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INFLAMMATORY SIGNALING IN MACROPHAGES: REGULATION BY G-PROTEIN COUPLED RECEPTOR KINASE-2 AND 5

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INFLAMMATORY SIGNALING IN MACROPHAGES: REGULATION BY G-PROTEIN COUPLED RECEPTOR KINASE-2 AND 5

By

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ABSTRACT

INFLAMMATORY SIGNALING IN MACROPHAGES: REGULATION BY G-PROTEIN COUPLED RECEPTOR KINASE-2 AND 5

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Sonika Patial

G-protein coupled receptor kinases (GRKs) are serine-threonine protein kinases which phosphorylate agonist bound G-protein coupled receptors (GPCRs) leading to their desensitization. Although originally discovered with regard to GPCR desensitization, recent studies have shown that GRKs play much wider roles than previously appreciated. In this regard, studies have shown that GRKs can phosphorylate non GPCR receptor as well as non receptor substrates. There is an ample amount of evidence in the literature showing that the expression and activity of GRKs is altered in several inflammatory disease conditions. For instance, the activity and expression levels of GRK2 were found to be significantly decreased in peripheral blood mononuclear cells (PBMC) of patients with rheumatoid arthritis whereas the expression levels of GRK2 and GRK5 were found to be enhanced in the neutrophils of sepsis patients. Moreover in our previous studies we found that stimulation of primary peritoneal macrophages with LPS under *in vitro* conditions causes an increase in the expression of GRK2. These studies point to a crucial role of GRKs in inflammatory signaling, however, the physiological importance of changes in the expression levels of GRKs is not well established. To enhance our understanding of the role of GRK2 and 5 in inflammatory signaling, we first investigated the mechanism by which GRK2 and 5 regulate TNFα-induced inflammatory signaling in mouse macrophage cell line.

Our results in this study demonstrated that both GRK2 and 5 positively regulate TNFα-induced NFκB signaling. Knockdown of GRK2 and 5 inhibited TNFα-induced NFκB signaling whereas overexpression of GRK2 and 5 substantially enhanced TNFα-induced NFκB activity. GRK2 and 5 were found to interact with and phosphorylate IκBα, an inhibitor of NFκB and this was found to be the biochemical mechanism of regulation of NFκB signaling pathway by GRK2 and 5.

To further elucidate the role of GRKs in inflammation under *in vivo* conditions, we utilized mice with homozygous GRK5 gene deletion. Primary peritoneal macrophages from $GRK5^{-/-}$ mice stimulated with LPS showed an inhibition of NFκB activity as compared to cells from $GRK5^{+/+}$ mice. Secretion of several LPS-induced inflammatory cytokines was found to be reduced in cell culture supernatants of $GRK5^{-/-}$ mice. Plasma levels of cytokines and chemokines were also found to be reduced which was associated with reduced liver injury in $GRK5^{-/-}$ mice compared to $GRK5^{+/+}$ mice suggesting that GRK5 positively regulates LPS-induced inflammatory signaling *in vivo* by modulating transcription factor NFκB. Since homozygous gene deletion of GRK2 is lethal in mice, we utilized Cre-loxP system to achieve a cell specific deletion of GRK2 whereby GRK2 was specifically deleted in the cells of myeloid lineage. LPS injection into these mice caused an increased expression of cytokines / chemokines in the plasma as well as in the primary peritoneal macrophage cell culture supernatants. GRK2 deficient mice also exhibited an increased lung and liver injury. Mechanistically, IKKβ-NFκB1 p105 /TPL2-MEK-ERK pathway was found to be negatively regulated by GRK2 which resulted in an increased expression of cytokines / chemokines in GRK2 deficient mice. Taken together, these results show that both GRK2 and 5 play a crucial role in LPS-induced inflammatory signaling under *in vivo* conditions by regulating NFκB and ERK signaling pathways.

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KEY TO ABBREVIATIONS

ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
cDNA	Complimentary DNA
CK	Casein Kinase
CMV	Cytomegalovirus
CRE	Cyclization recombination
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoretic mobility shift assay
GPCR	G-Protein coupled receptor
GRK	G-Protein coupled receptor kinase
GRK2	G-Protein coupled receptor kinase-2
GRK5	G-Protein coupled receptor kinase-5
GST	Glutathione S-transferase
HA	Hemaglutinin
HEK	Human Embryonic Kidney
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
HRP	Horseradish peroxidase
IAP1	Inhibitor of apoptosis 1

IKKβ	IκB kinase β
IL	Interleukin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IκBα	Inhibitor of NFκB
KCl	Potassium Chloride
LB	Luria Bertani
LoxP	Locus of crossover in P1
LPS	Lipopolysaccharide
$MgCl_2$	Magnesium Chloride
ml	Milliliter
mM	Millimolar
mRNA	Messenger RNA
NaCl	Sodium Chloride
NFκB	Nuclear factor-kappa B
nM	Nanomolar
PBS	Phosphate Buffered Saline
pH	Presence of active hydrogen
RIP1	Receptor interacting protein 1
RT	Reverse Transcriptase
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM	Standard error of mean
TAD	Transactivation domain
TBS-T	Tris-Buffered Saline-Tween 20
TNFR	TNF receptor
TNFα	Tumor necrosis factor-alpha
TRADD	Tumor necrosis factor receptor 1 associated death domain protein
TRAF	TNF receptor associated factor
UV	Ultraviolet
μg	Microgram
μl	Microliter
μm	Micrometer

CHAPTER 1

INTRODUCTION

G protein coupled receptor kinases (GRKs) are serine threonine kinases which play an important role in the desensitization of G protein coupled receptors (GPCRs). Agonist bound GPCRs are a target for phosphorylation by GRKs, thus leading to their rapid homologous desensitization. Although originally discovered with regard to this classical role of GPCR desensitization, recent studies have shown that GRKs can phosphorylate non-GPCR receptor as well as non-receptor substrates. Moreover, GRKs have also been shown to be involved in other phosphorylation-independent protein-protein interactions. In this regard, GRKs have been shown to regulate Toll like receptor 4 (TLR4) induced inflammatory signaling in macrophages [1]. Thus, it was hypothesized that GRKs are important regulators of inflammatory signaling in macrophages and therefore GRKs might play essential roles in the regulation of inflammatory diseases. This thesis research investigates the role of GRKs in inflammatory signaling mediated by TNF receptors in macrophages under *in vitro* conditions as well as the role of GRKs in TLR4-induced inflammatory signaling under *in vivo* conditions in a mouse model of sepsis.

This brief introduction provides an overview of the hypothesis tested in this thesis with a glance on the focus of subsequent chapters. Chapter two provides a comprehensive literature review which provides an in-depth discussion of the scientific literature surrounding the focus of this thesis project, including the classical and novel roles of GRKs as well as the involvement of GRKs in inflammation and immune response. Chapters three through five are organized based on the specific

aims of this thesis research project, each consisting of an abstract, introduction, materials and methods, results and discussion relevant to the individual aim. Chapter three focuses on the role of GRKs, in particular GRK2 and GRK5, in TNFα induced inflammatory signaling particularly in mouse macrophages under *in vitro* conditions. Chapter four investigates the role of GRK5 at the *in vivo* level in a mouse model of LPS induced sepsis using a homozygous GRK5 gene deletion. Chapter five tests the role of GRK2 in inflammatory signaling at the *in vivo* level whereby the focus was to generate a myeloid cell specific genetic deletion of GRK2 and investigating the role of this conditional deletion in a mouse model of LPS induced sepsis. Chapter six highlights the results obtained in this thesis project with a discussion on how these findings contribute to our current understanding of the novel roles of GRKs as well as the important role of GRKs in LPS mediated sepsis. Also, discussed are some of the limitations of this project as well as future directions.

CHAPTER 2

LITERATURE REVIEW

2.1: G protein coupled receptor kinases (GRKs)

G-protein coupled receptor kinases (GRKs) are serine/threonine kinases which were originally discovered with regard to G-protein coupled receptor (GPCR) desensitization [2]. They are the key modulators of GPCR signaling and play an important role in fine tuning the GPCR signaling mechanism preventing the overstimulation of GPCRs even in the presence of continuous agonist stimulation.

2.1.1: Historical perspectives of GRK discovery

Studies of mechanisms involved in the homologous desensitization of β2-adrenergic receptor (β2AR) and rhodopsin (the prototypic light receptor) led to the discovery of GRKs. In particular, rhodopsin played an important role in our current understanding on the desensitization mechanism of GPCRs by GRKs due to its availability in greater quantities. Rhodopsin undergoes light dependent phosphorylation, first discovered *in vivo* in the 1970's [3, 4]. Subsequently, it was shown that the kinetics of rhodopsin phosphorylation correlate with the quenching of cGMP phosphodiesterase activity, suggesting that rhodopsin phosphorylation has a role in its desensitization [5]. Rhodopsin kinase (GRK1) was then purified and shown to phosphorylate the receptor at multiple serine and threonine residues.

The occurrence of agonist-induced phosphorylation and desensitization of β2AR even in the absence of protein kinase A (in kin$^-$ S49 lymphoma cells) and alpha

subunit of Gs (in cyc^- cells) suggested the presence of another kinase, subsequently identified as β-adrenergic receptor kinase (βARK) [6, 7]. In the mid 1980's, βARK was also discovered as a functional homologue to rhodopsin kinase and this further suggested the existence of a multigene family of protein kinases which phosphorylate agonist bound GPCRs [8]. Subsequently, crude βARK preparations were made and were shown to mediate agonist dependent phosphorylation and uncoupling of the receptor *in vitro*. A cDNA encoding βARK (or GRK2) was subsequently sequenced and cloned, which expressed an enzyme that preferentially phosphorylates the agonist-bound β2AR [9].

Pure preparations of βARK however did not lead to a significant loss of Gs coupling. This suggested that an essential cofactor needed for βARK mediated desensitization was lost during the purification process. This essential cofactor was subsequently discovered as an additional protein called arrestin. Four distinct members of arrestins are now known. Two out of these are restricted to phototransduction pathways (visual arrestin1 and visual arrestin 2). The other two i.e beta arrestin 1 (arrestin 2) and beta arrestin 2 (arrestin 3) are expressed ubiquitously and regulate the internalization of many GPCRs as well as also play an important role in cellular signaling [10].

2.1.2: GRK mediated desensitization of GPCRs

GRKs cause homologous desensitization of the receptors whereby only the agonist activated receptors are phosphorylated and desensitized [2],[11]. Agonist-induced desensitization of a GPCR is a multistep process. GRKs first phosphorylate

agonist bound GPCRs on serine and threonine residues located in the carboxy-terminal tail region and/or the third cytoplasmic loop. This process enhances the affinity of agonist bound-GPCR for binding to arrestins which then sterically inhibit the interaction of the receptors with the G-proteins [12] leading to rapid homologous desensitization. Finally, the GRK-arrestin system induces the internalization of inactivated receptors to endosomal compartments via endocytosis.

Endocytosis of receptors has a fundamental role in receptor biology although this process is not necessary for homologous desensitization [13]. Internalization of receptors can have three consequences viz. (1) Dephosphorylation of the receptor, leading to resensitization and recycling back to the cell surface (2) Targeting of the receptor to lysosomes leading to degradation (3) or activation of other signaling pathways. Furthermore, endocytosis can occur via clathrin coated pits, caveolea or other uncoated vesicles.[14, 15]. For clathrin dependent internalization, phosphorylation of receptors by GRKs and subsequent binding by arrestins appears to be essential [12]. Moreover, arrestin and clathrin dependent endoycytosis is used by a majority of GPCRs.

2.1.3: Structure and distribution of GRKs

The GRK family consists of seven different genes and based on sequence and functional similarities they are divided into three subfamilies [16]. The rhodopsin kinase subfamily comprises GRK1 (rhodopsin kinase) and GRK7 (cone opsin kinase) that are found exclusively in retinal cells. The GRK2 subfamily comprises pleckstrin homology domain containing GRK2 (β-ARK1) and GRK3 (β-ARK2). Their

membrane recruitment depends on interaction with the Gβγ subunits of G proteins and phosphatidyl-inositol 4,5-biphosphate. The GRK4 subfamily comprises GRK4, GRK5 and GRK6 which are predominantly localized at the membrane either due to palmitoylation on C-terminal cysteine residues (GRK4/6) or interaction between a positively charged domain at the C-terminus with the negatively charged membrane phospholipids (GRK5) [17]. GRK4 is predominantly found in testes [18] and to a lesser extent also in brain and kidney [19] whereas GRK2, GRK3, GRK5 and GRK6 are widely expressed (**Table 2.1**).

All GRKs have a similar structural organization possessing an N-terminal domain, a central catalytic domain (with homology to other serine-threonine kinases) and a C-terminal domain. The N-terminal domain of 183-188 amino acids includes a region of homology to regulators of G-protein signaling (RGS) proteins that are known to act as GTPase activating proteins for many Gα subunits. The carboxyl terminal domain is of variable length and contains key determinants important for localization and translocation to the membrane either by post translational modifications or by sites of interaction with lipids or membrane proteins. In this regard, GRK1 and GRK7 are isoprenylated, GRK4 and GRK6 are palmitoylated at one or more cysteine residues clustered within the last 15-20 amino acids of the C-terminus, which is responsible for their membrane localization whereas GRK5 is localized in the membrane by virtue of a phosphatidyl inositol 4,5 biphosphate binding

Table 2.1: Classification of G protein coupled receptor kinases (GRKs)

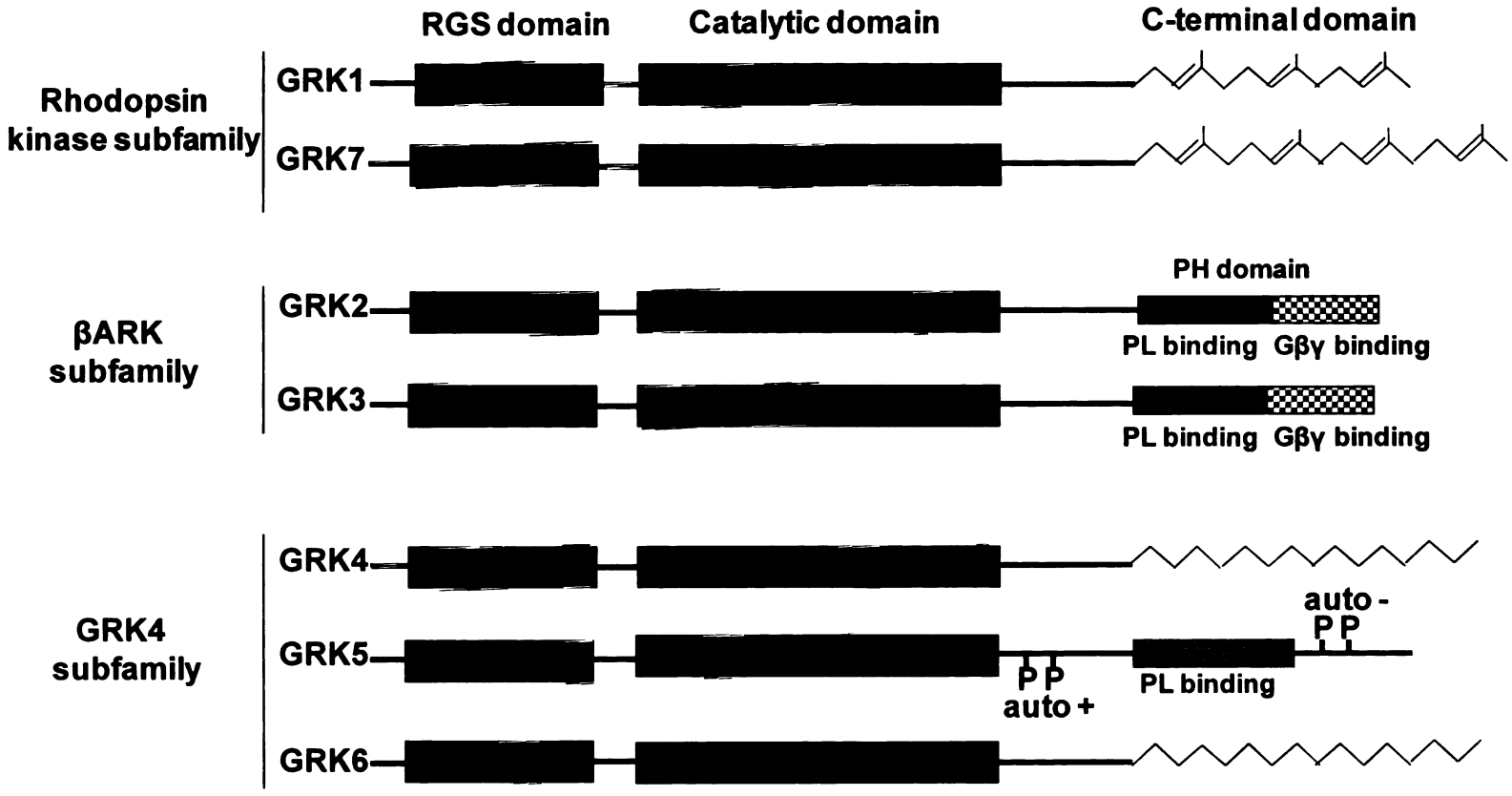
GRKs have been subdivided into three subfamilies based on the sequence and functional similarities. 1. Rhodopsin kinase subfamily which comprises GRK1 (Rhodopsin kinase) and GRK7 (cone opsin kinase). 2. GRK2 subfamily which comprises GRK2 (β-ARK1) and GRK3 (β-ARK2). 3. GRK4 subfamily which comprises GRK4, GRK5 and GRK6.

Rhodopsin kinase subfamily	GRK1
	GRK7
βARK subfamily	GRK2 (β-ARK1)
	GRK3 (β-ARK2)
GRK4 subfamily	GRK4
	GRK5
	GRK6

Figure 2.1: Comparison of Linear structure of GRKs

The seven isoforms of GRKs share a number of structural and functional similarities. The amino terminal domain (183-188 amino acids) includes a region of homology to regulators of G protein signaling (RGS) proteins. The central catalytic domain has a homology to other serine threonine kinases. The carboxyl terminal domain is of variable length and contains several key determinants for the localization and translocation of kinases to the membrane. This occurs either through post translational modifications or the presence of sites of interaction with lipids or membrane proteins. As shown in the figure, GRK1 and GRK7 undergo isoprenylation; GRK4 and GRK6 undergo palmitoylation: GRK5 has positively charged amino acid clusters at the C-terminus through which it binds to membrane phospholipids: GRK2 and GRK3 bear an extended C-terminus known as Pleckstrin homology domain (PH domain). This domain modulates the membrane targeting of GRK2 and GRK3.

PL, phospholipids; **auto (±)**, stimulatory or inhibitory autophosphorylation sites.


RGS domain
Catalytic domain
C-terminal domain
Rhodopsin kinase subfamily
GRK1
GRK7
PH domain
βARK subfamily
GRK2
PL binding Gβγ binding
GRK3
PL binding Gβγ binding
GRK4 subfamily
GRK4
auto -
P P
GRK5
P P
auto +
PL binding
GRK6

domain at the N-terminus and a polybasic region (amino acids 547-560) close to the C-terminus [20]. GRK2 and GRK3 bear an extended C-terminus which is involved in the modulation of the kinase targeting to the membrane. GRK2 and GRK3 are cytoplasmic proteins that translocate to the membrane upon receptor stimulation. Their C-terminus harbors a pleckstrin homology (PH) domain (residues 561-655) that partially overlaps with the Gβγ-binding region. It has been shown that free Gβγ sub-units can bind to GRK2 with a high affinity. Furthermore it has been shown that Gβγ is required for association of GRK2 to lipid vesicles in reconstituted systems [21].

Hence, it is believed that the interaction of Gβγ with GRK2 and GRK3 targets them to membranes at the site of GPCR activation. Binding of Gβγ also enhances the activity of GRK2 by causing allosteric activation of this kinase. The PH domain of GRK2 and GRK3 also interacts with phosphatidylinositol 4,5-biphosphate (PIP2) and other acidic phospholipids such as phosphatidyl serine (PS) which regulate its kinase activity [22]. Thus, Gβγ and lipids contribute synergistically to membrane localization and activation of GRK2 (**Figure 2.1**).

The GRK4-6 family of GRKs has been shown to contain a nuclear localization sequence (NLS). It was found that the nuclear localization of GRK5 is regulated by GPCRs. Binding of an agonist onto a GPCR leads to an elevation of intracellular calcium levels and activates a calcium sensor protein, calmodulin. Furthermore, GRK5 was found to bind to DNA under *in vitro* conditions [23]. More recently, it was shown that GRK5 can act as a histone deacetylase (HDAC) kinase independent of its actions on GPCRs [24].

2.1.4: Regulation of GRKs by other proteins

2.1.4.1 Regulation of GRKs by Caveolin, Clathrin and α-actinin

Caveolins control the basal kinase activity of GRKs: Caveolin is a 22-24 kDa integral membrane protein and is a prominent structural component of caveolae. Caveolae are specific cholesterol and glycosphingolipid-enriched plasma membrane structures in cells. A 20 aa region within the N-terminal domain of caveolin (known as scaffolding domain) has been found to mediate the association of caveolin with other proteins. Caveolins serve as scaffolding proteins for some GPCRs as well as certain mitogen activated protein kinases (MAPKs) and G proteins and help compartmentalize signaling pathways. Recent studies have shown that GRK2 is present in caveolin rich fractions of cellular membranes. GRK2 contains caveolin binding motifs in the PH domain (residues 567-584) as well as in the N-terminal domain (residues 63-71) while GRK3 and GRK5 can bind to caveolin only through N-terminal motifs. GRK2 binding to caveolin 1 or 3 inhibits GRK2 mediated phosphorylation suggesting caveolins can control the basal kinase activity of GRK2. In addition, it is possible that GRK-caveolin interactions may facilitate GRKs interactions with other signaling molecules [25].

