions: 1) investigating the response of intestinal epithelial cells to TNFα and how GRK2 can influence the cellular response and 2) identifying in a larger context how GRK2 influences the intestinal disease, colitis.

Although further studies are needed to fully understand the role for GRK2 in this tissue as well as the complete molecular mechanism of action in all of the cell types involved in colitis; we have shown that GRK2 plays a vital role in regulating intestinal inflammation and influencing the outcome of DSS induced colitis.

Early studies were targeted at the role for GRK2 in TNFα signaling in human colonic epithelial cells (SW480). TNFα is a pleiotropic signaling molecule but has been shown to significantly regulate wound healing in intestinal epithelial cells [1]. Knocking down GRK2 in these cells we observed that wound healing was significantly increased upon the addition of TNFα. Importantly, this increase in wound healing was independent of changes in proliferation and apoptosis in these cells. Further analysis into the mechanism behind these changes in wound healing revealed that knockdown of GRK2 altered the phosphorylation of map-kinase (MAPK) signaling in these cells, specifically ERK1/2, and ERK1/2 driven gene expression of MMP9. This is important because MMP9 has been demonstrated in other epithelial models to be critical in the onset and activation of epithelial migration and wound repair [2] and was equally necessary for the increased wound healing response in our studies. Migration through a matrigel layer (degradable by MMP9) was increased in the knockdown cells towards a serum stimulus

indicating that not only was there more MMP9 gene expression but also there was more active MMP9 in those cells which resulted in increased wound healing.

The mechanism behind this increase in ERK1/2 signaling and wound healing developed from experimental results indicating the presence of GRK2 in the mitochondria of these cells as well as mass spectrometry analysis indicating that GRK2 interacted with several mitochondrial proteins. The presence of GRK2 in the mitochondria altered ROS production, which has been shown to activate mitogen-associated signal transduction pathways including ERK1/2 [3,4]. Indeed, pharmacological inhibition of ROS in the knockdown cells resulted in the increased activation of ERK1/2 returning to control levels and this inhibition was also able to ablate the increase in wound healing seen after TNFα stimulation. This not only highlights the importance of GRK2 on TNFα signaling and epithelial wound healing but also elucidates the mechanism behind this protection in these cells. In addition to the *in vitro* studies, decreased levels of GRK2 in mice (heterozygous for GRK2) displayed increased wound healing *in vivo*, indicating that the results seen in our cell culture model may be translatable to whole body systems.

Further expanding on the data indicating the knock down of GRK2 improves TNFα driven epithelial wound healing *in vitro* and in an *in vivo* wound healing model, we examined the role of GRK2 in acute DSS induced colitis. Common characteristics of DSS induced colitis is gross epithelial damage and high levels of TNFα [5,6] establishing a strong correlation between our *in vitro* studies and our *in vivo* experiments. Indeed, in mice heterozygous for GRK2 we observed decreased weight loss and disease activity after an acute insult of colitis. It's important to note that *in vivo* intestinal inflammation involves several different cell types even though the injury is

initiated by DSS at the level of the intestinal epithelial cells and GRK2 is highly expressed in many different cell types including immune cells. Analyzing the levels of immune infiltration in the colonic lamina propria, mesenteric lymph nodes and bone marrow we were unable to detect any significant differences between the wild type and GRK heterozygous animals. Interestingly, myeloid specific GRK2 knockout animals also conveyed protection against DSS induced colitis indicating that it is most likely a combination of functional changes in the epithelium and myeloid cells in the colon providing protection rather than changes in immune infiltration. These functional changes present themselves in altered inflammatory cytokine and chemokine gene expression from the colon tissue and altered levels of IL-10 protein in the colon and spleen.

Therefore the data presented here suggest that GRK2 alters the response of intestinal epithelial cells to TNFα through regulation of the TNFα/ROS/ERK/MMP9 pathway ending in changes in wound healing in intestinal epithelial cells. The changes in the intestinal epithelium, in combination with functional changes in the myeloid cells, alter the progression of DSS induced colitis. This suggests that inhibiting GRK2 through pharmaceutical inhibitors may be promising avenues for novel therapeutics in the treatment of colitis.