Clathrin promotes arrestin independent internalization: Clathrin is a protein present in the internalized vesicles containing GPCRs. GRK2 can also interact with clathrin protein via a clathrin box located in the carboxyl terminal region of GRK2 [26]. β-arrestins bind to phosphorylated GPCRs and promote the internalization of the receptors by interacting with clathrin. Certain GPCRs, however, are only

slightly internalized due to their low affinity binding to β-arrestins. GRK2 bound to such receptors can facilitate GPCR internalization due to its ability to bind clathrin. This internalization is however, β-arrestin independent but depends on the presence of dynamin. [27].

Alpha-actinin inhibits GRKs: α-actinin is a protein that falls under the spectrin superfamily of actin crosslinking proteins. It is found most abundantly in muscle cells. It binds and crosslinks actin as well as lipid, creating a submembraneous meshwork consisting of crosslinked actin. α-actinin also binds various ion channels, cell adhesion molecules as well as transmembrane receptors, thereby incorporating them into the actin network creating signaling domains. It has been found that all GRKs can interact with α-actinin. α-actinins can completely inhibit GRKs, or modulate their activity, as well as substrate specificity [28].

2.1.4.2 Regulation of GRKs by Calcium binding proteins

Recoverin inhibits GRK1: Recoverin is a protein present in vertebrate photoreceptors, certain retinal cone bipolar cells and pineal glands, exhibiting a distribution quite similar to GRK1. Recoverin binds directly to GRK1 and inhibits it in a Ca^{2+}-dependent fashion. Calcium levels are very high during dark conditions and fall substantially under light conditions in the vertebrate photoreceptor systems. Hence, GRK1 would be complexed to recoverin and inactive when rhodopsin is in its inactive state under dark conditions. However, following rhodopsin activation, a drop in intracellular calcium levels causes the release of GRK1 from recoverin. GRK1 is now

disinhibited and can facilitate rapid phosphorylation and desensitization of rhodopsin [29].

Calmodulin inhibits GRKs: Calmodulin is a universal mediator of calcium signaling, which has been shown to inhibit the activity of GRK2, 3, 4, 5, and 6 albeit with different potencies [30]. Calmodulin binds directly to both GRK2 and GRK5, with different affinities, inhibiting the interaction of these enzymes with their agonist occupied receptors. GRK5 is highly sensitive to the presence of calcium bound calmodulin (IC50 ~ 50nM), whereas GRK2 is affected only at higher concentrations (IC50 ~ 2μM) [31]. Thus GRK2 and GRK5 mediated desensitization of GPCRs would be predicted to be inhibited in the presence of Ca^{2+}/calmodulin. GRK2 has a relatively low affinity for calmodulin suggesting that inhibition of GRK2 would occur only at sites highly enriched in calmodulin. However, the relatively high affinity of GRK5 for calmodulin suggests that this regulatory mechanism might be present at multiple locations.

2.1.4.3 Regulation of GRKs through phosphorylation by other kinases

Protein Kinase C (PKC) activates GRK2 but inhibits GRK5: GRKs activity, the protein stability as well as their ability to interact with other proteins is highly regulated by their own phosphorylation by other protein kinases. In this regard protein Kinase C (PKC) has been shown to phosphorylate both GRK2 and GRK5 in intact cells as well as under *in vitro* conditions. PKC phosphorylation of GRK2 enhances the translocation and interaction of GRK2 with the plasma membrane causing an increased activity towards receptor substrates. It does not, however, affect

the catalytic activity of GRK2 since GRK2 cannot phosphorylate soluble peptides under similar conditions. PKC phosphorylates GRK2 at serine 29 within the calmodulin binding motif of GRK2 [32]. It has been suggested that the inhibitory effect produced by calmodulin binding to GRK2 is abolished after phosphorylation by PKC, allowing the binding of GRK2 to the receptor substrate. In contrast to this, phosphorylation of GRK5 by PKC leads to the inhibition of its activity towards both receptors and soluble substrates suggesting a direct effect on the catalytic activity of GRK5. There are two major sites of phosphorylation by PKC in the C-terminal 26 amino acids of GRK5. The inhibitory autophosphorylation sites also reside in this region which are targeted in the presence of calmodulin. Thus Ca/Calmodulin and PKC both act as inhibitors by targeting identical inhibitory sites on GRK5. [20].

Protein kinase A (PKA) activates GRK2: Protein kinase A (PKA) is another kinase that can phosphorylate GRK2 at serine 685. This site is located near the Gβγ binding domain of GRK2. PKA phosphorylation of GRK2 at this site enhances the binding of GRK2 with the Gβγ subunits, facilitating the membrane targeting of GRK2 and further the interaction of GRK2 with activated receptors. This leads to an enhanced GRK2 activity towards β2-adrenergic receptors [33].

C-Src activates GRK2: c-Src, a tyrosine kinase has been shown to directly phosphorylate GRK2 under *in vitro* conditions as well as it promotes tyrosine phosphorylation of GRK2 upon stimulation of β2-adrenergic receptors [34, 35]. This process is however dependent on the ability of β-arrestins to recruit c-Src since β-arrestin mutants defective in c-Src binding are defective in this process. [36]. Tyrosine phosphorylation of GRK2 directly enhances its catalytic activity as is seen by its

increased activity towards both soluble and membrane bound substrates. Hence, recruitment of c-Src by β-arrestins to the activated GPCR results in GRK2 phosphorylation at tyrosine residues and increases its catalytic activity.

Extracellular signal regulated kinase 1 (ERK1) inhibits GRK2: ERK1, a MAPK, has also been shown to phosphorylate GRK2 at serine 670 under *in vitro* and *in situ* conditions [37, 38]. This phosphorylation site is present within the Gβγ binding domain of GRK2. Hence, ERK1 phosphorylation of this site impairs the interaction of GRK2 with Gβγ, inhibiting GRK2 translocation to the activated membrane receptors thus inhibiting its catalytic activity. This process triggers a negative feedback loop that prevents accumulation of an active pool of GRK2. Moreover, both kinases have been found to co-immunoprecipitate in an agonist dependent manner [38].

2.1.4.4 Regulation of GRKs by Gβγ subunits and phospholipids

GRK2 and GRK3 contain a PH domain (residues 561-655) in the carboxy-terminal region that partially overlaps with the Gβγ-binding region. Free Gβγ subunits bind to GRK2 with high affinity, and is required in reconstituted systems for association of GRK2 to lipid vesicles and GPCR phosphorylation [21]. This is important since both GRK2 and GRK3 are cytoplasmic proteins that transiently translocate to the membrane upon GPCR stimulation. Hence, it is suggested that the interaction of Gβγ with GRK2 and GRK3 targets these kinases to membrane sites where receptors are activated and Gβγ dimers are released. Gβγ binding increases the activity of GRK2 by promoting GPCR-mediated allosteric activation of GRK2.

PH domains of GRK2 and GRK3 can also interact directly with PIP2 and other acidic phospholipids, affecting their kinase activity [22, 39]. GRK2 phosphorylation of membrane receptors is enhanced by atleast two to three fold by binding of phosphatidyl serine (PS) and PIP2. Among these, PS directly affects the catalytic activity of GRK2 since PS also enhances the phosphorylation of soluble substrates. PIP2 appears to bind to the amino-terminus of the PH domain suggesting that Gβγ and lipids have a synergistic role in GRK2 translocation and activation. GRK5 is also regulated by lipids despite lacking the PH domain. GRK5 possesses two different regions rich in positively charged amino acids (amino terminal residues 22-29 and carboxyl terminal residues 547-560) that can bind to lipids [40]. The amino terminal binding site exhibits a high specificity for PIP2 in contrast to the carboxyl terminal binding site which does not exhibit a stringent specificity for binding to lipids. PIP2 binding onto the amino terminal binding site increases GRK5 phosphorylation of receptor substrates but does not affect phosphorylation of soluble peptides or GRK5 autophosphorylation. On the other hand, lipid binding onto the carboxyl-terminal binding site stimulates autophosphorylation of GRK5 and its activity towards different substrates. These lipid binding domains are highly conserved in the GRK4 subfamily and binding of PIP2 enhances receptor phosphorylation by GRK4 and GRK6 as well.

2.1.4.5 Regulation of GRKs by interaction with agonist activated GPCRs

The activity of GRKs is also regulated by interaction with agonist stimulated GPCRs. Activity of GRK1 towards a peptide substrate increases in the presence of activated rhodopsin receptor. This is because the association of GRK1 with the third

intracellular loop of rhodopsin causes kinase activation [41]. Similarly, the catalytic activity of GRK2 is regulated in the presence of either agonist activated β2-AR and rhodopsin, or synthetic peptides derived from intracellular loops. These interactions appear to occur through the N-terminal region of GRKs [42]. It is thought that interaction of the GRKs with the receptor causes a conformational change in the GRKs thus releasing an autoinhibitory constraint and causing kinase activation.

GRK2 and GRK3 has also been shown to interact to activated Gαq subunits with high affinity, leading to a reduced Gαq mediated phospholipase C-β activation both *in vitro* and in intact cells [43, 44]. Hence the interaction of GRKs with the GPCRs and Gαq subunits may serve two functions, 1) translocation of GRKs to the membrane and 2) phosphorylation independent termination of signal transduction mediated by GPCRs by interfering with the binding of stimulated GPCR and Gαq with their effectors [45].

2.1.5: Other novel interactions of GRKs and their physiological effects

Over the past few years it has become evident that the role of GRKs is not limited to GPCR phosphorylation, desensitization and internalization. Apart from these classical functions, GRKs have now been shown to interact to non GPCR receptor substrates and to a variety of signaling proteins, regulating cell signaling in a phosphorylation dependent as well as independent manner. In this regard, GRKs have been shown to phosphorylate other non GPCR membrane receptors such as Platelet derived growth factor (PDGF) receptor [46] as well as non receptor substrates such as tubulin [47, 48], synnucleins [49], phosducin [50], ribosomal protein P2 [51], ezrin-

radixin-moesin (ERM) family protein ezrin [52] and NFκB1 P105 [1]. In addition, GRKs have been shown to regulate several signaling pathways in a phosphorylation independent manner by interacting with a variety of signaling as well as trafficking proteins. The novel interactions of GRKs along with their functional consequencies are summarized:

PDGFRβ receptor: The platelet-derived growth factor receptor-β (PDGFRβ) plays a crucial role in regulating the proliferation and survival of mesenchymal cells. It has been found that GRK2 phosphorylates PDGFRβ receptor on Ser1104 which is responsible for its desensitization in physiological systems. GRK2 mediated phosphorylation of Ser1104 on PDGFRβ promotes its dissociation from the PDZ domain containing NHERF protein (Na^+/H^+ exchange regulatory factor) which is involved in potentiating PDGFRβ dimerization and activation, thus causing receptor desensitization [46].

Tubulin: In a quest to search for other proteins that might interact with GRK2, a GST fusion protein containing the C-terminus of GRK2 was used. GRK2 was found to bind to the cytoskeletol protein tubulin through its carboxy terminal domain (residues 467-689) by this method. Furthermore, GRK2 can phosphorylate tubulin and phosphorylation of rhodopsin is inhibited by tubulin in a dose dependent manner [47]. However, the physiological relevance of this interaction / phosphorylation is not very clear yet although it is possible that the GRK2-tubulin interaction might have a role in regulating the desensitization of GPCRs by GRK2. Furthermore, it is also speculated that GRKs might play a role in regulating microtubule dynamics and cytoskeletol reorganization.

Synuclein: To identify new substrates for GRKs, bovine calf brain extracts were prepared and were used in phosphorylation reactions. Synuclein, a protein highly expressed in brain was identified as a substrate by this method. Lipids and Gβγ subunits, which stimulate phosphorylation of GPCRs by GRKs, also activate synuclein phosphorylation by GRKs. Furthermore, all GRKs can phosphorylate synuclein. Phosphorylation of synuclein by GRKs blocks the interaction of synuclein with phospholipids. This in turn reduces the inhibitory effect on Phospholipase D2 (PLD2), an enzyme involved in regulating vesicular trafficking. Hence, it is possible that agonist bound GPCRs activate GRKs which then phosphoryate GPCRs targeting them for endocytosis as well as also phosphorylate synuclein thus activating PLD2 which is involved in vesicle formation needed for endocyotsis as well as recycling of receptors.

Raf kinase inhibitor protein (RKIP): RKIP is a protein that belongs to a family of phosphatidylethanolamine-binding proteins (PEBPs) that serve as inhibitors of kinase signaling pathways. RKIP is a physiological inhibitor of Ras-Raf-MEK-ERK pathway involved in several cellular processes such as differentiation, proliferation, cell survival and cell transformation. RKIP has been shown to interact with GRK2 [53]. GPCR stimulation leads to the activation of PKC which can phosphorylate RKIP on serine 153. Phosphorylation of RKIP leads to an increase in its affinity towards GRK2 which then leads to its dissociation from its target Raf1, prolonging the activation of ERK kinase. The interaction of GRK2 with RKIP blocks the kinase activity of GRK2, impairing the desensitization process, and leading to a prolonged signaling.

Phosphoinositide 3-kinase (PI3K): PI3Ks are a conserved family of lipid kinases whose function is to catalyze the addition of a phosphate on the third position of inositol ring. Stimulation of GPCRs causes activation of PI3Ks increasing the levels of D-3 phosphatidyl inositols which are potent signaling molecules. A direct protein-protein interaction has been shown for PI3Kγ and GRK2 through 197 amino acid residues PIK domain in PI3K [54]. GRK2 and PI3Kγ form a cytosolic complex and translocate to the membrane in an agonist dependent manner. This interaction plays an important role in receptor sequestration as it has been shown that inhibition of kinase activity of PI3K results in attenuation of β-adrenergic receptor sequestration. Furthermore, cardiac specific overexpression of the PIK domain causes disruption of the GRK2-PI3K interaction under *in vivo* conditions, preserving β-adrenergic receptor signaling even after prolonged catecholamine administration. This helps restore myocardial contractibility to normal in heart failure conditions [55].

Akt: Akt is a serine threonine kinase which directly interacts with GRK2. The region of interaction has been determined to be GRK2 C-terminus (aa 492-689). This interaction leads to an inhibition of Akt phosphorylation. A study using rats with portal hypertension showed an enhanced expression of GRK2 in their sinusoidal endothelial cells. Enhanced GRK2 was found to lead to an inhibition of Akt phosphorylation. Since Akt phosphorylation is required for the production of NO by eNOS, inhibition of NO production was found to lead to portal hypotension. [56].

GPCR-kinase interacting proteins (GIT proteins): The GIT family of proteins were found to be binding partners of GRKs [57]. Their structure consists of a zinc-finger motif and several ankyrin repeats in the N-terminal portion with a GAP

(GTPase activating protein) domain in the first 45 amino acids. The GIT proteins are active as GAPs on ARF6 protein present on the plasma membrane. Yeast two hybrid screen has identified GIT1 and GIT2 as binding partners for GRK2, 3, 5 and GRK6 [57]. However, the functional significance of this interaction is not very clear as yet.

MEK: GRK2 and MEK1 has also been shown to be present in the same multimolecular complex. Furthermore, it was found that elevated levels of GRK2 inhibit chemokine mediated induction of ERK whereas decreased levels of GRK2 promote chemokine mediated ERK activity. MEK activity was not found to be affected though. Furthermore, neither the kinase activity nor the interaction of GRK2 to G protein subunits is necessary for this effect. [58].

Heat shock protein 90 (Hsp 90): Hsp 90 is a highly conserved chaperone protein that interacts with a wide variety of signaling proteins. GRK2 has been found to interact with Hsp 90 and it has been shown that the disruption of this interaction causes an increased degradation of GRK2 via the proteasome pathway. This suggests that the interaction of GRKs with the heat shock proteins plays an important role in the folding and maturation of GRKs [59].

2.2 Physiological and Pathological roles of GRKs

Development of genetically modified mouse models with either a targeted deletion of a particular GRK or overexpressing a GRK transgene has given us a great insight into the roles of individual GRKs in various signaling pathways as well as in an intact animal.

2.2.1: Physiological roles of GRKs as determined by knockout / transgenic mice

GRK1 is required for rhodopin desensitization: GRK1 or rhodopsin kinase was the first GRK to be discovered [60] and was found to phosphorylate light activated rhodopsin at C-terminal residues. It is expressed in retina and in mammalian pineal gland. A better idea of the role of GRK1 under *in vivo* conditions came with the generation of mice deficient in GRK1 ($GRK1^{-/-}$) [61]. Deficiency of GRK1 led to an elimination of the light dependent phosphorylation of rhodopsin causing the single photon response to become larger and longer than normal. Also, a day of constant light caused the rods in these mice to undergo apoptotic degeneration. Furthermore, the cone response recovered around 30-50 times slower than normal when the mice were exposed to a bright conditioning flash.

GRK2 plays an essential role in embryonic development: Similar to GRK1, studies with GRK2 knockout mice confirmed an important role of GRK2 in embryonic cardiac development and function. GRK2 ($GRK2^{-/-}$) homozygous knockout mice embryos die during gestation. Examination of these embryos revealed that there was a pronounced hypoplasia of the ventricular myocardium as well of the interventricular septum due to which the atrial and ventricular cavities appeared to be unusually large [62]. Furthermore, it was found that there was a 70% decrease in left ventricular ejection fraction suggesting an impaired heart function. These mice, however, developed normally with no apparent adult cardiac phenotype at baseline except a modestly enhanced contractile function when GRK2 was specifically deleted in cardiac myocytes [63]. This study suggests that GRK2 does not play a specific role

in cardiac development, rather it might have a much more broader role in embryonic development. A specific role for GRK2 in embryonic development has not been identified yet though. Contractile response to β-adrenergic receptor and angiotensin II receptor stimulation is attenuated by myocardical overexpression of GRK2 [64]. Vascular smooth muscle (VSM) specific overexpression of GRK2 similarly attenuates β-adrenergic receptor signaling resulting in increased resting blood pressure [65].

GRK3 knockout mice exhibit supersensitivity to olfactory stimuli: GRK3 knockout mice have a normal embryonic as well as postnatal development. GRK3 deletion ($GRK3^{-/-}$) has shown that GRK3 plays a role in the regulation of olfactory receptors. Cilia preparations from $GRK3^{-/-}$ mice do not show a fast agonist induced desensitization which is normally seen after stimulation with odorants [66].

GRK4 regulates dopamine-1 receptor: Transgenic mice overexpressing GRK4 were generated whereby it was found that the transgenic mice overexpressing wild type GRK4γ showed a normal phenotype in contrast to the mice carrying a naturally occurring polymorphism A142V in GRK4γ which showed increased kinase activity and hypertension. The diuretic and natriuretic effects of D1 agonist were also found to be impaired in these mice suggesting that GRK4 regulates dopamine-1 receptor in kidney [67].

GRK5 is required for M2 muscarinic receptor desensitization: GRK5 deletion ($GRK5^{-/-}$) caused an increase in cholinergic responses such as hypothermia, hypoactivity, tremor, salivation and antinociception [68]. Furthermore, central M2 muscarinic receptors have been found to be resistant to desensitization in the absence

of GRK5. It was also found that β2-adrenergic receptor induced airway smooth muscle relaxation was reduced in GRK5$^{-/-}$ mice [69]. Transgenic mice with cardiac specific overexpression of GRK5 show enhanced β-adrenergic receptor desensitization in comparison to non-transgenic control mice. However, there was no change in the contractile response to angiotensin II receptor stimulation [70]. Vascular smooth muscle (VSM) specific overexpression of GRK5 showed an increase in blood pressure which was also found to be gender dependent i.e. male mice showed a much larger increase in blood pressure as compared to that in female mice [71]. However, unlike the VSM specific overexpression of GRK2, overexpression of GRK5 in VSM did not cause vascular or cardiac hypertrophy.

GRK6 plays an essential role in desensitization of chemokine receptors: T cells from GRK6 knockout mice (GRK6$^{-/-}$) show an impaired chemotactic response to CXCL12 (ligand for CXC chemokine receptor 4 CXCR4) [72] whereas neutrophils from the same mice showed an enhanced chemotactic response to CXCL12 [73] as well as to leukotriene B4. Furthermore, acute migration of neutrophils from the bone marrow in response to G-CSF has been found to be impaired suggesting that this might be due to CXCR4-mediated retention of neutrophils in the bone marrow [73] (**Table 2.2**).

2.2.2: Pathological roles of GRKs

Change in GRKs expression and activity have been found in several pathologies suggesting an important role of GRKs in particular disease conditions. Reduced response to β-receptor agonist and a loss of cardiac contractility in human

chronic heart failure has been shown to be linked to an increased expression of GRK2 and reduced expression of β1 receptor [74, 75]. Furthermore, the activity as well as the expression of GRK2 in lymphocytes is significantly enhanced in hypertensive subjects as compared to normotensive controls [76]. This increase negatively correlates with β-agonist stimulated lymphocyte adenylyl cyclase activity [77]. Reduced adenylyl cyclase activity correlates with a reduced vasodilator response to β-adrenergic agonist stimulation of vascular smooth muscle receptors in hypertensive state [78].

A significant decrease in expression as well as activity of GRK2 and GRK6 has been shown in peripheral blood mononuclear cells (PBMCs) of patients with rheumatoid arthritis as compared to those from healthy individuals [79]. This reduced expression and activity of GRKs correlates with an increased sensitivity to β2-adrenergic stimulation as β-agonist induced cAMP production has also been found to be increased in leucocytes from these patients. Furthermore, it was found that beta blockers and receptor antagonists for a chemokine, MCP1 (Monocyte Chemoattractant Protein 1) can reduce the severity of disease in chronic arthritis [80, 81]. This is important since chemokine receptors which fall under classical GPCRs, as well as other GPCRs such as those for prostaglandins and substance P play an important role in inflammation in Rheumatoid arthritis. This suggests that cell type specific decrease in GRKs activity might be responsible for sustained activation of G-protein coupled pro-inflammatory receptors and hence defects in cellular metabolism. Rat models of

Table 2.2: Phenotypic characteristics of GRK Knockout/Transgenic mice

Modified from:

Premont, RT and Gainetdinov, RR. Physiological roles of G-protein coupled receptor kinases and arrestins. *Annual Review of Physiology* 2007 69:511-534

Metaye, T., Gibelin, H., Perdrisot, R., Kraimps, GL. Pathophysiological roles of G-protein coupled receptor kinases. *Cellular Signaling* 2005 17:917-928

Knockout	Phenotype
GRK1	• Prolonged response and light-induced apoptosis in rods • Slow resensitization in cones
GRK2 double allele deletion	• Embryonic lethal, thin myocardium syndrome in embryos
GRK2 single allele deletion	• Enhanced myocardial contractile response to a β-agonist • Enhanced chemotaxis of T-lymphocytes • Altered progression of experimental autoimmune encephalomyelitis
GRK2 cardiac specific deletion	• Enhanced inotropic sensitivity to isoproterenol
VSM specific overexpression of GRK2	• VSM βAR signaling attenuated • Diminished βAR mediated dilation • High blood pressure
VSM specific deletion of GRK2	• Enhanced βAR mediated dilation • Does not rescue high blood pressure • Increased α_{1D} AR stimulation
GRK2 ablation in myeloid cells	• Earlier onset of HI (Hypoxia-Ischemia) brain injury
GRK3	• Enhanced airway response to a cholinergic agonist • Loss of kappa opioid tolerance • Lack of olfactory receptor desensitization
GRK4	• Normal fertility and sperm function. No obvious phenotype
GRK5	• Excessive opposition of airway smooth muscle relaxation after β-agonist administration • Enhanced hypothermia, hypoactivity, tremor, and salivation by oxotremorine
GRK5 myocardial overexpression	• Attenuation of contractility and heart rate in response to a β-agonist
GRK6	• Enhanced locomoter- stimulating effect of cocaine and amphetamine • Impaired chemotaxis of T-lymphocytes • Enhanced chemotaxis of bone-marrow-derived neutrophils and impaired acute neutrophil mobilization in response to G-CSF • Altered central dopamine receptor regulation

arthritis also showed that changes in GRK2 and GRK6 levels occur after induction of arthritis. GRK2 has also been found to be reduced in platelets from patients with depression whereby treatment with antidepressants has been found to cause an upregulation in GRK2 levels [82]. Lung homogenates from Cystic fibrosis patients show a decreased β-agonist stimulated adenylyl cyclase activity and a simultaneous increase in GRK activity as well as mRNA and protein for both GRK2 and GRK5 [83] suggesting that an increase in GRKs might be responsible for alteration in β2-adrenergic receptor density.

2.3: GRKs and inflammation

GRKs play an important role in inflammation and disease. Firstly, GRK2, 3, 5 and 6 have been found to be expressed at particularly high levels in immune cells [84] and their levels in immune cells are dynamically regulated in response to inflammation suggesting an important role for these kinases in immune activity. Secondly, targeted deletion of GRKs *in vivo* affects the progression of various acute as well as chronic inflammatory diseases again suggesting an important role for GRKs in disease. Furthermore, chemokine receptor signaling has also been shown to be tightly regulated by GRKs under *in vitro* conditions suggesting that GRKs play an important role in inflammation.

2.3.1: GRKs expression and inflammation: Mak *et al.*, showed an increased expression of GRK2 and GRK5 in the lungs of rats treated with IL-1β, a pro-

inflammatory cytokine, and this effect was found to be abolished when the rats were treated with an anti-inflammatory steroid, dexamethasone [85]. Pro-inflammatory cytokines as well as oxygen radicals have also been found to reduce the levels of GRK2 under *in vitro* conditions [79, 86]. LPS has been shown to downregulate chemokine induced expression of GRK2 and GRK5 in neutrophils (PMNs) [87]. Moreover, pro-inflammatory cytokines such as IL-1, TNF-α and IFN-γ regulate GRK2 expression at the transcriptional level by regulating the GRK2 promoter [88]. In one study, our lab found that stimulation of mouse peritoneal macrophages with ligands for TLR2, TLR3, TLR4 and TLR7 caused an upregulation in the levels of GRK2. Conversely, stimulation of mouse peritoneal macrophages with ligands for TLR2, TLR4 and TLR7 caused a significant decrease in the GRK5 and GRK6 protein levels [89]. In another study, it was found that Lipoteichoic acid (LTA), a known TLR2 agonist, increased the expression of GRK2 in neutrophils. This corresponded with a downregulation in the expression of chemokine receptor CXCR2 in neutrophils in sepsis and an impaired migration of neutrophils into an infectious focus *in vivo* and reduced chemotaxis *in vitro* [90]. These studies suggested that GRKs have an important role in inflammation since TLRs are important mediators of inflammatory response.

2.3.2: GRKs deletion and inflammation: Heterozygous GRK2 knockout mice ($GRK2^{+/-}$) have been found to have an advanced onset of multiple sclerosis with increased numbers of inflammatory cells in the spinal cord as compared to control mice [91]. However, overexpression of GRK2 in vascular smooth muscle cells has

been found to induce hypertension and cardiac hypertrophy [65]. Another finding shows that arachidonic acid induced acute ear inflammation but not PMA-induced inflammation increased substantially in $GRK6^{-/-}$ and $GRK6^{-/+}$ mice suggesting a specificity towards an agent causing inflammation rather than a general effect of GRK6 deficiency on the chemotactic activity of neutrophils [92].

2.3.3: GRKs regulation of chemokine signaling and inflammation: GRKs can also regulate chemokine responses in inflammation. Increased signaling by chemokine receptors plays an important role in disease pathology, regulating the migration of neutrophils, monocytes and T-cells. In this regard, T-cells from $GRK2^{+/-}$ mice have been found to have enhanced CCR5 agonist induced calcium mobilization, PKB and ERK1/2 signaling and migration towards CCR5 agonist under *in vitro* conditions [93] Spleenocytes from $GRK2^{+/-}$ mice were also found to have an enhanced CCR2 mediated signaling to ERK1/2 [58].

Hence, it is clear that the alterations in expression and activity of GRKs as well as the role of GRKs in regulating chemokine mediated responses play an important role in inflammatory diseases and should be taken into account when developing therapeutic strategies for such conditions (**Table 2.3**).

2.4: LPS and TNFα induced inflammation

2.4.1 LPS induced inflammation

Table 2.3: Involvement of GRKs in diseases

Slightly modified from

Metaye, T., Gibelin, H., Perdrisot, R., and Kraimps, J.L. (2005) Cellular signaling 17(8), 927-928

GRK	Disease	GRK modification
GRK1	Oguchi disease	Genetic mutation associated with a loss of GRK activity
GRK2	Chronic heart failure	Increased expression of mRNA and protein
GRK4γ	Hypertension	Genetic variants with a marked stimulation of GRK4γ activity
GRK2	Hypertension	Increased protein expression
GRK2/GRK6	Rheumatoid arthritis	Decreased protein expression
GRK2/GRK6	Opiate addiction	Decreased protein expression
GRK2	Differentiated thyroid carcinoma	Increased enzymatic activity
GRK2/GRK4γ/δ	Ovarian cancer	Increased protein expression
GRK3	Hyperfunctioning thyroid nodule	Increased protein expression
GRK2	Depression	Increase in membrane fixation
GRK3	Bipolar disorder	Single nucleotide polymorphism in the promoter region of GRK3
GRK2/GRK5	Cystic fibrosis	Increased expression of mRNA and protein
GRK2/GRK3/ GRK5	Left ventricular overload diseases	Increased expression of mRNA and protein
GRK2/GRK6	Cardiopulmonary bypass in cardiac diseases	Decreased protein expression

The mammalian innate immune system defends against bacterial or viral infection and essentially forms the first line of host defense. Toll like receptors (TLR) are crucial components of the innate immune system, and recognize a wide variety of microbial products known as pathogen associated molecular patterns (PAMPs) present in bacteria, viruses and other pathogens. Of the 10 human TLRs, the best characterized is TLR4, which recognizes gram negative bacterial outer membrane structural component known as lipopolysaccaride (LPS) activating immune cells such as monocytes and macrophages leading to the induction of endotoxic shock in mammals.

2.4.1.1: Toll like receptor 4 (TLR4) signaling

TLR4 works in concert with a coreceptor protein CD14 plus a secreted protein MD2 to transmit cell signals whereby CD14 is required for presentation of LPS to TLR4-MD2 [94]. Formation of LPS–TLR4-MD2 complex is required for the activation of downstream signals. LPS recognition causes TLR4 to undergo oligomerization and recruitment of downstream adapter proteins through interaction with the TIR domain (Toll-interleukin-1 receptor). The TIR domain is highly critical for signal transduction since it has been found that a single point mutation in the TIR domain can abolish the response to LPS [95]. TLR4 utilizes five TIR domain containing adapter proteins. These are MyD88 (Myeloid differentiation primary response gene 88), TIRAP (TIR domain-containing adapter protein, also known as Mal or MyD88-adapter-like), TRIF (TIR domain- containing adapter inducing IFN-β), TRAM (TRIF-related adapter molecule), and SARM (Sterile alpha and HEAT-armadillo motifs-containing protein) [96].

TLR4 signaling has been divided into MyD88 dependent and MyD88-independent (TRIF-dependent) pathways. Under MyD88 dependent signaling, upon LPS stimulation, MyD88 activates a death domain-containing kinase IRAK-4 (IL-1 receptor-associated kinase-4). IRAK-4 leads to the activation of TRAF6 (TNF receptor associated factor 6). TRAF6 activates TAK1 (Transforming growth factor-B-activated kinase 1) which then activates IKK (IκB kinase) and MAPK pathways [97, 98]. IκB kinase finally leads to the activation of NFκB which controls the expression of pro-inflammatory cytokines and chemokines as well as various other genes involved in innate and adaptive immunity. Activation of MAPK causes induction of AP-1, another transcription factor which is also involved in the expression of pro-inflammatory cytokines and chemokines [99]. It has been found that NFκB and MAPK are activated through MyD88 independent pathway as well.

MyD88 independent signaling is carried on by TRIF which is another TIR-domain containing protein. MyD88-independent pathway also leads to the activation of NFκB, however, this activation occurs later than the MyD88-dependent activation. It was shown by Covert et al. that NFκB activation by MyD88-independent pathway requires protein synthesis which leads to a delay in activation. It was later discovered that TRIF-dependent pathway activates TNFα expression and secretion in an NFκB-independent manner through a TRIF-dependent pathway specific transcription factor IRF3. The secreted TNFα then binds onto its receptors and activates NFκB. This suggested that the activation of NFκB by TRIF-dependent pathway occurs by a secondary response through TNFα, thus resulting in an autocrine pathway for delayed NFκB activation [100] (**Fig. 2.2**).

LPS challenge leads to a quick production of TNFα peaking at 1.5 hours. Excessive production of pro-inflammatory cytokines such as TNFα not only enhances immune responses required for fighting against invading pathogens but can also produce deleterious effects that perturb regular hemodynamic and metabolic balances in the system. TNFα is produced in very high levels during the early phase of the response to LPS induced endotoxemia. TNFα in turn can affect the expression levels of TLR4 suggesting some feedback regulation [101].

2.4.2 TNFα-a potent pro-inflammatory cytokine

Tumor necrosis factor-alpha (TNFα) is a pro-inflammatory cytokine primarily secreted by activated macrophages and monocytes. It is expressed as a 26kDa type II membrane-bound protein that self-associates into a bioactive homotrimer [102, 103] Normally TNFα is kept in balance by other anti-inflammatory factors. But in case of inflammation, this balance is shifted and TNFα levels increase which in turn cause upregulation of adhesion molecules on the endothelium (such as ICAM1 and VCAM1) and cause stimulation of fibroblasts leading to their proliferation, and recruitment of leukocytes to the site of inflammation. TNFα also stimulates the production and release of other cytokines and chemokines from macrophages.

TNFα mediates its diverse effects through two cell surface receptors: p55TNFR1 and p75TNFR2. TNFR1 is constitutively expressed on almost all nucleated cells whereas TNFR2 is expressed mainly on immune and endothelial cells [104]. The TNFR1 contains a death domain (DD) which is lacking in TNFR2 [105].

Figure 2.2: LPS activates NFκB and MAPK pathways

Stimulation of TLR4 receptor by LPS causes TLR4 to dimerize and recruit MyD88. Myd88 binds IRAK4 which in turn phosphorylates IRAK1. IRAK1 binds TRAF6 which in turn binds a preformed complex bound to the membrane consisting of TAB1/TAK1/TAB2. Active TAK1 then phosphorylates and activates the IKK complex which in turn phosphorylates and activates IκBα which undergoes proteasomal degradation releasing NFκB subunits (p50 and p65) which then translocate to the nucleus to initiate gene transcription. LPS also activates MAP kinases namely c-Jun N-terminal kinase (JNK), p38 and ERK1/2 MAP kinase.

In macrophages, however, stimulation with LPS leads to IKKβ mediated phosphorylation and degradation of NFκB1 p105 releasing associated p50 subunit which translocates to the nucleus to modulate gene transcription. p105 degradation also liberates the associated MAP3 kinase (TPL-2) which then activates ERK kinase. "Images in this dissertation are presented in color".

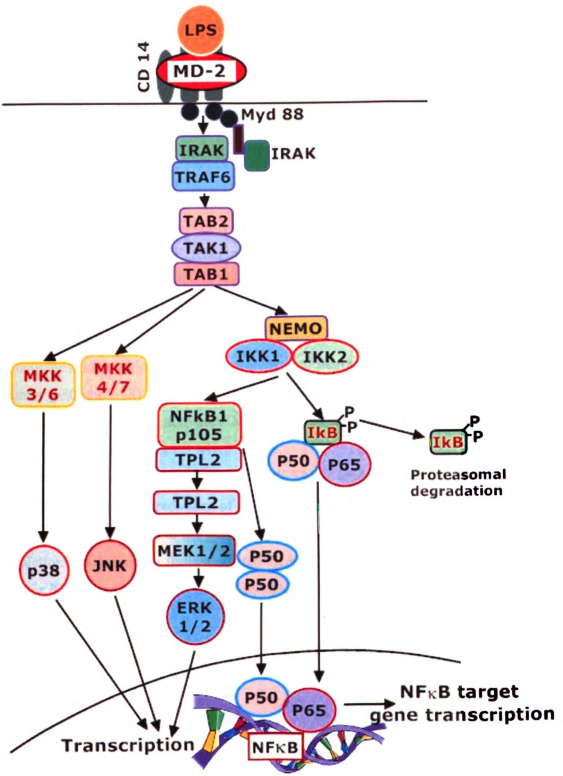
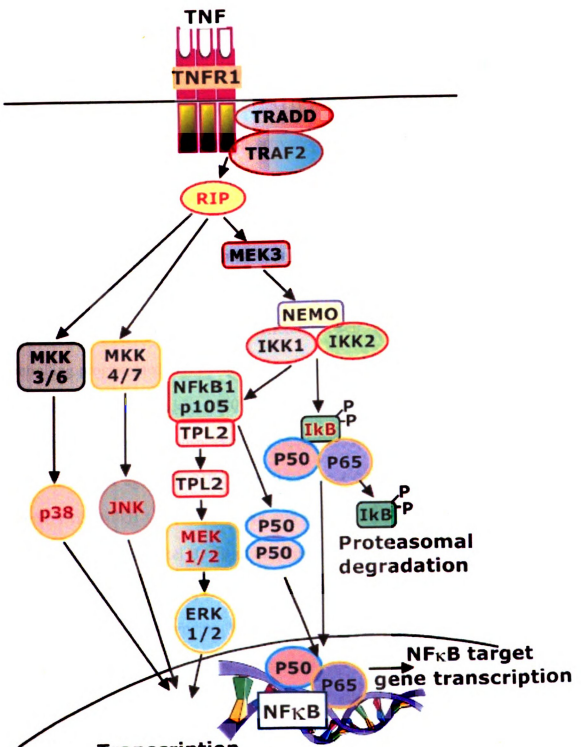

LPS
CD 14
MD-2
Myd 88
IRAK
IRAK
TRAF6
TAB2
TAK1
TAB1
NEMO
IKK1
IKK2
MKK 3/6
MKK 4/7
NFkB1 p105
TPL2
TPL2
MEK1/2
ERK 1/2
p38
JNK
IkB
P
P
P50
P65
IkB
P
P
Proteasomal degradation
P50
P50
P50
P65
NFκB
NFκB target gene transcription
Transcription

Figure 2.3: TNFα stimulates NFκB and MAPK pathways

Binding of TNFα onto TNF receptor causes receptor trimerization, which facilitates recruitment of adapter protein TRADD. TRADD leads to the recruitment of RIP1 to the signaling complex. RIP1 acts as an adaptor that facilitates recruitment of IKK complex to TNFR. IKKβ then phosphorylates and activates IκBα which undergoes proteasomal degradation releasing NFκB subunits (p50 and p65) which then translocate to the nucleus to initiate gene transcription. TNFα also activates MAP kinases namely c-Jun N-terminal kinase (JNK), p38 and ERK1/2 MAP kinase.

In macrophages, however, stimulation with TNFα leads to IKKβ mediated phosphorylation and degradation of NFκB1 p105 releasing associated p50 subunit which translocates to the nucleus to modulate gene transcription. p105 degradation also liberates the associated MAP3 kinase (TPL-2) which then activates ERK kinase. "Images in this dissertation are presented in color".


TNF
TNFR1
TRADD
TRAF2
RIP
MEK3
NEMO
IKK1
IKK2
MKK 3/6
MKK 4/7
NFkB1 p105
TPL2
IkB
P
P
P50
P65
TPL2
IkB
P
P
p38
JNK
MEK 1/2
P50
P50
Proteasomal degradation
ERK 1/2
NFκB target gene transcription
P50
P65
NFκB
Transcription

Stimulation of TNFR1 leads to the recruitment of DD-containing adapter molecule, Tumor necrosis factor receptor 1 associated death domain protein (TRADD) [106] followed by binding of DD-containing Ser/Thr kinase, Receptor interacting protein-1 (RIP1) [107]. This signaling complex is then bound by other adapter proteins such as TNF receptor associated factor 2/5 (TRAF2/5) and Inhibitor of apoptosis 1 (c-IAP1) which subsequently leads to the activation of NFκB and MAPK pathways **(Fig. 2.3)** [108].

2.5: NFκB transcription factor in inflammation

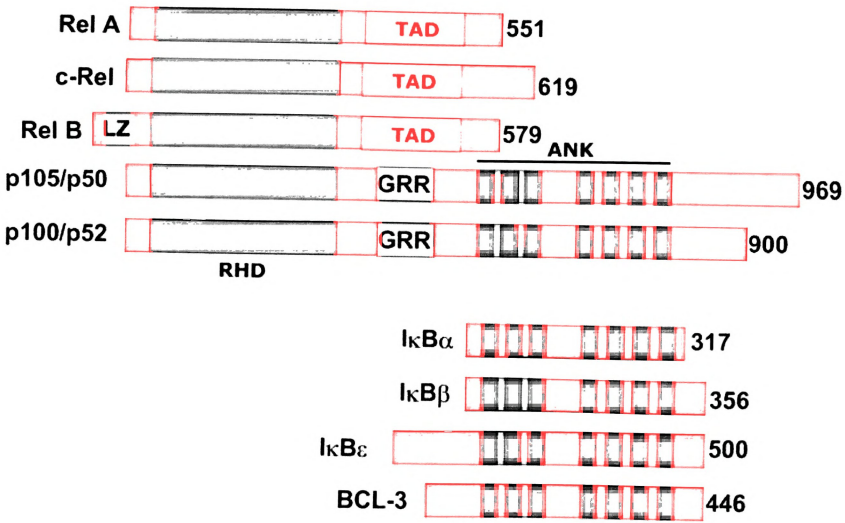
NFκB regulates transcription of genes involved in inflammation, the innate and adaptive immune responses, cell proliferation, cell adhesion, genes involved in controlling programmed cell death (apoptosis), and genes involved in cellular stress response and tissue remodeling [109-113]. It was discovered as a transcription factor that binds to the intronic enhancer of the kappa light chain gene (the κB site) in B cells around 20 years ago. The NFκB family of transcription factors consists of five members viz. NFκB-1 (p105 precursor and p50), NFκB2 (p100 precursor and p52), Rel A (p65), c-Rel and Rel B. All of these possess a Rel homology domain (RHD) at the N-terminus which is required for homo and hetero dimerization as well as for sequence specific DNA binding. Rel A, Rel B and cRel also contain a transcription activation domain (TAD) at their C-terminus which is absent in the p50 and p52 subunits. Hence, p50 and p52 interact with other factors to positively regulate transcription. NFκB is sequestered in the cytoplasm in the form of an inactive complex with IκB family of inhibitory protein under unstimulated conditions [114]. The IκB

family consists of three classical members viz. IκBα, IκBβ, and IκBε all of which are characterized by the presence of multiple ankyrin repeats that are important for binding to NFκB dimers and interfere with the nuclear localization signals present in the NFκB. NFκB1 (p105) and NFκB2 (p100) are synthesized as large precursors that contain RHD at their N-terminal and multiple ankyrin repeats in their C-terminal halves due to which they function as IκB like proteins. Proteolysis of the C-terminus of these precursors yields mature P50 and P52 subunits [115].

Classical or canonical pathway of NFκB activation typically involves IKKβ (subunit of IκB kinase complex)-dependent phosphorylation and subsequent 26S proteasomal degradation of IκBα, IκBβ, and IκBε. The kinetics of phosphorylation and degradation of IκBβ and IκBε are however much slower than that of IκBα **(Fig. 2.4)**. The proteasomal degradation leads to the release and translocation of NFκB (most commonly the P50-Rel A dimer) to the nucleus [116] where it causes an increased transcription of genes encoding for cytokines, chemokines, adhesion molecules (ICAM1 or Intercellular adhesion molecule 1, VCAM1 or vascular cell adhesion molecule 1, ELAM or endothelial leucocyte adhesion molecule 1), matrix metalloproteinases (MMPs), cyclo-oxygenase 2 (COX2), inducible nitric oxide synthase (iNOS) as well as inhibitors of apoptosis all of which are important part of an innate immune response [117]. As further elaborated in chapters 3 and 4, our studies both in cultured cell line as well as primary cells implicate GRK5 as an important mediator of NFκB pathway by potentially phosphorylating the same residues as that of IKKβ. These results suggest that GRK5 might be a better and alternative target to

Figure 2.4: Mammalian NFκB and IκB family members

The Nuclear factor κB (NFκB) family has five members. These are Rel A (P65), c-Rel, Rel B, p105/p50 (NFκB1) and p100/p52 (NFκB2). At the amino terminal, there is a structurally conserved Rel homology domain (RHD). RHD contains the dimerization, nuclear localization (N) and DNA-binding domains. Rel A, Rel B and c-Rel also have a carboxy terminal non homologous transactivation domain (TAD). Rel B also contains a leucine zipper motif (LZ). Inhibitor of NFκB (IκB) family has IκBα, IκBβ, IκBε and Bcl3 as members all of which have multiple ankyrin repeats (ANK). p105 and p100 contain RHDs at the amino terminus and ANK repeats at the carboxyl-terminus due to which they act like IκB proteins. P105 and p100 undergo proteolytic processing to generate p50 and p52 as NFκB proteins. The number of amino acids in each protein are shown on the right. "Images in this dissertation are presented in color".