FUTURE DIRECTIONS

Future experiments will be needed to further characterize the role of GRK2 in both the epithelial cells as well as in the *in vivo* colitis model. In particular, our studies show that GRK2 is able to localize to the mitochondria both basally and under inflammatory conditions and likely influences the metabolism of these cells, specifically in ROS generation. Our immuno-precipitation and mass spectrometry identification of binding partners of GRK2 in the SW480 cells identified several mitochondrial proteins that may be responsible for these alterations in ROS generation. However, it remains unclear whether or not GRK2 is directly interacting with these proteins or interacts through a mediator as well as how exactly this interaction influences mitochondrial functionality. Therefore, future studies will need to elucidate the exact mechanism of interaction between these proteins including whether this is driven via GRK2 kinase activity, GRK2s phosphorylation status or through direct interaction with GRK2 and these proteins. Mutagenesis studies can begin to elucidate this interaction. Moreover, the mechanism in which GRK2 alters the activity of this protein and the overall role for GRK2 in epithelial mitochondria have not been yet been studied. In other cell types, GRK2 has been shown to alter mitochondrial biogenesis [7] but future studies will need to analyze specific elements of mitochondrial function including biogenesis, ATP production, ROS production, electron transport and how GRK2 regulates these processes in epithelial cells.

The role that GRK2 plays in the intestinal epithelium involving the regulation of acute colitis remains to be fully understood. Even though GRK2 is protective in the heterozygous model, the role of GRK2 in the intestinal epithelium in this disease is complicated by the whole body heterogeneity of the animal model. To more strongly

correlate the *in vitro* and *in vivo* results an animal model with GRK2 knocked specifically out of the intestinal epithelium is necessary. Therefore, the DSS colitis experiments need to be expanded into floxed GRK2^{VillinCre} animals knocking out GRK2 from only the epithelial cells expressing villi in the small and large intestine [8]. This will more strongly demonstrate the role of GRK2 in the intestinal epithelium and its influence on wound healing and the outcome of colitis.

The inflammatory response to DSS induced colitis involves a large variety of cells including innate and adaptive immune cells as well as epithelial cells. Our work has begun to get at the mechanistic role of GRK2 in simply one of the cell types. Our lab and others in the field continually investigate the role of GRK2 in multiple cell and tissue types (see Chapter 1) but the stimuli and environment of colitis are not always represented. This work can continue in not only in the epithelial cells but also expand into the immune populations investigating the mechanisms of GRK2 regulation in response to TNFα, microbial dissemination, other inflammatory stimuli, etc that are associated with colitis and epithelial damage.

Finally, GRK2 inhibitors are currently being developed by others in the GRK field [9] and testing these inhibitors in IBD model would move this work closer to translational work and potentially provide an avenue for targeting GRK2 in human IBD.

REFERENCES

REFERENCES

1 Leppkes, M., Roulis, M., Neurath, M. F., Kollias, G. and Becker, C. (2014) Pleiotropic functions of TNF-α in the regulation of the intestinal epithelial response to inflammation. Int. Immunol. **26**, 509–515.

2 Bove, P. F., Hristova, M., Wesley, U. V., Olson, N., Lounsbury, K. M. and van der Vliet, A. (2008) Inflammatory levels of nitric oxide inhibit airway epithelial cell migration by inhibition of the kinase ERK1/2 and activation of hypoxia-inducible factor-1 alpha. J. Biol. Chem. **283**, 17919–28.

3 Zhang, J., Jin, N., Liu, Y. and Rhoades, R. A. (1998) Hydrogen Peroxide Stimulates Extracellular Signal-regulated Protein Kinases in Pulmonary Arterial Smooth Muscle Cells. Am. J. Respir. Cell Mol.Biol. **19**, 324–332.

4 Chen, K. C., Zhou, Y., Xing, K., Krysan, K. and Lou, M. F. (2004) Platelet derived growth factor (PDGF)-induced reactive oxygen species in the lens epithelial cells: the redox signaling. Exp. Eye Res. **78**, 1057–1067.

5 Neurath, M. F. (2014) New targets for mucosal healing and therapy in inflammatory bowel diseases. Mucosal Immunol. **7**, 6–19.

6 Iizuka, M. and Konno, S. (2011) Wound healing of intestinal epithelial cells. World J. Gastroenterol.

7 Fusco, A., Santulli, G., Sorriento, D., Cipolletta, E., Garbi, C., Dorn, G. W., Trimarco, B., Feliciello, A. and Iaccarino, G. (2012) Mitochondrial localization unveils a novel role for GRK2 in organelle biogenesis. Cell. Signal. **24**, 468–475.

8 Madison, B. B., Dunbar, L., Qiao, X. T., Braunstein, K., Braunstein, E. and Gumucio, D. L. (2002) Cis elements of the villin gene control expression in restricted domains of the vertical (crypt) and horizontal (duodenum, cecum) axes of the intestine. J. Biol. Chem. **277**, 33275–83.

9 Guccione, M., Ettari, R., Taliani, S., Da Settimo, F., Zappalà, M. and Grasso, S. (2016) G-Protein-Coupled Receptor Kinase 2 (GRK2) Inhibitors: Current Trends and Future Perspectives. J. Med. Chem. **59**, 9277–9294.