Rel A
TAD
551
c-Rel
TAD
619
Rel B
LZ
TAD
579
ANK
p105/p50
GRR
969
p100/p52
GRR
900
RHD
IκBα
317
IκBβ
356
IκBε
500
BCL-3
446

that of IKKβ, since IκBα phosphorylation and NFkB activation is only partially affected.

However, there is a more recently discovered alternative pathway of NFκB activation which involves IKKα (subunit of IκB kinase complex)-dependent phosphorylation and degradation of P100 and subsequent activation of p52:RelB dimers [118]. Furthermore, IKKβ dependent degradation of IκBα has been found to occur within minutes whereas the kinetics of IKKα dependent degradation of p100 is slower and requires several hours.

IκBα is the best studied IκB family member and is known to regulate the classical RelA:p50 heterodimers. In response to inflammatory stimuli such as LPS or TNFα, it undergoes rapid degradation and is finally resynthesized in an NFκB dependent fashion which constitutes a negative feedback loop whereby newly synthesized IκB enters the nucleus and associates with deacetylated RelA:p50 heterodimers shuttling them back to the cytoplasm [119, 120].

LPS and TNFα act as important stimulators of NFκB signaling pathway. NFκB transcription factor is activated in tissues such as liver, lungs and spleen within 4 hours of intra-peritoneal challenge of LPS [121]. It activates more than 150 genes, most of which are pro-inflammatory in function. Deficient NFκB activation in intestinal epithelium has been linked to an increased inflammation under *in vivo* conditions suggesting that a defect in NFκB signaling can lead to immunosuppression which triggers and also maintains inflammation [122].

NFκB has been found to be highly activated in several inflammatory disease conditions such as sepsis, rheumatic diseases, inflammatory bowel disease, multiple

sclerosis etc. as well as in other diseases such as cancer and diabetes as detected by electrophoretic mobility shift assays as well as tissue staining using NFκB specific antibodies of biopsies from these patients. These changes are in turn accompanied by an increased recruitment of inflammatory cells to the tissues. Hence, although a prompt activation of NFκB is required for a good immune response, it should also be terminated properly to prevent from reaching a stage of tissue damage, organ failure and finally death.

Inhibition of NFκB activation has been shown by several studies to be effective in controlling inflammatory disease conditions [123]. Several drugs used to treat inflammatory disease conditions have effects on NFκB activity. Anti TNFα drugs have been found to be highly effective in controlling inflammation and are widely used against rheumatoid arthritis. Moreover, anti-inflammatory drugs such as corticosteroids, aspirin and other non-steroidal anti-inflammatory drugs (NSAIDS) although do not directly target NFκB, they do control the expression of genes regulated by NFκB [124].

In sepsis, inhibition of NFκB activation prevents LPS-induced iNOS and COX2 mRNA and protein expression and activity. NFκB inhibition also corrects cardiovascular functional abnormalities as well as help restore systemic hypotension in sepsis [125]. Inhibition of IKK activity also reduces cytokine production and decreases infiltration of neutrophils in lungs, liver and colon thus improving the rate of survival in polymicrobial model of sepsis [126]. Inhibition of NFκB activation further reduces LPS induced increases in microvascular permeability. In liver, inhibition of NFκB activation has been found to suppress LPS induced cytokine and adhesion

molecule expression, reduce influx of neutrophils as well as prevent LPS induced increase in microvascular endothelial permeability [127].

Although NFκB is an attractive target for therapeutic inhibition under certain disease conditions, considering its role in normal cellular physiology as well as in mounting effective immune response, its inhibition might have serious effects. Thus, although it has been suggested by several studies that inhibition of NFκB activation *in vivo* leads to reduced inflammatory responses in sepsis, it also favors apoptosis in immune cells which may lead to immunosuppression and fatal outcome in severe sepsis.

As for instance, it was found that inhibiting NFκB exacerbated acute inflammation but helped attenuate chronic inflammation in the intestinal tract. NFκB protects against epithelial cell apoptosis which was reduced leading to an aggravation of an acute inflammatory response. In chronic inflammation however the risk of epithelial cell apoptosis is absent, thus NFκB inhibition is beneficial under such conditions [128]. In another study, it was found that mice with a targeted deletion of IKKβ in myeloid cells are more susceptible to endotoxic shock due to an increased production of plasma IL-1β suggesting the possible complications that could arise from IKKβ inhibition [129]. Blockage of NFκB could also compromise normal host defenses. It was shown that mice were unable to clear opportunistic infections such as those involving Listeria monocytogenes after NFκB inhibition [130]. Another study showed that blocking NFκB pathway in LPS model of sepsis inhibited inflammatory as well as injury promoting responses thereby improving survival. On the other hand, blocking NFκB pathway in a bacterial model of sepsis inhibited host defense

responses in addition to inhibiting inflammatory and injury promoting responses. Thus the beneficial effects of inhibiting inflammatory responses would be compromised by the impairment of bacterial clearance capacity. Hence, the best approach would be to identify specific targets in the NFκB signaling pathway in different disease conditions for therapeutic targeting rather than considering global inhibition of NFκB.

2.6: MAPKs in inflammation

Cells continuously respond to signals in the extracellular environment by sensing through cell surface receptors and further transmitting the signals to the cytosol and nucleus by activating signal transduction pathways. MAPKs constitute a family of highly conserved serine-threonine kinases that play a role in several cellular processes such as cell proliferation, cell survival/apoptosis, differentiation as well as cell stress and inflammatory conditions.

2.6.1: ERK1/2 kinase

ERK1/2 is one of the three MAP kinases that is induced under inflammatory conditions by LPS as well as TNFα. Activation of ERK cascade may be responsible for monocyte and macrophage reprogramming and thus dysregulation of cytokine cascade. It has been suggested that MEK-ERK1/2 pathway is activated in a Raf-1 dependent manner by LPS as demonstrated by the inhibition of LPS induced TNFα production upon repressing Ras or Raf-1 [131]. Furthermore, inhibition of MEK in monocytes causes reduction in the LPS induced production of certain pro-inflammatory cytokines such as TNFα, IL-1, IL-8 and PGE2 which suggests that ERK plays a crucial role in inflammatory gene expression [132]. ERK inhibitors have been

found to be useful in reducing inflammation in an ear edema model in mice as well as experimental osteoarthritis model in rabbits [133].

In macrophages however, activation of ERK1/2 MAP kinase requires a serine-threonine kinase TPL2 (Tumor progression locus 2) also known as COT. TPL2 in turn is present in a complex with NFκB1 p105. Stimulation with LPS causes phosphorylation and proteasomal mediated degradation of NFκB1 p105. This releases p50 as well as TPL2. In unstimulated macrophages, TPL2 MEK kinase activity is blocked due to its association with p105. Released TPL2 functions as a MAP3 kinase which phosphorylates MAP2 kinase MEK1/2 which in turn phosphorylates and activates MAP kinase ERK1/2 [134]. It has been found that LPS upregulation of TNFα and COX2 is reduced in TPL2 deficient macrophages due to a defective ERK1/2 activation suggesting an important role of this pathway in mediating an immune response [135, 136]. Thus, in macrophages LPS stimulation of IKK complex plays a role in activating both NFκB and ERK pathways via NFκB1 p105 regulation (**Figure 2.2**). Interestingly, as described in the studies in chapter 5, GRK2 appears to be a negative regulator of this pathway in primary peritoneal macrophages. Previous studies in Raw264.7 macrophage cell line however have shown that NFκB1 p105-ERK pathway is regulated by GRK5. The implications of our current findings in primary cells are further discussed in chapter 5.

2.6.2: P38 MAPK

The p38 mitogen activated protein kinase is activated by diverse stimuli such as UV light, heat shock, certain mitogens, LPS as well as pro-inflammatory cytokines

such as IL-1 and TNFα. It has been shown that TNFα stimulation of neutrophils causes an enhanced p38-MAPK dependent phosphorylation and activation of PLA2 which is a primary regulator of arachidonate signaling [137]. Hence, p38 directly affects arachidonate signaling which can both stimulate and suppress inflammatory responses. P38 also plays a role in the production and release of cytokines such as TNFα and IL-1 [138]. P38 also plays a major role in the movement of neutrophils to inflammatory sites. P38 is involved in increasing the expression of ICAM-1 (adhesion molecule) on vascular endothelium which is required for binding of neutrophils through L-selection in the initial steps in the migratory process. Furthermore, p38 is required in the efficient generation of reactive oxygen species by NADPH oxidase activity in response to multiple agonists once the neutrophils reach their destination [139]. P38 has also been suggested to play a role in some chronic neuronal diseases such as Alzheimer's where it is found to be activated in association with the neurofibrillary tangle-bearing neurons containing the aggregated paired helical filament protein tau [140]. P38 is also required for the angiogenesis in inflammation in a murine model of collagen induced arthritis [141]. P38 is required for the upregulation of B1 bradykinin receptors. These receptors are present at very low levels in most tissues. However, they are strongly upregulated after various types of tissue injury. It acts as a potent vasodilator resulting in increased capillary permeability which causes accumulation of extracellular water bed in conditions such as acute respiratory distress syndrome [142]. Several studies have shown the role of p38 MAPK in sepsis. In one such studies, it was shown that the p38 MAPK activity was markedly increased in splenic and peritoneal macrophages and inhibition of p38

MAPK markedly improves survival in a polymicrobial ceacal ligation and puncture (CLP) model of sepsis [143].

2.6.3: JNK kinase

JNK is the third important MAP kinase that is activated under inflammatory stimuli. LPS has been shown to activate JNK kinase in THP1 monocytic and Raw 264.7 cells as well as other cell types. JNK kinase induces transcription factors namely AP-1, c-Jun, ATF-2, and Elk-1, all of which are important mediators of inflammatory gene transcription. JNK activation of AP-1 is important for synthesis of TNFα, as well as proliferation and differentiation of T-cells. JNK is found to be activated in joint synoviocytes suggesting an association to rheumatoid arthritis possibly due to an enhanced production of TNFα. However, JNK deficiency has been found to reduce the progression of experimental autoimmune encephalitis (EAE), due to an increase in the expression of an anti-inflammatory cytokine IL-10 by macrophages [144].

2.7: Septic shock

Encounter with microbes or their components such as LPS causes the body to initiate an innate immune response, the purpose of which is to defend the body against infection. Sepsis or septic shock is a very complex clinical condition which results when the normal host response to infection goes unchecked leading to tissue damage and ultimately multiple organ failure. Sepsis is a leading cause of death amongst critically ill patients [145]. In United States, it accounts for nearly 250,000 deaths annually [145]. The initial clinical features are characterized by fever, transient

hypotension, decreased urine output and thrombocytopenia which progresses into profound hypotension, coagulation abnormalities and multiple organ failure. Mononuclear cells play a key role in this process by releasing an array of pro-inflammatory cytokines and chemokines such as IL-1, IL-6, TNFα as well as other molecules such as reactive oxygen species (ROS), platelet activating factor (PAF) and nitric oxide (NO). These pro-inflammatory cytokines play an important role in sepsis by inducing a complex network of secondary responses to fight infection.

2.7.1 Animal models of sepsis

There are three primary methods of inducing sepsis in animals: a) By injection of an exogeneous toxin such as LPS, b) By altering the animal's endogenous protective barrier. This involves methods such as inducing an intestinal leakage as is done by ceacal ligation and puncture (CLP) or by colon ascendens stent peritonitis (CASP) and c) By infusing exogenous bacteria.

Endotoxin (LPS) is a component of the outer membrane of gram negative bacteria and plays an essential role in the pathogenesis of sepsis. LPS administration / injection induces systemic inflammation with increase in pro-inflammatory cytokines such as TNFα and IL-1 but without bacteremia. The CLP model involves performing a surgery whereby ceacum is ligated distal to the ileocecal valve and then punctured using a needle which leads to the release of fecal contents into the peritoneum causing polymicrobial bacteremia and sepsis. The severity of sepsis produced can be adjusted based on the length of the ligated cecum as well as the size / number of punctures. The

bacterial infusion model of sepsis can approximate introducing a single pathogen in a controlled manner thus allowing reproducible infection.

Each of these methods has its advantages and disadvantages as listed in **Table 2.4.** For example, LPS injection model is very simple and also a sterile method. Furthermore the dose of LPS can be titrated. This model can be used to mimic early sepsis as is seen in human patients where there is little hemodynamic compromise. This is very useful to study systemic and renal responses during initial phases of sepsis due to the fact that a lower dose of LPS does not cause any systemic hypotension despite decreasing glomerular perfusion. CLP model has an advantage that it shows a similar cytokine profile as is seen in human sepsis. Furthermore, multiple bacterial species are observed in circulation similar to human sepsis. The disadvantage being the strain variability and also that the standard CLP model does not develop reproducible acute lung or kidney injury as seen in human sepsis.

Despite the fact that sepsis is mostly characterized by the presence of multiple species of bacteria, human sepsis can also be caused by a single species. Bacterial infusion model can be used to study the sepsis caused by a single species as well as for mimicking pneumonia and other nosocomial infections. The single pathogen can be introduced in a controlled manner. This model essentially provides complimentary information in a pathogen specific manner.

Out of these three different models of sepsis, LPS injection or infusion has been widely used model in sepsis research. Infusion or injection of LPS induces a systemic inflammatory response that resembles several initial clinical

Table 2.4: Animal models of sepsis and their advantages verses disadvantages

Slightly modified from

Doi, K., Leelahavanichkul, A., Yuen, P.S., and Star, R.A. (2009) *The Journal of clinical investigation* 119(10), 2868-2878

Animal model	Advantage	Disadvantage
LPS injection	Simple and sterile with some similarities to human sepsis pathophysiology	Early and transient increase in inflammatory mediators more intense than in human sepsis
CLP or CASP	Early silent period; moderate and delayed peak of mediators; multiple bacterial flora	Age and strain variability; early hemodynamic period in some models
Infusion or instillation of exogenous bacteria	Early hyperdynamic state	No change in intrarenal microcirculation; need large animals; labor intensive

characteristics of sepsis although there is no bacteremia. As compared to human sepsis, LPS injection/infusion however causes an earlier induction of cytokines. The cytokine levels are also higher in this case as compared to that observed in human sepsis. However, an exception to this is meningococcal sepsis as cytokine levels observed in LPS injection/infusion model are comparable to what is seen in meningococcal sepsis [146, 147].

2.7.2 Different animal species as models for sepsis

In general small animals are preferred for sepsis research due to the fact that they can be generated as genetically similar, are relatively inexpensive and can be maintained as pathogen free. However, large animals have also been utilized for this purpose. For example, pig model has been used due to the similarity of its cardiovascular, renal and gastrointestinal anatomy and physiology to that of humans. Primates, in particular baboons are immunologically similar to humans thus making them good models to study the cytokine response[148]. There are species differences in the sensitivity to endotoxins. Moreover, within a particular species also there are differences in response based on sex, maturity levels, diet as well as estrus states. Furthermore, transgenic animal models with either deletions or overexpression of specific gene products are being widely used today to study the roles of particular genes.

2.7.3 Neutrophils and Monocyte / macrophages in sepsis

Cells of myeloid lineage are known to play an important role in sepsis. Neutrophils are the first and most abundant cells which arrive at the infection site. These cells have large stores of proteolytic enzymes as well as have a machinery to generate reactive oxygen species (ROS) and reactive nitrogen species (RNS) which can degrade internalized pathogens. Damage to the host tissues in sepsis can also occur due to premature neutrophil activation during migration and by extracellular release of cytotoxic molecules as well as amplification of acute inflammatory responses. Neutrophils constitutively favor apoptosis which is important for resolution of inflammation and cell turnover. It is important for neutrophils to undergo apoptosis soon after they kill microbes using ROS, RNS and proteolytic enzymes since delayed clearance of neutrophils can also contribute to organ injury. Apoptotic clearance of cells induces anti-inflammatory effects in tissues. Neutrophils from patients with sepsis has been shown to have a prolonged *in vitro* survival as well as increased cellular activation [149]. Although molecules such as ROS and RNS have beneficial physiologic functions such as their involvement in intracellular signaling for cytokines, redox regulation as well as defense mechanisms against pathogens, overproduction or reduced scavenging of these molecules can cause oxidative / nitrosative stress which plays a key role in enhancing sepsis [150].

2.7.4 Coagulation defect in sepsis

One of the hallmark clinical features of sepsis is the disorders of coagulation. Pro-inflammatory cytokines, in particular IL-1 and IL-6 induce coagulation during sepsis. IL-10, on the other hand regulates coagulation by inhibiting the expression of

tissue factor (TF) on monocytes. A more severe form of coagulation is Disseminated intravascular coagulation (DIC) which is seen in 30-50% of the cases. Coagulation is initiated by the microbial components by inducing the expression of tissue factor on mononuclear and endothelial cells. The tissue factor then activates a series of proteolytic signaling cascades resulting in the formation of thrombin from prothrombin ultimately generating fibrin from fibrinogen. Furthermore, high plasma concentrations of Plasminogen-activator inhibitor type-1 (PAI-1) prevent formation of plasmin from plasminogen thereby impairing the normal regulatory fibrinolytic mechanisms (breakdown of fibrin by plasmin). Also, it has been found that a state of sepsis induces downregulation of antithrombin, protein C and tissue factor pathway inhibitor, all of which are naturally occuring anticoagulants. These proteins also possess anti-inflammatory properties: For eg. activated protein C directly inhibits the production of TNFα by inhibiting the transcription factors NFκB and AP-1 in monocytes [151]. Hence, there is an increased production and reduced removal of fibrin which causes the deposition of fibrin clots in small blood vessels, impairing tissue perfusion and ultimately resulting in an organ failure. In our sepsis studies, we observed that the blood in our myeloid specific GRK2 deleted mice starts undergoing coagulation after around 12 hours of LPS injection. Complete blood count for the blood showed no significant differences except eosinophilia in GRK2 deleted mice as compared to control mice (Discussed in chapter five). However, the importance of this observation is not clear as yet.

2.7.5 Multiple organ failure

The end stage in sepsis is characterized by multiple organ failure leading ultimately to death. However, the pathogenesis of multiple organ failure is highly complex and incompletely understood. Tissue hypoxia and hypoperfusion play an important role. In addition to hypoxia, dysoxia is also seen which is characterized by a state of inability to utilize the available oxygen. Furthermore, adequate oxygenation of tissues is also compromised due to the development of tissue exudates in sepsis. Microvascular occlusion occurs due to a widespread deposition of fibrin leading to tissue hypoperfusion.

Cellular infiltration of tissues, in particular by neutrophils damage tissues by releasing lysosomal enzymes as well as superoxide derived free radicals. TNFα and some other cytokines such as IL-1 causes an increased expression of inducible nitric oxide synthase (iNOS) leading to an enhanced production of nitric oxide (NO) which contributes further to vascular instability and also causes direct myocardial depression seen in sepsis [152]. Sepsis is characterized by high cardiac output and a reduced peripheral resistance due to dilatation of systemic resistance vessels which causes a progressive systemic hypotension leading finally to organ dysfunction due to impaired organ perfusion [153]. It is believed now that multiple organ dysfunction occurs due to a combination of factors such as NO overproduction, antioxidant depletion, decreased ATP concentration, and mitochondrial dysfunction [154].

Hypothesis and Specific Aims

GRKs, in particular GRK2 and GRK5 are highly expressed in immune cells. Furthermore, several studies have established that the expression levels of GRKs change in the immune cells such as neutrophils and macrophages under inflammatory disease conditions such as rheumatoid arthritis and sepsis. However, the physiological importance of such changes in the expression levels of GRKs is not well established. To understand the physiological importance of GRKs in immune cell signaling as well as their role in inflammatory diseases, I propose the following overall hypothesis:

Hypothesis: GRKs are important regulators of inflammatory signaling in macrophages and therefore GRKs might play essential roles in the regulation of inflammatory diseases.

Each of the chapters in this dissertation is dedicated to address the following specific aims:

Aim 1: To determine the biochemical mechanisms by which GRKs regulate TNFα-induced signaling pathway in mouse macrophages

The role of GRKs in TNFα signaling was examined using siRNA mediated knockdown as well as by overexpression techniques, using cultured macrophage cell line

Aim 2: To determine the role and mechanism by which GRKs regulate inflammation in a mouse model of disease (Endotoxic shock)

Subaim 1: To study the role of GRK5 in inflammation in a GRK5 knockout mice using endotoxic shock model

GRK5 knockout mice were procured from Jackson labs and the role of GRK5 in inflammation was investigated by comparing the levels of pro-inflammatory cytokines, survivability etc. between the knockout and wt control mice

Subaim 2: To generate and characterize a myeloid cell specific GRK2 knockout mice and to study the role of GRK2 in inflammation using endotoxic shock model

A GRK2 deletion specifically in myeloid cells was achieved using a Cre-loxP technology and the role of GRK2 in inflammation was investigated in these mice by comparing the levels of pro-inflammatory cytokines, survivability etc. between the knockout and littermate control mice.

Importance:

The findings from these studies will have a great impact on our current understanding on the roles of GRKs in inflammatory signaling. The *in vivo* studies in knockout mice will further strengthen our *in vitro* findings as well as establish a previously unidentified role of GRKs in sepsis.

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CHAPTER 3

G-protein coupled receptor kinases mediate TNFα-induced NFκB signaling via direct interaction with and phosphorylation of IκBα

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ABSTRACT

Tumor necrosis factor-α (TNFα) is a multifunctional cytokine involved in the pathophysiology of many chronic inflammatory diseases. TNFα activation of the nuclear factor κB (NFκB) signaling pathway particularly in macrophages has been implicated in many diseases. We demonstrate here that G-protein coupled receptor kinase-2 and 5 (GRK2 and 5) regulate TNFα-induced NFκB signaling in Raw264.7 macrophages. RNAi knockdown of GRK2 or 5 in macrophages significantly inhibits TNFα-induced IκBα phosphorylation and degradation, NFκB activation, and expression of the NFκB-regulated gene, macrophage inflammatory protein-1β. Consistent with these results, over-expression of GRK2 or 5 enhances TNFα-induced NFκB activity. In addition, we show that GRK2 and 5 interact with IκBα via the N-terminal domain of IκBα and that IκBα is a substrate for GRK2 and 5 *in vitro*. Furthermore, we also find that GRK5 but not GRK2 phosphorylates IκBα at the same amino acid residues (Ser32/36) as that of IKKβ. Interestingly, associated with these results, knockdown of IKKβ in Raw264.7 macrophages did not affect TNFα-induced IκBα phosphorylation. Taken together, these results demonstrate that both GRK2 and 5 are important and novel mediators of a non-traditional IκBα-NFκB signaling pathway.

INTRODUCTION

Tumor necrosis factor-alpha (TNFα) is a pleiotropic cytokine secreted by immune cells, in particular by monocytes and macrophages and mediates a number of biological activities ranging from cell proliferation, differentiation and death to inflammation and innate and adaptive immune responses [1]. Importantly, TNFα has been implicated in the pathogenesis of chronic inflammatory diseases such as rheumatoid arthritis and Crohn's disease and several drugs targeting the TNFα system are already in clinical practice [2]. TNFα mediates its diverse effects through two cell surface receptors: p55TNFR1 and p75TNFR2. TNFR1 is constitutively expressed in most nucleated cells whereas TNFR2 is expressed mainly in immune and endothelial cells. Stimulation of TNFR1 leads to the recruitment of several death domain containing adapter proteins such as TRADD (Tumor necrosis factor receptor 1 associated death domain protein) and RIP1 (Receptor interacting protein-1). This signaling complex interacts with adapter proteins such as TRAF2/5 (TNF receptor associated factor 2/5) and c-IAP1 (Inhibitor of apoptosis 1) which subsequently leads to the activation of signaling pathways that regulate many of the important biological activities of TNFα [3]. Especially important from a physiological as well as pathological perspective is the major role of the "Nuclear factor κ B (NFκB)" signaling pathway in chronic inflammatory disease [4].

The NFκB family of transcription factors regulate genes involved in inflammation, innate and adaptive immune responses, cell proliferation, cell adhesion, programmed cell death (apoptosis), and cellular stress response and tissue remodeling (reviewed in [5]). The NFκB family members include p65 (RelA), p50, RelB, cRel

and p52. These NFκB transcription factors are sequestered in the cytoplasm in the form of homo- or heteromeric complexes with the inhibitory proteins of the IκB family. The IκB family members include IκBα, IκBβ, Iκβε, p105 (NFκB1), and p100 (NFκB2). Activation of the NFκB pathway typically involves the phosphorylation of an IκB member primarily by the IκB kinase (IKK) complex. The phosphorylated IκB then undergoes ubiquitination and subsequent proteolysis leading to the release and translocation of NFκB into the nucleus where it affects gene transcription (reviewed in [5]. Amongst the various IκB members, IκBα plays a critical role in the early activation of NFκB after ligand stimulation [6]. IκBα binds to NFκB subunits p50 and p65 under unstimulated conditions. Upon stimulation IκBα undergoes rapid phosphorylation by IKKβ, followed by ubiquitination and degradation. IκBα degradation releases the NFκB subunits p50 and p65, which then translocate into the nucleus to evoke NFκB-dependent gene transcription (reviewed in [5]. In addition to being physiological substrates for the IKK complex of enzymes, the IκB family members have also been shown to be substrates for other kinases [7]. In this context, we demonstrated recently that p105 is a substrate for G-protein coupled receptor kinase-5 (GRK5) and that GRK5 phosphorylation of p105 regulates Toll-like receptor-4-induced p105 phosphorylation in macrophages [8].

G-protein coupled receptor kinases (GRKs) are serine/threonine kinases originally discovered for their role in the phosphorylation of G-protein coupled receptors (GPCRs) (reviewed in [9]). The seven mammalian GRKs are divided into three subfamilies based on sequence and functional similarities. The rhodopsin kinase subfamily (GRK1 and GRK7); the GRK2 subfamily (GRK2 and GRK3) and the

GRK4 subfamily (GRK4, 5 and 6). All GRKs have a similar structural organization possessing N-terminal, catalytic and C-terminal domains. Interestingly, recent studies have shown that GRKs have functions that go beyond their role in GPCR phosphorylation. For example, GRKs have been shown to phosphorylate a number of cytoplasmic and nuclear proteins as well as additional classes of membrane-localized receptors [10-12] Moreover, yeast-two hybrid analysis identified NFκB1 p105 as a GRK2-interacting protein (http://www.signalinggateway.org/data/Y2H/cgi-bin/y2h.cgi) while we found that GRK5 but not GRK2 regulates p105 function in macrophages [8].

To better understand the role of GRKs in NFκB signaling in macrophages and to identify the potential role of these kinases in the regulation of other IκB members, we tested the role of GRK2 and 5 in the regulation of IκBα in the context of TNFα signaling in macrophages. Surprisingly, in contrast to the regulation of p105, our studies reveal that both GRK2 and 5 regulate the TNFα-induced IκBα-NFκB pathway in Raw264.7 mouse macrophages. We further demonstrate that IκBα directly interacts with GRK2 and 5 and is differentially phosphorylated by GRK2 and 5. Our observations add new insight into NFκB signaling as well as demonstrate novel GPCR-independent roles for GRKs.

MATERIALS AND METHODS:

Materials

Mouse TNFα was from PeproTech Inc. (Rocky Hill, NJ). IRDye® 700 labelled NFκB oligonucleotide probes were from LI-COR Biosciences (Lincoln, NE). Protease inhibitor cocktail tablets were from Roche Diagnostics (Indianapolis, IN). Luciferase assay buffer and substrate were from Promega (Madison, WI). All other reagents were from Sigma unless otherwise noted.

Antibodies: Anti-P50, P65, Lamin B, Protein A/G PLUS-agarose beads, Actin-HRP and polyclonal IκBα antibody were from Santa Cruz Biotechnology (Santa Cruz, CA), anti-mouse IκBα and phospho-IκBα antibodies were from Cell Signaling Technology (Boston, MA), HA monoclonal antibody was from Covance and HA polyclonal antibody from Sigma. GRK2 and 5 monoclonal antibodies were from Upstate Biotechnology.

Plasmids: pcDNAGRK2, pcDNAGRK2-K220R, pcDNAGRK5 and pcDNAGRK5-K215R have been described previously [13, 14]. HA-GRK2 was created by excising GRK2 from pRK5-GRK2 construct with EcoRI and SalI and then inserting into pHA-CMV vector. Expression plasmid pCMX-IκBα (Addgene plasmid 12331) was from Dr. I. M. Verma [15], HA-IKKβ (Addgene plasmid # 15470) from Dr. H. Nakano [16], Flag-IKKγ (Addgene plasmid # 11970) from Dr. J. D. Ashwell [17], and Flag-IκBα from Dr. G. Pei [18]. Adenoviruses expressing GRK2 and GRK5 were kindly provided by Dr. W. J. Koch.

Cell Culture

Raw 264.7 macrophages and HEK293T cells were obtained from ATCC and were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37° C in 5% CO_2.

RNA interference

Control siRNA pool (against luciferase gene not expressed in macrophages), GRK2 siRNA pool (against mouse GRK2), GRK5 siRNA pool (against mouse GRK5) and IKKβ siRNA pool (against mouse IKKβ) were purchased from Dharmacon (Dharmacon Research Inc., Lafayette, CO). Raw264.7 macrophages were transfected with siRNA using Amaxa nucleofector (Program D-032) as described previously [8]. The cells were analyzed for knockdown by Western blotting after 48 hours of transfection.

Luciferase assay

HEK293T cells were transfected with an NFκB promoter luciferase plasmid (pELAM luciferase plasmid [19, 20] kindly provided by Dr. E. Latz, University of Massachusetts) and LacZ-expression plasmid [21] (kindly provided by Dr. Philip B. Wedegaertner, Thomas Jefferson University) together with either vector (control) or GRK2/5 wild type or GRK2-K220R/GRK5-K215R kinase deficient expression plasmids. Cells were stimulated with TNFα for 8 hours, lysates prepared and analysed for NFκB luciferase activity using Promega Luciferase Reporter Assay kit according to manufacturer's instructions. β-Gal activity was determined as described previously [21]. NFκB luciferase activity was expressed as a ratio of luciferase to beta-

galactosidase activity. All experiments were performed in triplicate and repeated at least 3-5 times.

Nuclear Extracts

Nuclear extracts were prepared as described in Nuclear extraction kit (Panomics, CA). Briefly, control or GRK2 knockdown cells in a 60 mm plate were lysed in 500 µl of the buffer A mix [buffer A (100 mM HEPES, pH 7.9, 100 mM KCl, 100 mM EDTA), 100 mM DTT, 10 µl protease inhibitor cocktail and 0.38% NP-40]. Sonication was done to disrupt the cell clumps. The lysate was then centrifuged at a maximum speed (15,000 x *g*) for 3 min at 4°C. Supernatants (cytosolic fraction) were collected and the pellet suspended in 100 µl of buffer B mix [buffer B (100 mM HEPES, pH 7.9, 2 M NaCl, 5 mM EDTA), protease inhibitor cocktail and 100 mM DTT). The lysates were kept on a rocking platform for ~2 hours, after which the lysates were centrifuged at high speed for 20 min and the supernatants (nuclear fraction) were collected for further analysis.

Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assays (EMSA) were performed using nuclear extracts [6]. Briefly, ~9 fmol of double stranded oligonucleotide probes corresponding to the human consensus NFκB sequence end labeled with IRDye® 700 were incubated with 5 µg of nuclear extracts for 20 min in the dark at room temperature. Samples were then subjected to electrophoresis using 6% non-denaturing polyacrylamide gels and then analysed on LI-COR's odyssey.

Co-immunoprecipitation and Western Blotting

HEK293T cells were transiently transfected using Fugene (Roche) with the indicated expression plasmids in 60-mm cell culture dishes. The cells were washed twice with ice-cold phosphate-buffered saline after 48 hours and were lysed in 400 µl of cold lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% NP-40 containing protease and phosphatase inhibitors). Lysates were then incubated with antibody and protein A/G PLUS-agarose beads for 1 hour at 4° C. The beads were washed three times with lysis buffer and the immune complexes eluted with 2X SDS sample buffer. The eluates were then run on SDS-PAGE and transferred to nitrocellulose. Western blotting was then performed as described previously with either fluorescently tagged secondary antibodies using Licor or with HRP-conjugated secondary antibodies using chemiluminescence [22].

Glutathione S-transferase (GST) protein expression and purification

GST (GE HealthCare Biosciences, NJ), GST-IκBα and GST-IκBα(Δ1-75) [23] (kindly provided by Dr. Junan Li, Ohio State University) in pGEX bacterial expression plasmids were expressed and purified as described by the manufacturer (Amersham Biosciences). Briefly, overnight bacterial cultures were diluted 1:100 in 100 ml of fresh LB-ampicillin, grown for 2-3 hours until the OD reached 0.5-0.9. The culture was then induced with IPTG (1 mM) and incubated for another 3 hours after which the cells were pelleted (6000 x *g* for 10 min at 4° C) and resuspended in ice cold PBS. Suspended cells were then disrupted using a sonicator and solubilized in 1% Triton-X-100. The cells were then centrifuged at 12,000 x *g* for 10 min at 4° C and the supernatants collected. These supernatants were incubated with 2 ml of the 50% slurry of Glutathione Sepharose 4B equilibrated with PBS for 30 min and the resin

was transferred to a column (Empty Disposable PD-10 column). The column was washed 3 times with PBS after which the fusion proteins were eluted by adding 1 ml of elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0). The purity and integrity of the fusion proteins were analysed and confirmed by SDS-PAGE and Coomassie blue staining.

Overlay assay

Interaction between GST fusion proteins and GRK2 or 5 were determined using an overlay assay [24]. Briefly, GST-fusion proteins (5 μg) were resolved on a 10% SDS-PAGE gel and transferred to nitrocellulose membranes. Membrane was then blocked with 5% w/v fat-free milk in TBS-T and incubated overnight at 4° C in lysates (~400 μg total protein) of HEK293T cells expressing vector, HA-GRK2, or GRK5. Blots were then washed three times with TBS-T (Tris- Buffered saline-Tween 20) and immuno blotting performed using appropriate antibodies. Blots were then stained with Ponceau to confirm equivalent amount of GST-fusion protein loading.

Phosphorylation assay

In vitro phosphorylation reactions were performed using purified GRK2 and GRK5 with IκBα as a substrate [8]. Purified GST-IκBα or GST (~200 nM each) were incubated with 25 nM GRK2 or GRK5 at 30° C for 15 min in the presence or absence of 2 mg/ml soybean phosphatidylcholine and 60 nM purified Gβγ in 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 5 mM $MgCl_2$, 0.2 mM ATP, 1–2 μCi of [^{32}P]ATP in a final volume of 20 μl. Reactions were stopped by the addition of 5 μl of SDS sample buffer and incubation at room temperature for 30 min. Samples were then electrophoresed on a 10% SDS-polyacrylamide gel, the gel was dried, and ^{32}P-labeled IκBα was

visualized by autoradiography and quantified by excising the bands and scintillation counting.

Real time Q-RT-PCR

RNA extraction and real-time Q-RT-PCR were performed as described previously [22]. Briefly, total RNA was extracted using TRIzol reagent (Invitrogen). After sodium acetate–ethanol precipitation and several ethanol washes, the RNA integrity was verified by formaldehyde-agarose gel electrophoresis. Synthesis of cDNA was performed with reverse transcriptase (RT) with the total RNA using the superscript II kit with Oligo-dt (12–18) primers as recommended by the manufacturer (Invitrogen). cDNA was amplified by PCR in a final reaction volume of 25 μl using SYBR Green Supermix (Invitrogen) with 10 pmol of each primer for MIP1β and cyclophilin (for normalization) (primers obtained from IDT DNA Technologies, primer sequence available upon request). Real-time PCR was performed using MX3000P (Stratagene) thermocycler and data analyzed using MX3000P software.

Statistical analysis

All values are represented as mean±SEM. Data were analyzed and statistics performed using GRAPHPAD PRISM software (San Diego, California) using student's t-test (for comparing two groups) and ANOVA (for comparing three or more groups). P value of less than 0.05 was considered significant.

RESULTS

GRK2 mediates TNFα-induced NFκB activation in Raw264.7 macrophages

Previous studies have shown that of the seven GRKs, GRK2 and 5 are expressed at relatively high levels in immune cells including macrophages [25, 26]. Furthermore, recent studies have shown that GRKs can regulate GPCR as well as non-GPCR signaling in macrophages [8]. Because TNF receptor-induced NFκB signaling plays a major role in macrophage biology and has been implicated in many chronic inflammatory diseases [4, 27], we explored the role of GRKs in TNFα-induced NFκB signaling in macrophages. For this, we first tested the effect of GRK2 knockdown using siRNA pool, on TNFα-induced IκBα phosphorylation and degradation. Treatment of control macrophages with TNFα caused a time-dependent increase in IκBα phosphorylation (at serines-32/36) and a subsequent decrease in IκBα levels (**Fig. 3.1A**). IκBα phosphorylation was maximal at 5 min after TNFα stimulation, whereas, the consequent decrease in IκBα levels was maximal at 15 min after stimulation in control cells. However, the TNFα-mediated increase in IκBα phosphorylation and decrease in IκBα levels were both significantly blocked in GRK2 knockdown macrophages. Compared to the maximal stimulation of 95±5% at 5 min in control cells, IκBα phosphorylation reached only 40±2% in GRK2 knockdown cells (**Fig. 3.1B**). Consistent with the effects on IκBα phosphorylation, IκBα degradation was also inhibited in GRK2 knockdown cells compared to control cells. At 15 min after treatment IκBα levels reached 62±7% of untreated levels (=100%) in control cells, whereas it reached only 94±5% in GRK2 knockdown cells (**Fig. 3.1C**). Importantly, IκBα levels did not differ between control and GRK2 knockdown cells in

the absence of TNFα treatment (0.136±0.057 in control cells v/s 0.134±0.057 in GRK2 knockdown cells), suggesting that the effect of GRK2 knockdown on IκBα levels is specific for stimulated conditions.

Because IκBα phosphorylation and degradation leads to subsequent release and translocation of the NFκB subunits, primarily p50 and p65, we next examined the nuclear levels of the NFκB subunits in control and GRK2 knockdown cells before and after TNFα treatment. As with IκBα phosphorylation/degradation, TNFα-induced p50 and p65 nuclear translocation were significantly inhibited in GRK2 knockdown cells compared to control cells (**Fig. 3.1D**). These effects of GRK2 are specifically due to a decrease in nuclear translocation and not a decrease in expression levels of these proteins since the cytosolic expression of p50 and p65 did not differ between control and GRK2 knockdown cells. Associated with these findings, we also observed that the NFκB binding activity (assessed by EMSA) was inhibited in GRK2 knockdown macrophages (**Fig. 3.1E**).

Furthermore, to rule out the non-specific effects of the siRNA, we repeated these experiments using individual siRNAs and obtained similar results (**Fig. 3.2A**). Interestingly, the effect of GRK2 knockdown on IκBα was specific for TNFα-induced pathway because LPS-induced IκBα degradation was not inhibited by GRK2 knockdown (**Fig. 3.2B**).

Over-expression of GRK2 causes an increase in TNFα induced IκBα phosphorylation

To further rule out the possibility of non-specific effects of RNAi, we tested the effects of GRK2 over-expression in TNFα-induced IκBα phosphorylation in

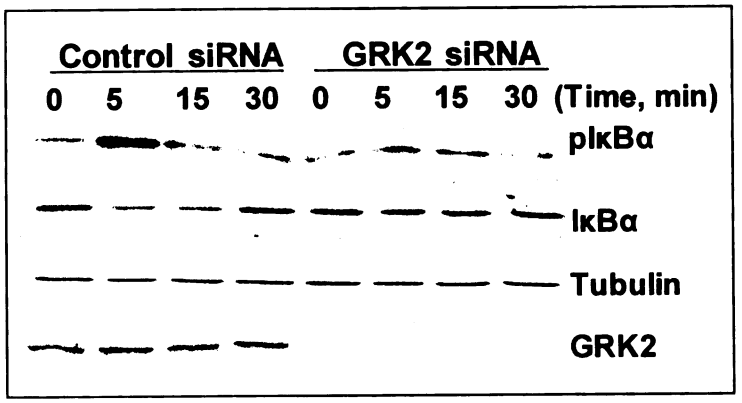
Figure 3.1: GRK2 knockdown in macrophages inhibits TNFα-induced IκBα phosphorylation and degradation

A, B and C. Raw 264.7 macrophages were transfected with either control siRNA or GRK2 siRNA using Amaxa's nucleofector. Forty-eight hours after transfection, cells were serum starved for ~3 hours, and stimulated or not with TNFα (25 ng/ml) for the indicated time points. Lysates were extracted and run on SDS-PAGE, transferred to nitrocellulose and then immunoblotted with primary antibodies against pIκBα, IκBα and tubulin. Secondary antibodies were fluorescently labeled and the blots were developed using Odyssey's Licor as described before [8]. Representative blots for pIκBα, IκBα, tubulin and GRK2 are shown in (**A**). Quantitation is shown in (**B**) and (**C**). ***$p<0.001$ compared to control; *$p<0.05$ compared to control. N=7.

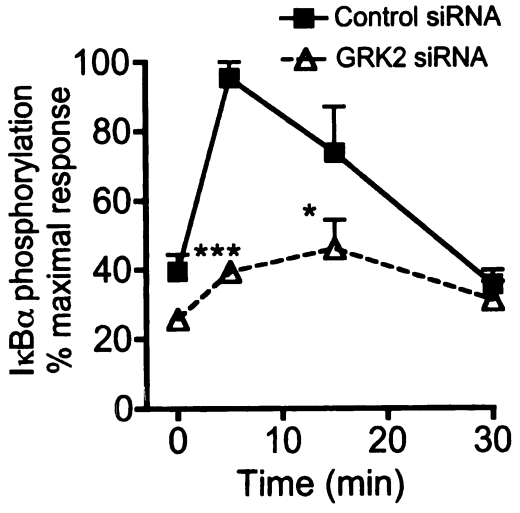
D. Raw264.7 macrophages with control or knockdown levels of GRK2 were treated with TNFα as indicated. Nuclear lysates were then extracted and immunoblotted for NFκB P50 and P65 (Rel A) as shown. Nuclear protein lamin B is shown as a loading control. A representative experiment is shown.

E. Nuclear extracts (from **D** above) were incubated with IR^{700} dye-labeled NFκB oligonucleotide probes and electrophoretic mobility-shift assay was performed as described in the Materials and Methods. Gels were scanned on Licor Odyssey (Licor Biosciences) to detect binding. A representative experiment is shown.

A.


Control siRNA
GRK2 siRNA
0 5 15 30 0 5 15 30 (Time, min)
pIκBα
IκBα
Tubulin
GRK2

B.


Control siRNA
GRK2 siRNA
IκBα phosphorylation
% maximal response
100
80
60
40
20
0
***
*
0
10
20
30
Time (min)

C.

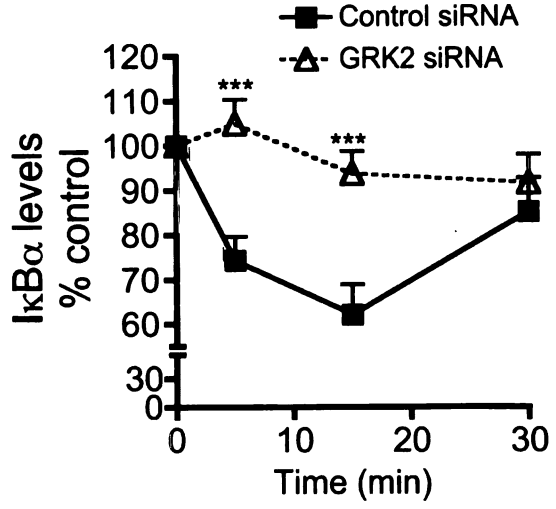
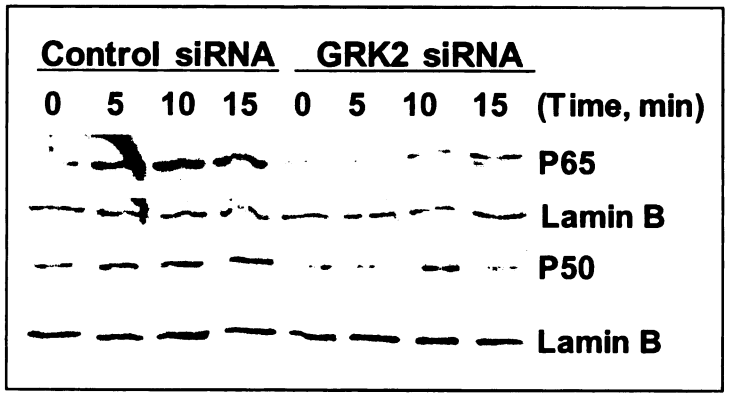

Control siRNA
GRK2 siRNA
IκBα levels
% control
120
110
100
90
80
70
60
30
0
***
***
0
10
20
30
Time (min)

Figure 3.1. Continued....

D.



E.

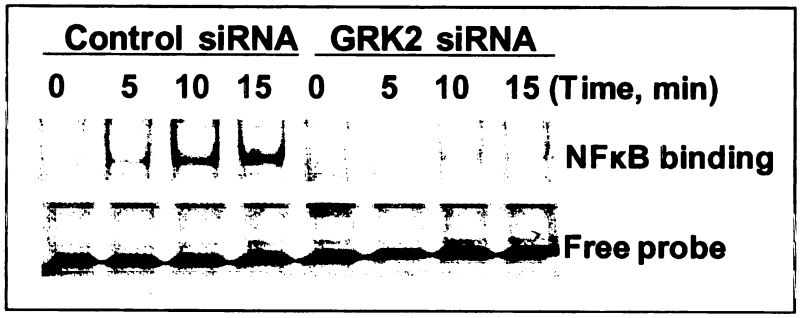
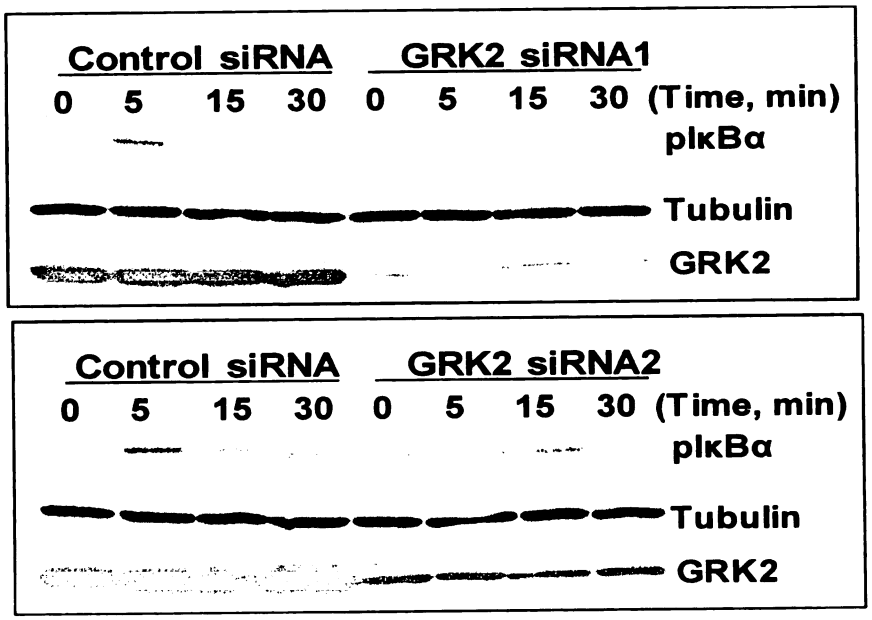


Figure 3.2: Effect of different GRK2 siRNA oligos on TNFα-induced IκBα phosphorylation

A. Raw 264.7 macrophages were transfected with either control or GRK2 specific individual oligos (siRNA GRK2 #1 **(top panel)** and siRNA GRK2 #2 **(bottom panel)** using Amaxa's nucleofector. Forty-eight hours after transfection, cells were serum starved for ~3 hours, and stimulated or not with TNFα (25 ng/ml) for the indicated time points. Lysates were extracted and run on SDS-PAGE, transferred to nitrocellulose and then immunoblotted for pIκBα and tubulin. Representative blots from three similar experiments are shown.

B. Raw 264.7 macrophages were transfected with either control siRNA or GRK2 siRNA using Amaxa's nucleofector. Forty-eight hours after transfection, cells were serum starved for ~3 hours, and stimulated or not with LPS (1μg/ml) for the indicated time points. Lysates were extracted and run on SDS-PAGE, transferred to nitrocellulose and then immunoblotted with primary antibodies against IκBα and tubulin. Secondary antibodies were fluorescently labeled and the blots were developed using Odyssey's Licor as described before [8]. Quantitation is shown as percent control.

A.


Control siRNA
GRK2 siRNA1
0 5 15 30 0 5 15 30 (Time, min)
pIκBα
Tubulin
GRK2
Control siRNA
GRK2 siRNA2
0 5 15 30 0 5 15 30 (Time, min)
pIκBα
Tubulin
GRK2

B.

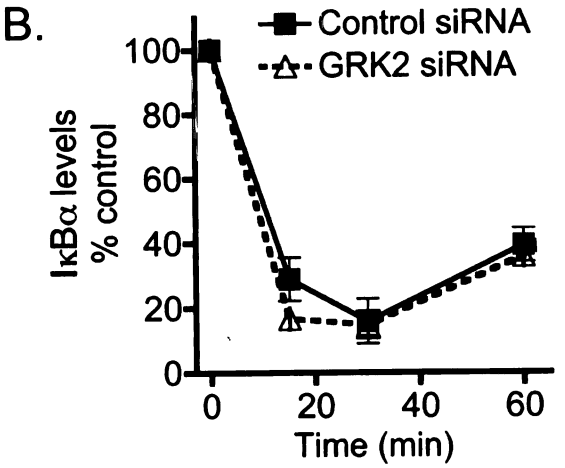
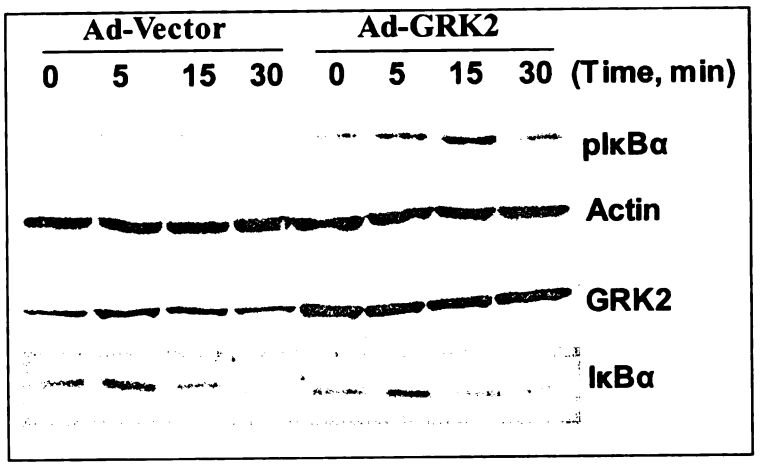

Control siRNA
GRK2 siRNA
IκBα levels
% control
100
80
60
40
20
0
0
20
40
60
Time (min)

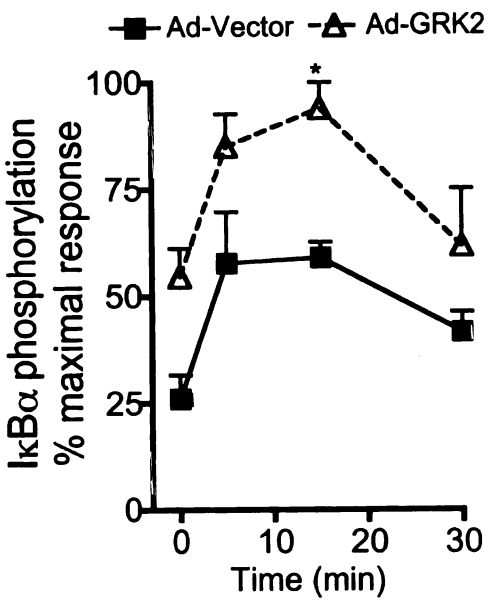
Figure 3.3: GRK2 over-expression enhances TNFα-induced IκBα phosphorylation in macrophages

Raw264.7 macrophages were infected with adenoviruses encoding empty vector or GRK2 at 50,000 viral particles/cell. Forty-eight hours after infection, cells were serum starved for 3-4 hours, and stimulated with TNFα (25 ng/ml) for indicated times. Lysates were immunoblotted for pIκBα, IκBα and tubulin. A representative blot is shown in (**A**) and quantitation in (**B**). *$p<0.05$ compared to vector. N=3.

A.


Ad-Vector
Ad-GRK2
0 5 15 30 0 5 15 30 (Time, min)
pIκBα
Actin
GRK2
IκBα

B.


Ad-Vector
Ad-GRK2
IκBα phosphorylation
% maximal response
100
75
50
25
0
*
0 10 20 30
Time (min)

macrophages. Raw264.7 macrophages were transfected with adenoviruses expressing vector or GRK2 and the effect of TNFα-induced IκBα phosphorylation tested as described earlier. As predicted, over-expression of GRK2 significantly enhanced TNFα-induced IκBα phosphorylation compared to vector controls (**Fig. 3.3**).

Role of GRK5 in TNFα-induced IκBα phosphorylation and degradation

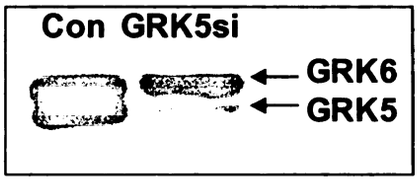
We next tested whether other GRKs can regulate IκBα phosphorylation/degradation. We especially focused on GRK5 since we previously showed that GRK5 inhibits LPS-induced p105 phosphorylation in macrophages [8]. Interestingly, similar to the effects of GRK2, knockdown of GRK5 using siRNA pool (**Fig. 3.4A**) significantly inhibited TNFα-induced IκBα phosphorylation and degradation (**Fig. 3.4B, C & D**). TNFα-stimulated IκBα phosphorylation (Ser32/36) in GRK5 knockdown cells reached only 27±5% of the maximal response after 5 min compared to 100% in control cells (**Fig. 3.4C**). Similarly, IκBα levels after 15 min of TNFα stimulation reached 62±6% of untreated levels in control cells but only 99±5% in GRK5 knockdown cells (**Fig. 3.4D**). Unlike GRK2 knockdown, basal levels of IκBα were somewhat elevated after GRK5 knockdown (0.361±0.080 in control vs. 0.563±0.071 in GRK5 knockdown cells). Similar to GRK2 siRNAs, individual siRNAs against GRK5 also gave similar results to that of the pool (**Fig. 3.5A**). Interestingly, over-expression of GRK5 using adenovirus only modestly enhanced IκBα phosphorylation (**Fig. 3.5B**).

Role of GRKs is specific for IκBα-NFκB pathway

Figure 3.4: GRK5 knockdown inhibits TNFα-induced IκBα phosphorylation and degradation in macrophages

Raw 264.7 macrophages were electroporated with either control or GRK5 siRNA as indicated. Forty-eight hours after electroporation, cells were serum starved for ~3 hours, and stimulated with TNFα (25 ng/ml) for the indicated times. Lysates were immunoblotted for pIκBα, IκBα and tubulin and blots developed using Licor's Odyssey. Representative blots for GRK5 knockdown is shown in (**A**), pIκBα and IκBα in (**B**). Quantitation for IκBα phosphorylation and IκBα degradation are shown in (**C**) and (**D**) respectively. ***$p<0.001$ compared to control. N=6.

A.


Con GRK5si
GRK6
GRK5

B.

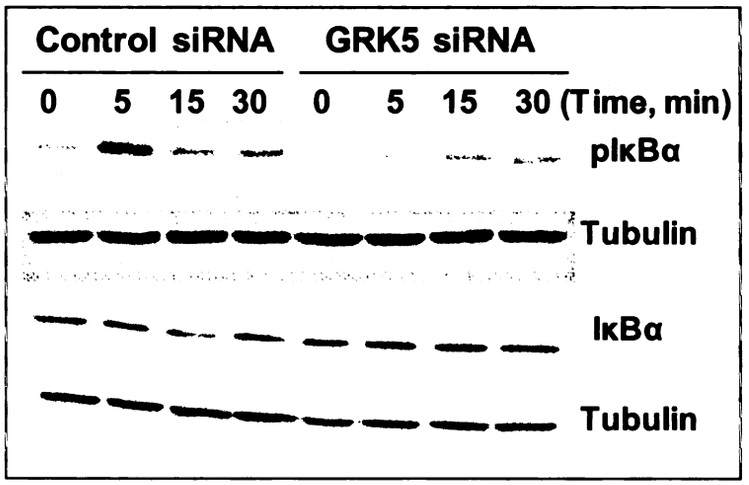

Control siRNA
GRK5 siRNA
0 5 15 30 0 5 15 30 (Time, min)
pIκBα
Tubulin
IκBα
Tubulin

Figure 3.4. Continued....

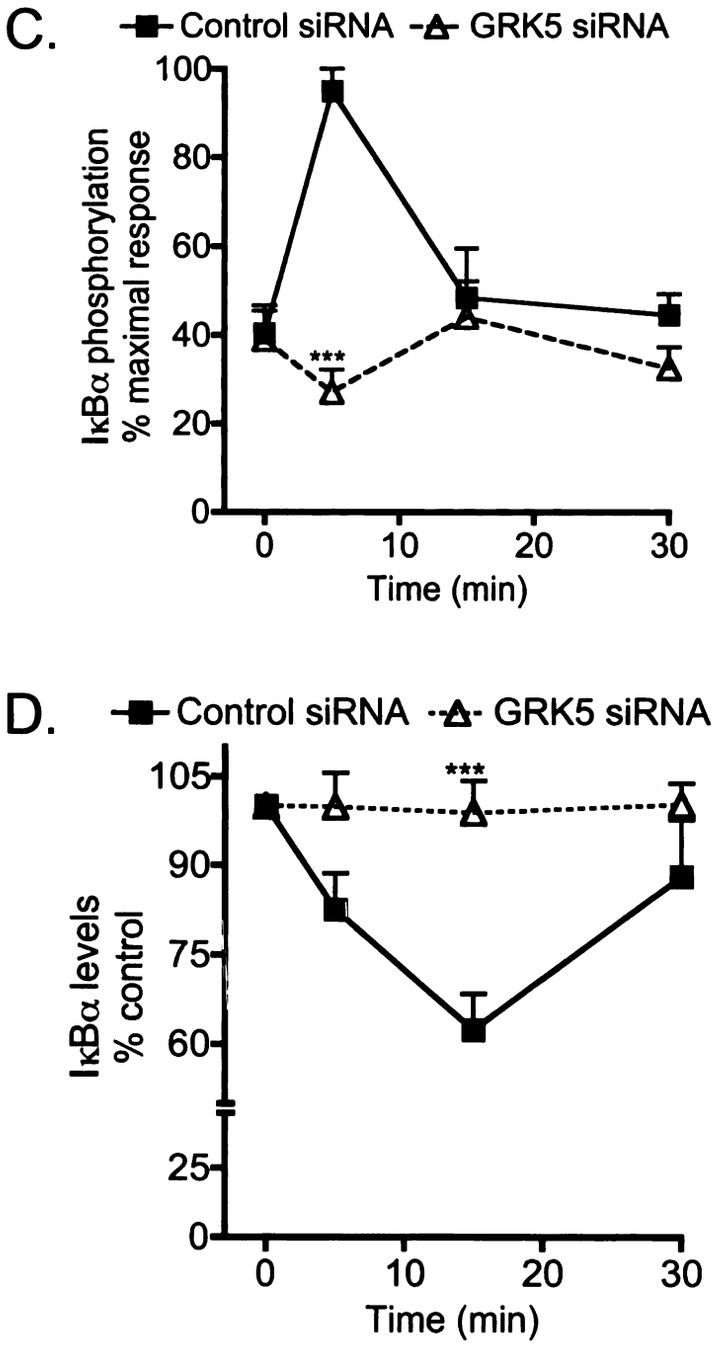
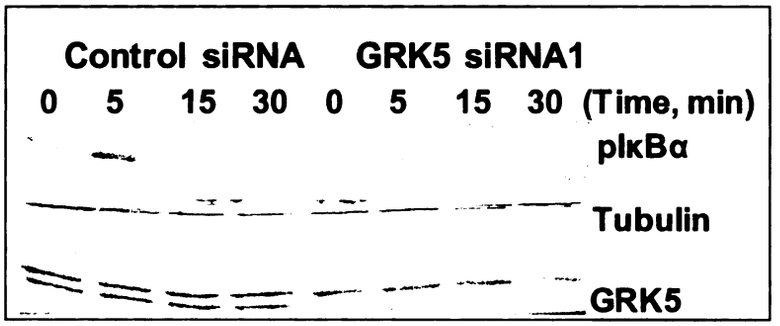


Figure 3.5 Effect of a different GRK5 siRNA oligo on TNFα-induced IκBα phosphorylation

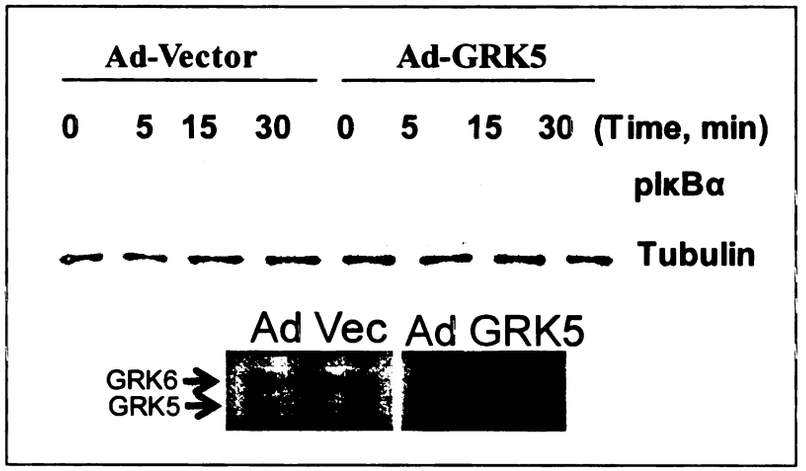
A. Raw 264.7 macrophages were transfected with either control or GRK5 specific individual oligo (siRNA GRK5 #1) using Amaxa's nucleofector. Forty-eight hours after transfection, cells were serum starved for ~3 hours, and stimulated or not with TNFα (25 ng/ml) for the indicated time points. Lysates were extracted and run on SDS-PAGE, transferred to nitrocellulose and then immunoblotted for pIκBα and tubulin. Representative blots from three similar experiments are shown.

B. Raw264.7 macrophages were infected with adenoviruses encoding empty vector or GRK5 (kindly provided by Dr. W. Koch, Thomas Jefferson University) at 1000 viral particles/cell. Forty-eight hours after infection, cells were serum starved for 3-4 hours, and stimulated with TNFα (25 ng/ml) for indicated times. Lysates were immunoblotted for pIκBα, and tubulin. A representative blot from two such experiments is shown.

A.


Control siRNA
GRK5 siRNA1
0 5 15 30 0 5 15 30 (Time, min)
pIκBα
Tubulin
GRK5

B.


Ad-Vector
Ad-GRK5
0 5 15 30 0 5 15 30 (Time, min)
pIκBα
Tubulin
Ad Vec
Ad GRK5
GRK6
GRK5

In addition to IκBα phosphorylation, TNFα treatment also induces NFκB1 p105 (another member of the IκB family) phosphorylation at Ser932 [28]. Previous studies have shown that GRK2 and 5 can interact with p105 and that GRK5 negatively regulates LPS-induced p105 phosphorylation [8]. To determine whether the observed effects of GRK2/5 knockdown are specific for TNFα-induced IκBα phosphorylation or whether p105 phosphorylation can also be regulated in a similar manner, we examined TNFα-induced p105 phosphorylation in control and GRK2/5 knockdown macrophages. Interestingly, unlike IκBα phosphorylation, GRK2/5 knockdown did not affect TNFα-induced p105 phosphorylation (at Ser932) (**Fig. 3.6A and 3.6B**). This suggests that the role of GRK2/5 is specific for TNFα-induced IκBα-NFκB pathway.

Regulation of NFκB-dependent gene expression by GRK2 and 5 in macrophages

IκBα-NFκB pathway was recently shown to be critical for the expression of MIP1β (macrophage inflammatory protein 1β), one of the major chemokines expressed by macrophages [29]. Therefore, we tested whether the role of GRK2 and 5 on the TNFα-induced NFκB pathway extended downstream of NFκB activation to MIP1β mRNA expression. Treatment of control macrophages with TNFα induced MIP1β mRNA expression by ~4-5-fold. This increase in MIP1β expression was significantly blocked in both GRK2 and 5 knockdown macrophages (**Fig. 3.7A and 3.7B**). These results demonstrate that GRK2 and 5 regulate TNFα-induced IκBα-NFκB pathway as well as its physiological gene expression target in macrophages.

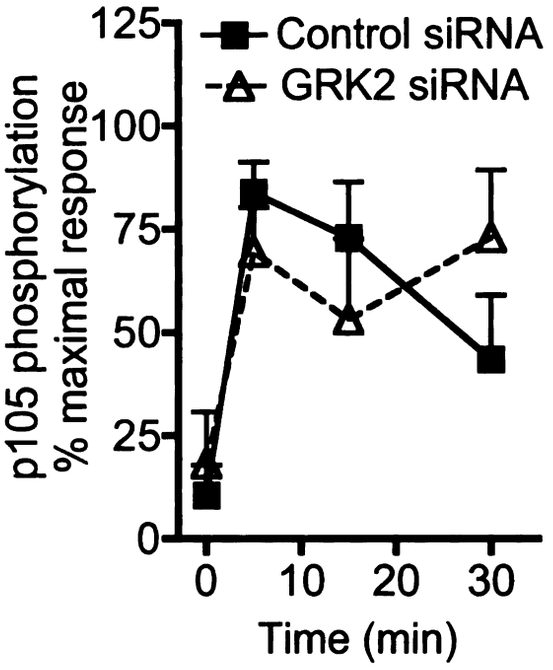
Kinase activity of GRK2 and 5 is required for TNFα-induced NFκB-dependent gene transcription

Figure 3.6 Role of GRKs is specific for IκBα-NFκB pathway

A. Raw 264.7 macrophages were transfected with either control siRNA or GRK2 siRNA using Amaxa's nucleofector. Forty-eight hours after transfection, cells were serum starved for ~3 hours, and stimulated or not with TNFα (25 ng/ml) for the indicated time points. Lysates were extracted and run on SDS-PAGE, transferred to nitrocellulose and then immunoblotted with primary antibody against phospho-p105. For quantitation, p-p105 bands were normalized as described before [8]. Quantitation is shown as percent maximal response. N=4.

B. Raw 264.7 macrophages were transfected with either control siRNA or GRK5 siRNA using Amaxa's nucleofector and treated as described above in **A.** Quantitation is shown as percent maximal response. N=4.

A.


125
100
75
50
25
0
Control siRNA
GRK2 siRNA
p105 phosphorylation
% maximal response
0
10
20
30
Time (min)

B.

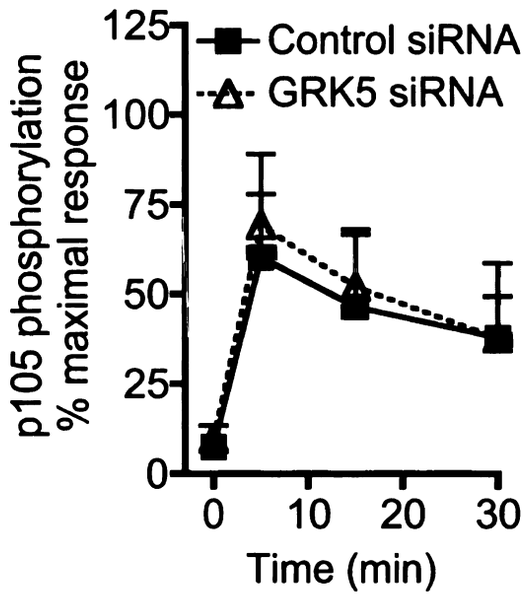
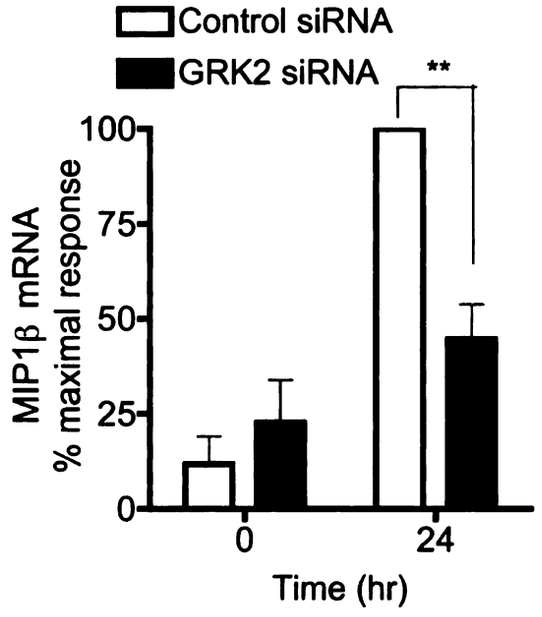

125
100
75
50
25
0
Control siRNA
GRK5 siRNA
p105 phosphorylation
% maximal response
0
10
20
30
Time (min)

Figure 3.7 Expression of Macrophage inflammatory protein-1β (MIP1β), an NFκB-regulated gene, is inhibited by GRK2 or GRK5 knockdown in macrophages

A. Control or GRK2 knockdown Raw264.7 macrophages were treated or not with TNFα for 24 hours and RNA extracted as described in the Methods. Quantitative real-time RT-PCR was performed using MIP1β specific primers. MIP1β mRNA levels were normalized to cyclophilin levels. ** $p<0.01$. N=3.

B. Control or GRK5 knockdown Raw264.7 macrophages were treated with TNFα and mRNA levels of MIP1β and cyclophilin determined as described in (**A**) above. *** $p<0.001$; **$p<0.01$. N=3.

A.


Control siRNA
GRK2 siRNA
**
100
75
50
25
0
MIP1β mRNA
% maximal response
0
24
Time (hr)

B.

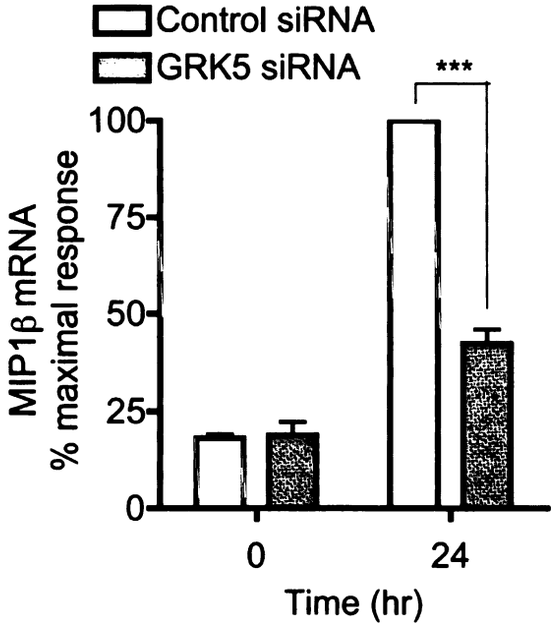
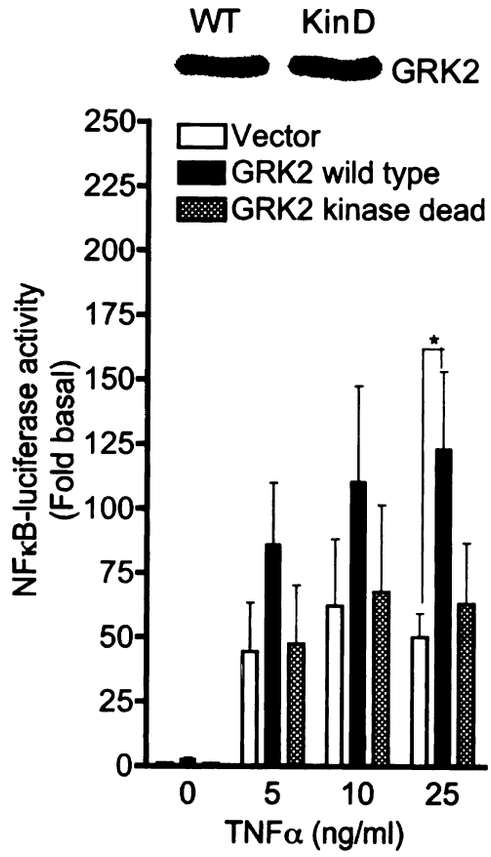

Control siRNA
GRK5 siRNA
***
100
75
50
25
0
MIP1β mRNA
% maximal response
0
24
Time (hr)

Figure 3.8 Kinase activity of GRK2 and 5 is essential for mediating TNFα-induced NFκB transcriptional activation

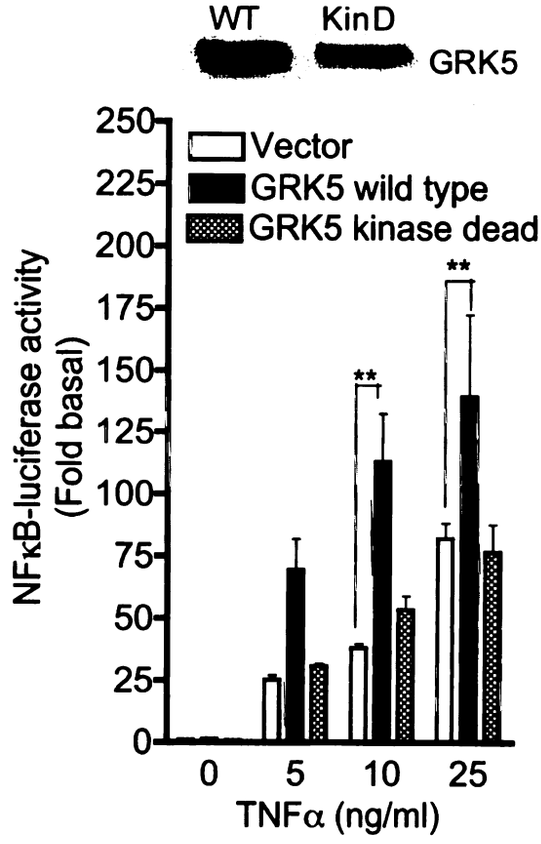
A. HEK293T cells were transfected with vector, GRK2 wild type or GRK2-K220R (kinase deficient mutant) expression plasmids along with pELAM-luciferase and LacZ. Forty hours after transfection, cells were stimulated (in triplicates) with different concentrations of TNFα (as indicated) for 8 hours. Lysates were prepared and analysed for luciferase and β-galactosidase activities. Quantitation was performed after normalizing luciferase activity to β-galactosidase activity. Data is expressed as fold basal of the vector transfected cells. Immunoblots showing expression levels of GRK2 wild type and GRK2 kinase deficient mutant are shown in the top panel. *$P<0.05$. N=4.

B. HEK293T cells were transfected with vector, GRK5 wild type or GRK5-K215R (kinase deficient mutant) expression plasmids along with pELAM-luciferase and LacZ. Cell treatments, activity assays and quantitation were performed as described in (**A**) above. Immunoblots showing expression levels of GRK5 wild type and GRK5 kinase deficient mutant are shown in the top panel. **$P<0.01$ N=4.

A.


WT
KinD
GRK2
Vector
GRK2 wild type
GRK2 kinase dead
NFκB-luciferase activity
(Fold basal)
250
225
200
175
150
125
100
75
50
25
0
*
0
5
10
25
TNFα (ng/ml)

B.


WT
KinD
GRK5
Vector
GRK5 wild type
GRK5 kinase dead
NFκB-luciferase activity
(Fold basal)
250
225
200
175
150
125
100
75
50
25
0
**
**
0
5
10
25
TNFα (ng/ml)

To further explore the biochemical mechanism by which GRK2 and 5 mediate TNFα-induced NFκB activity, we next tested whether the kinase activity is essential for the observed effects of the GRKs. For this, we over-expressed vector or wild type or kinase dead GRK2 or GRK5 in HEK293T cells along with an NFκB reporter plasmid (pELAM luciferase) and LacZ (for transfection normalization). Forty-hours after transfection, cells were serum starved (~3 hours) and were stimulated (or not) with TNFα for 8 hours and luciferase and β-galactosidase activity determined as described in the methods. In vector transfected cells, TNFα stimulation significantly increased NFκB-luciferase activity (**Fig. 3.8**). As predicted, over-expression of wild type GRK2 or GRK5 significantly enhanced TNFα-induced NFκB activity while over-expression of kinase-inactive GRK2 or GRK5 (GRK2-K220R or GRK5-K215R) had no effect (**Fig. 3.8A and 3.8B**). This demonstrates that the kinase activities of GRK2 and 5 are required for the observed effects of GRKs in TNFα-induced NFκB signaling. Interestingly, as observed with IκBα phosphorylation in macrophages, over-expression of GRK2 but not GRK2-K220R enhanced NFκB-luciferase activity even in the absence of ligand stimulation (1.0 ± 0.51 activity in vector cells compared to 2.35 ± 1.07 in GRK2 and 0.70 ± 0.32 in GRK2-K220R expressing cells). In contrast, over-expression of GRK5 or GRK5-K215R did not significantly affect basal NFκB activity (1 ± 0.14 activity in vector cells compared to 1.51 ± 0.17 in GRK5 and 0.86 ± 0.04 in GRK5-K215R expressing cells).

Interaction of GRK2 and 5 with IκBα

To define the biochemical mechanisms that mediate GRKs' actions, we first tested whether GRKs affect IKKβ expression or activity. For this purpose, we knocked down and over-expressed GRK2/5 in Raw264.7 macrophages and HEK293T cells

respectively and examined the levels of IKKβ. Neither knockdown nor over-expression of GRK2/5 affected IKKβ levels in the presence or absence of TNFα (data not shown). To further rule out the effect of GRKs on IKKβ activity, we performed an *in vitro* IKKβ kinase assay using IKKβ immunoprecipitated from cells over-expressing GRK2/5. *In vitro* phosphorylation of GST-IκBα by immunoprecipitated IKKβ was not affected by over-expression of either GRK2 or GRK5 (data not shown). Similarly, the interaction of IκBα and IKKβ was not affected by over-expression of GRKs in HEK293T cells (data not shown). Based on these results we hypothesized that GRKs might mediate their effects via directly interacting with and phosphorylating IκBα. To first examine if GRK2 or 5 can interact directly with IκBα, we tested the ability of GST or GST-IκBα to bind GRK2 and 5 using an overlay blot assay. For this, bacterially expressed and purified GST or GST-IκBα or GST-IκBα(76-302) were run on SDS-PAGE, and transferred to nitrocellulose. The membranes were then incubated with HEK293T lysates over-expressing either vector, GRK2 or GRK5 and tested for the ability of GRKs to specifically bind to GST-IκBα. As hypothesized, both GRK2 (**Fig. 3.9A and 3.9B**) and GRK5 (**Fig. 3.10A and 3.10B**) bound to GST-IκBα with no significant binding to GST. In addition, neither GRK2 (**Fig. 3.9A**) nor GRK5 (**Fig. 3.10A**) interacted appreciably with an N-terminal deletion mutant of IκBα [IκBα(76-302)], suggesting that both GRKs primarily interact with IκBα at the N-terminus. We also tested the ability of GRKs to interact with IκBα in intact cells and found that immunoprecipitation of IκBα co-immunoprecipitated both GRK2 and 5 (**Fig. 3.9C and 3.10C**) in HEK293T cells; **Fig. 3.9D** in human monocytic cells THP1). Taken together, these results suggest that IκBα is

Figure 3.9 GRK2 interacts with the N-terminus of IκBα

A. Purified GST-IκBα (full length), GST-IκBα (76-302) and GST alone, (5 μg each) were resolved on a SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated overnight in HEK293T lysates over-expressing HA-GRK2 **(A)** or vector **(B)**. Membrane was washed and probed for HA-GRK2 binding to GST-IκBα by immunoblotting using anti-HA antibody. Western blot of the overlay assay is shown in the top panel and a Ponceau stain of the overlay blot is shown in the bottom. Blots are representative of at least four individual experiments.

C. GRK2 and IκBα were co-expressed in HEK293T cells and the lysates were immunoprecipitated using IκBα polyclonal antibody and the blots were then subjected to immunoblotting as indicated.

D. Lysates from THP1 monocytic cells were immunoprecipitated with IκBα polyclonal antibody. Immunoprecipitates were then denatured using sample buffer, boiled and separated on a 10% SDS-PAGE gel. Immunoblotting was performed for GRK2 as shown.

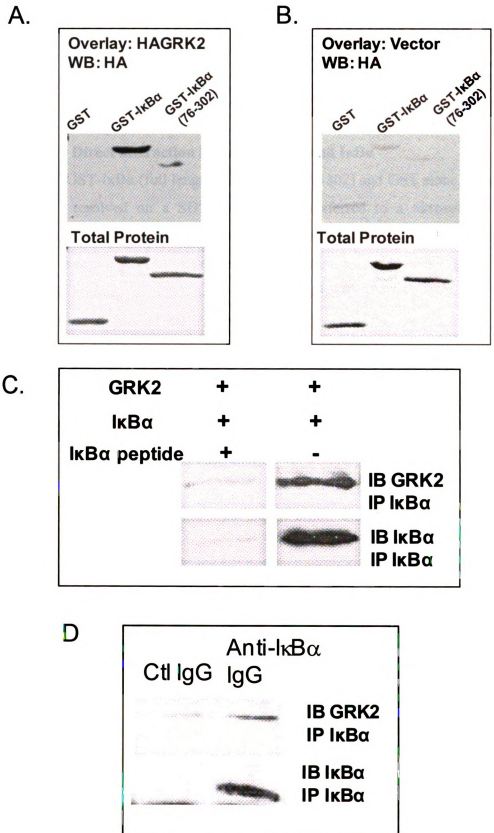
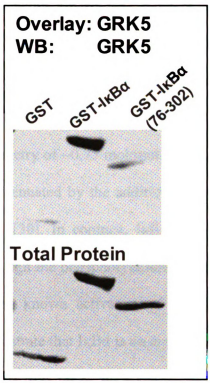

A.
Overlay: HAGRK2
WB: HA
GST
GST-IκBα
GST-IκBα (76-302)
Total Protein
B.
Overlay: Vector
WB: HA
GST
GST-IκBα
GST-IκBα (76-302)
Total Protein
C.
GRK2 + +
IκBα + +
IκBα peptide + -
IB GRK2
IP IκBα
IB IκBα
IP IκBα
D
Anti-IκBα
Ctl IgG IgG
IB GRK2
IP IκBα
IB IκBα
IP IκBα

Figure 3.10 Direct interaction between GRK5 and IκBα

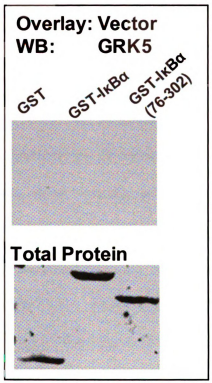
A. Purified GST-IκBα (full length), GST-IκBα (76-302) and GST alone, (5 μg each) were resolved on a SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated overnight in HEK293T lysates over-expressing GRK5 **(A)** or vector **(B)**. Membrane was washed and probed for GRK5 binding to GST-IκBα by immunoblotting using anti-GRK5 antibody. Western blot of the overlay assay is shown in the top panel and a Ponceau stain of the overlay blot is shown in the bottom. Blots are representative of at least four individual experiments.

C. GRK5 and IκBα were co-expressed in HEK293T cells and the lysates were immunoprecipitated using IκBα polyclonal antibody and the blots were then subjected to immunoblotting as indicated.

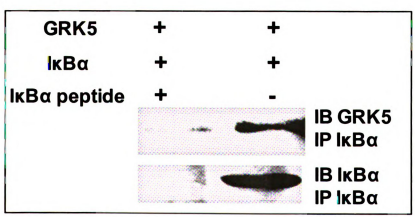
A.


Overlay: GRK5
WB: GRK5
GST
GST-IκBα
GST-IκBα (76-302)
Total Protein

B.


Overlay: Vector
WB: GRK5
GST
GST-IκBα
GST-IκBα (76-302)
Total Protein

C.


GRK5 + +
IκBα + +
IκBα peptide + -
IB GRK5
IP IκBα
IB IκBα
IP IκBα

a direct interaction partner for both GRK2 and GRK5.

Phosphorylation of IκBα by GRK2 and 5

Experiments using kinase-dead mutants of GRK2 and 5, as well as the experiments described above, suggest that IκBα may be a substrate for GRKs in TNFα-induced NFκB signaling. To directly test this, we performed *in vitro* phosphorylation assays using purified GRK2 and 5 with IκBα as the substrate. GRK5 effectively phosphorylated IκBα to a stoichiometry of ~0.75 mol/mol (**Fig. 3.11B**) and interestingly, phosphorylation was effectively attenuated by the addition of phospholipids, which normally activate GRK5 (**Fig. 3.11D**) [30]. In contrast, IκBα was a relatively poor substrate for GRK2 (**Fig. 3.11A**), although the phosphorylation was enhanced by the addition of Gβγ subunits and phospholipids, known activators of GRK2 (**Fig. 3.11C**) [31]. Taken together, these results demonstrate that IκBα is an *in vitro* substrate for both GRK2 and GRK5.

To identify the GRK phosphorylation sites in IκBα and to examine if IκBα is differentially phosphorylated by GRK2 and 5, we first tested whether the known IKKβ phosphorylation sites (Ser32/36) are also phosphorylated by GRK2 or GRK5. Indeed, previous studies have shown that these two residues are targeted by additional kinases such as ribosomal S6K and CK II, especially in NFκB pathways that are largely IKKβ-independent [32, 33]. To test this, we assessed the ability of GRK2 and GRK5 to phosphorylate a GST-IκBα S32/36A mutant. Our results show that GRK2 mediated phosphorylation of wild type and mutant GST-IκBα was comparable, suggesting that these sites are not phosphorylated by GRK2 (**Fig. 3.11E**). In contrast, GRK5 mediated phosphorylation of the IκBα mutant was decreased ~60% compared to wild type IκBα

Figure 3.11 IκBα is a substrate for GRK2 and GRK5 *in vitro*

A and B. Purified GRK2 and GRK5 were prepared as described before [30]. 200 nM purified GST-IκBα or GST alone were incubated with 25 nM GRK2 or GRK5 in buffer containing 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.1 mM [γ^{32}P] ATP, 5 mM $MgCl_2$ and the phosphorylation reactions were done for various time points as indicated. Reaction was stopped with SDS buffer, electrophoresed on a 10% SDS-PAGE, the gel dried and subjected to autoradiography. Phosphorylated IκBα bands were then counted on a liquid scintillation for quantification.

C and D. The reactions were incubated in the absence or presence of liposomes as indicated and phosphorylation reactions performed for 30 min and stoichiometry quantified as described above. GRK2 phosphorylation samples also contained Gβγ in addition to liposomes.

E and F. GRK2 and GRK5 were incubated with 200 nM purified GST-IκBα (wild type or mutant S32A/S36A) in a kinase reaction and phosphorylated IκBα quantified using liquid scintillation as described in the methods.

G. Purified GRK2, GRK5 or IKKβ were incubated with GST-IκBα as a substrate as described above without [γ^{32}P]ATP. Reaction was stopped with SDS buffer, electrophoresed on a 10% SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblotting was performed using an antibody that specifically detects phospho-IκBα(ser32).

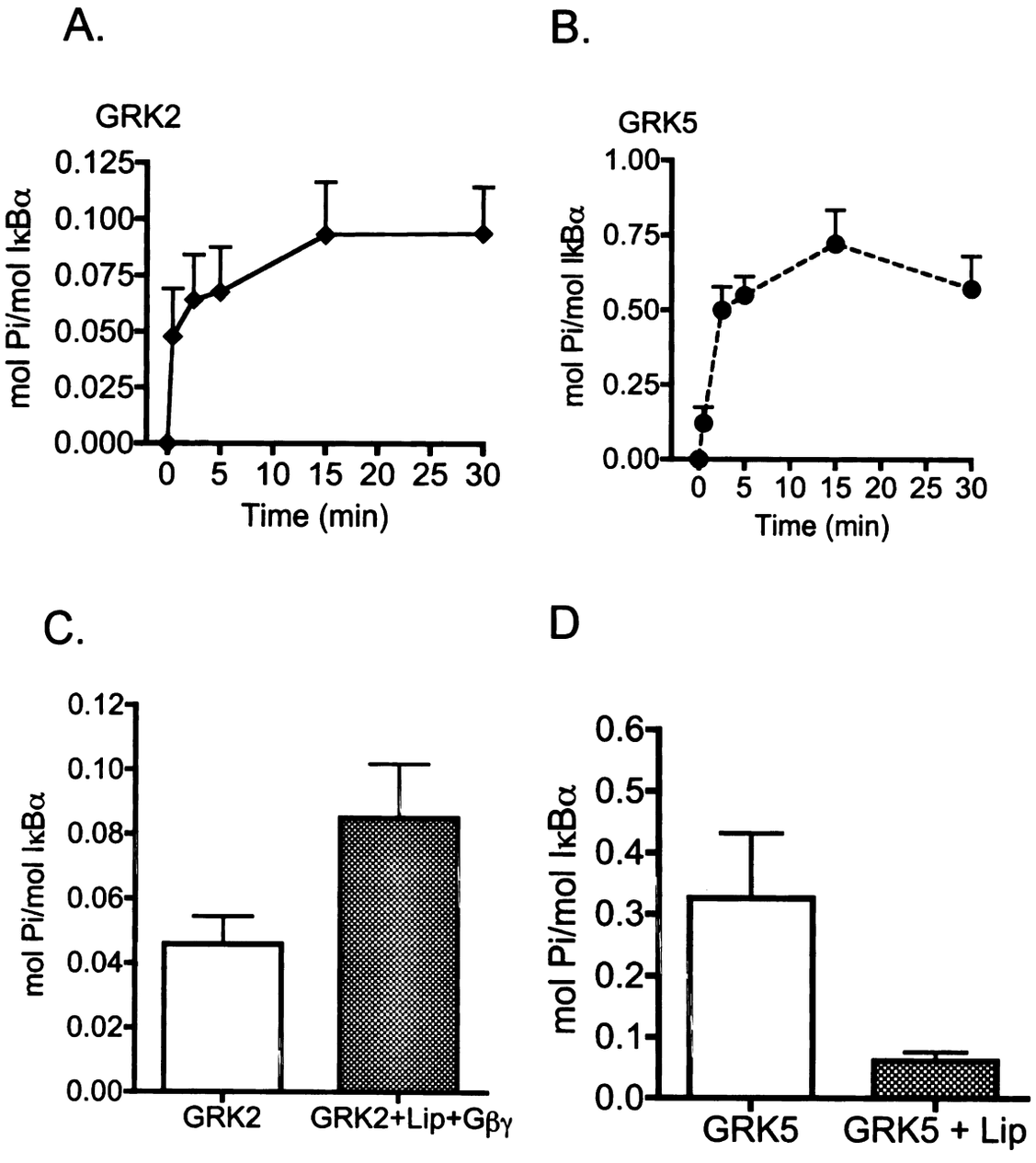

A.
GRK2
0.125
0.100
0.075
0.050
0.025
0.000
mol Pi/mol IκBα
0 5 10 15 20 25 30
Time (min)
B.
GRK5
1.00
0.75
0.50
0.25
0.00
mol Pi/mol IκBα
0 5 10 15 20 25 30
Time (min)
C.
0.12
0.10
0.08
0.06
0.04
0.02
0.00
mol Pi/mol IκBα
GRK2
GRK2+Lip+Gβγ
D
0.6
0.5
0.4
0.3
0.2
0.1
0.0
mol Pi/mol IκBα
GRK5
GRK5 + Lip

Figure 3.11 Continued...

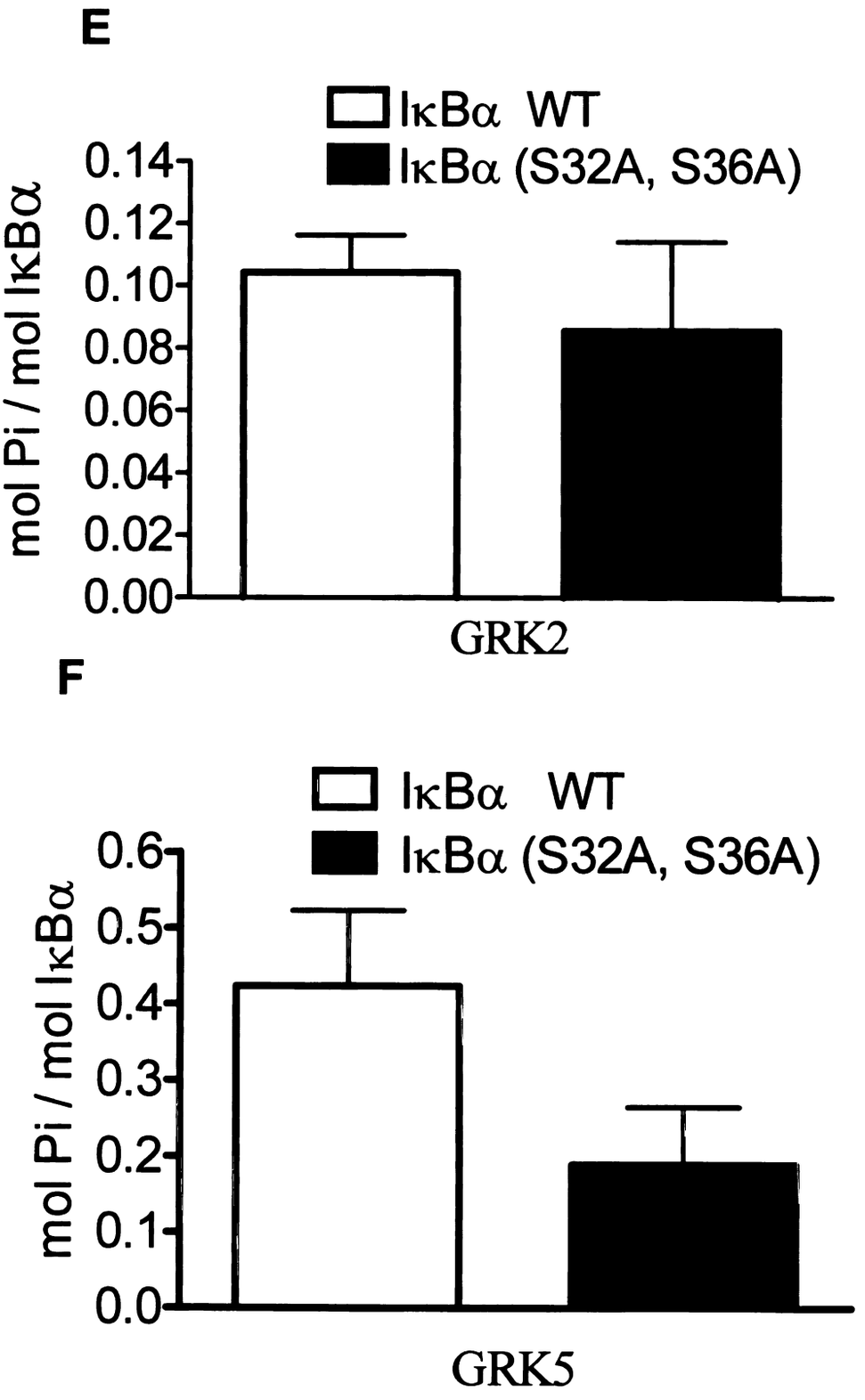
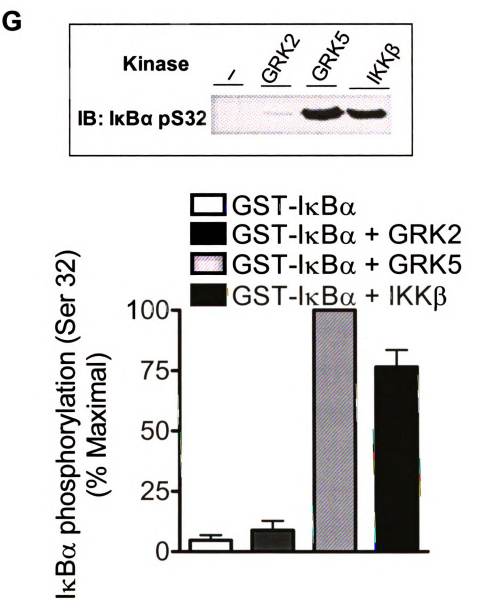


Figure 3.11 Continued…



(**Fig. 3.11F**), suggesting that GRK5 phosphorylates one or both of these sites. This result was confirmed using an antibody that recognizes phosphoSer32 in IκBα. For these studies, GST-IκBα was initially phosphorylated *in vitro* using GRK2, GRK5 or IKKβ and the samples were run on SDS PAGE and immunoblotted using anti-IκBα-phospho-Ser32. These results show that Ser32 is selectively phosphorylated by GRK5 but not GRK2 and that GRK5 mediated phosphorylation of IκBα is comparable to that seen with IKKβ (**Fig. 3.11G**). Overall, these results reveal that Ser32 is phosphorylated by GRK5 and that GRK2 and GRK5 phosphorylate distinct residues.

IKKβ knockdown does not inhibit TNFα-induced IκBα phosphorylation in Raw264.7 macrophages

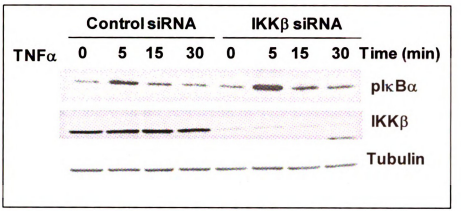
Similar to GRK5, other kinases such as casein Kinase II and ribosomal S6 kinase have been shown to phosphorylate IκBα at Ser32/36. Interestingly, these kinases were shown to selectively regulate IκBα-NFκB pathway in an IKKβ-independent manner. Therefore, we hypothesized that because of the role of GRK2 and 5 as "IκBα kinases", IKKβ may be dispensable in Raw264.7 cells, particularly for TNFα-induced IκBα phosphorylation. To test this hypothesis, we knocked down IKKβ in Raw264.7 macrophages and tested the effect of TNFα on IκBα phosphorylation (Ser32/36). As predicted, we found that TNFα-induced IκBα phosphorylation is not significantly affected by knockdown of IKKβ, suggesting that IKKβ may be redundant in this system (**Fig. 3.12A**). However, it is possible that the level of knockdown is not sufficient to inhibit IκBα phosphorylation because of the residual IKKβ kinase activity present. To rule out this possibility, we further tested the ability of IKKβ knockdown to inhibit LPS-induced IκBα phosphorylation. Interestingly, our results demonstrate that LPS-induced

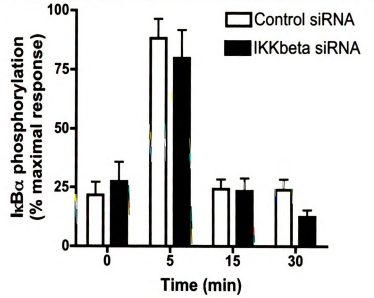
Figure 3.12 IKKβ knockdown does not inhibit TNFα-induced IκBα phosphorylation but inhibits LPS-induced phosphorylation

A. Raw 264.7 macrophages were electroporated with either control or IKKβ siRNA as indicated. Forty-eight hours after electroporation, cells were serum starved for ~3 hours, and stimulated with TNFα (25 ng/ml) for the indicated times. Lysates were immunoblotted for pIκBα (Ser32/36) and tubulin for normalization. Representative blots are shown at the top and quantitation at the bottom. N=4.

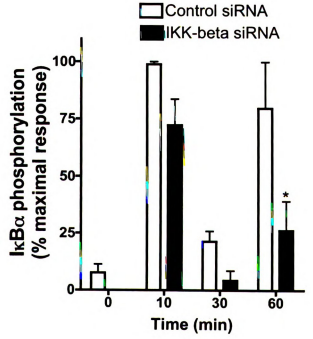
B. Raw 264.7 macrophages were electroporated with either control or IKKβ siRNA as indicated. Forty-eight hours after electroporation, cells were serum starved for ~3 hours, and stimulated with LPS (1 μg/ml) for the indicated times. Lysates were immunoblotted for pIκBα (Ser32/36) and tubulin for normalization. Quantitation is shown. N=4.

A.


Control siRNA
IKKβ siRNA
TNFα
0 5 15 30 0 5 15 30 Time (min)
pIκBα
IKKβ
Tubulin


Control siRNA
IKKbeta siRNA
IκBα phosphorylation (% maximal response)
100
75
50
25
0
0 5 15 30
Time (min)

B.


Control siRNA
IKK-beta siRNA
IκBα phosphorylation (% maximal response)
100
75
50
25
0
*
0 10 30 60
Time (min)

IκBα phosphorylation (at Ser32/36) is inhibited by IKKβ knockdown and this effect was particularly evident at later time points (**Fig. 3.12B**). These results suggest that the IKKβ plays a crucial role in LPS-induced IκBα phosphorylation, but not in TNFα-induced IκBα-NFκB pathway in Raw264.7macrophages. Taken together, our results demonstrate a critical role for GRK2 and 5 in the regulation of TNFα-induced IκBα-NFκB pathway in Raw264.7 macrophages and suggest that IκBα phosphorylation by GRKs might be an essential step in this regulation.

DISCUSSION

G-protein coupled receptor kinases were first discovered for their role in GPCR phosphorylation and desensitization [34, 35]. Recent studies, however, have revealed a number of non-GPCR substrates for GRKs [10, 11]. Although GRKs mediate their cellular effects for the most part through their catalytic activity, recent studies have also proposed a kinase-independent role for GRKs in cellular signaling (via protein-protein interaction with the RH domain). In this regard, GRK2 has been shown to interact with MEK1 and regulate ERK activation in a kinase-independent manner [36]. GRK2 has also been shown to interact with other proteins such as PI3K [37], Akt [38], and GIT [39] and regulate a number of cell biological effects. In addition to the receptor and cytosolic substrates, GRKs, have also been found to phosphorylate nuclear proteins. A physiologically important role for the nuclear localized GRK5 was recently identified by Martini *et al* [40] who showed that the nuclear GRK5 is a HDAC kinase the mediates the epigenetic regulation of gene expression in cardiomyocytes. Taken together these studies suggest that the role of GRKs is much broader than previously appreciated.

Studies have just begun to emerge on the potential role for GRKs in the regulation of various components of the NFκB pathway. In this regard, we demonstrated that GRK5 stabilizes LPS-stimulated p105 levels in macrophages [8]. More recently, while this manuscript was in preparation, a similar stabilizing role for GRK5 in maintaining IκBα levels in endothelial cells was shown [41]. Surprisingly in the present study we find that GRK2 and GRK5 are important regulators of IκBα-NFκB signaling and mediate TNFα-induced IκBα phosphorylation. In addition, in contrast to the findings of Sorriento *et al* [41] in endothelial cells, our results demonstrate that GRK2 and GRK5 mediate TNFα-

induced NFκB-dependent gene transcription in macrophages. Our results further suggests that even with in a given cell type, the role of GRKs is selective for a particular ligand.

Our data further demonstrate that the role of GRK2 and GRK5 on TNFα-induced NFκB signaling is dependent on the kinase activities because the kinase-deficient GRKs failed to mediate NFκB activation. Also, *in vitro* kinase assays indicate that IκBα may be differentially phosphorylated by GRK2 and 5. Our results show that the presence of Gβγ subunits and liposomes significantly enhances the phosphorylation of IκBα by GRK2. Gβγ subunits have clearly been demonstrated to be important in the translocation of GRK2 to the plasma membrane for GPCR phosphorylation. Whether a similar role for Gβγ subunits in TNFα signaling exists, is presently not known. However, Kawamata *et al* [42] showed recently that TNFα signaling is mediated by activation of G-proteins in adipocytes. In addition, TNFα treatment of THP-1 monocytic cells has been shown to mediate GRK2 translocation to the membrane and affect β-adrenergic receptor desensitization [43], suggesting possible regulation of TNFα-induced GRK2 activity by G-proteins. In contrast to the role of lipids in GRK2 activity, GRK5 phosphorylation of IκBα appears to be effectively inhibited in the presence of lipids even though previous studies have clearly shown that lipids activate GRK5 [30]. Thus it is possible that GRK5 phosphorylation of IκBα is regulated by biochemical mechanisms that are distinct from its phosphorylation of other substrates such as synucleins [30] as well as from that of IκBα phosphorylation by GRK2. Whether these differences in the phosphorylation of IκBα by GRK2 and 5 translate into regulation of IκBα in different sub-cellular environments in not known and will be tested in future studies. Other studies have clearly

shown that IκBα-NFκB complexes can be present in different sub-cellular environments [44, 45] and therefore, these complexes could be potentially regulated by GRK2 or 5 depending on the local cellular environment.

IKKβ has been identified as the primary kinase that phosphorylates IκBα. However, there is now extensive evidence that other kinases including IKKα, CK II and ribosomal S6K can phosphorylate IκBα at the same sites as that of IKKβ. This redundancy can in part be explained by the receptor- and cell type-specific regulation of IκBα-NFκB pathways [7, 32, 46]. For example, studies have shown that UV light-induced IκBα degradation is mediated by phosphorylation of IκBα by CK II [7]. Also, PMA-induced IκBα phosphorylation has been shown to involve ribosomal S6K [32]. Similarly, recent studies have shown IKKβ-dependent and –independent pathways that regulate IκBα-NFκB pathway in human macrophages in response to specific ligands [47]. Our studies clearly suggest that GRKs while necessary for TNFα-induced IκBα-NFκB pathway, are not involved in LPS-induced IκBα-NFκB signaling. Interestingly, knockdown of IKKβ does not affect TNFα-induced IκBα phosphorylation (at Ser32/36), but does inhibit LPS-induced IκBα phosphorylation, suggesting that in Raw264.7 macrophages, GRKs play the role of IκBα kinases selectively for TNFα signaling. Our *in vitro* kinase reactions further support our findings in macrophages in that, GRK5 is able to phosphorylate some residues in IκBα that are similar to that of IKKβ. Thus it is possible that GRK5 specifically might function in a similar capacity to that of IKKβ. If GRK5 can function as an IκBα kinase, then what is the role of GRK2? It appears from our macrophage experiments that GRK2 is also necessary for TNFα-induced IκBα

phosphorylation (Ser32/36). However, *in vitro* GRK2 does not phosphorylate Ser32/36 and therefore appears to phosphorylate a different set of residues. In cells, GRK2 phosphorylation of these unidentified residues appears to be necessary for phosphorylation of Ser32/36 since knockdown of GRK2 inhibits IκBα phosphorylation at these two sites. It is also possible the level of IKKβ knockdown obtained might not be sufficient to define IKKβ-independent regulation because of the residual kinase activity present in the knockdown cells. If this is the case, GRKs might work co-operatively with IKKβ in the phosphorylation of IκBα. In this regard, although Ser32/36 in IκBα are the well-characterized IKK phosphorylation sites, there is strong evidence that IKK also phosphorylates less well-characterized sites at the c-terminus [48]. Therefore, in our experiments, we cannot rule out the role of IKKβ on phosphorylation of these other sites. Our results, however, indicate that IKKβ knockdown can certainly inhibit LPS-induced IκBα phosphorylation (on Ser32/36) and therefore, suggest that LPS and TNFα signal to NFκB activation via different mechanisms in Raw264.7 macrophages.

In conclusion, our studies unravel important biochemical roles for GRK2 and 5 in TNFα-induced IκBα phosphorylation and NFκB signaling. Because regulation of the NFκB pathway appears to be receptor-specific as well as cell type-specific, further broad and unbiased proteomic approaches are necessary to identify macrophage-specific and TNFR-specific signaling complexes that mediate NFκB activation. These studies will undoubtedly identify therapeutic targets for inhibiting TNFα signaling in chronic inflammatory diseases.

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CHAPTER 4

G-protein coupled receptor kinase 5 (GRK5) mediates Toll-like receptor-4-induced NF-κB pathway in macrophages and is necessary for the production of inflammatory cytokines and chemokines *in vivo*

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ABSTRACT

G-protein coupled receptor kinase-5 (GRK5), a serine-threonine kinase is one of the seven GRK family members, the primary function of which is to regulate the desensitization of G-protein coupled receptors. Recent studies have shown that GRK5 also regulates NFκB pathway stimulated by non-GPCRs. This study was undertaken to determine the potential role of GRK5 in inflammatory signaling in primary macrophages and *in vivo* using $GRK5^{-/-}$ mice. Consistent with our previous findings in macrophage cell lines, we demonstrate here that TLR4-induced IκBα-NFκB pathway is inhibited in primary macrophages from $GRK5^{-/-}$ mice compared to cells from $GRK5^{+/+}$ mice. Our results also indicate that this role of GRK5 is specific for IκBα-NFκB pathway because activation of other signaling pathways, including ERK, JNK and p38 are similar between the two genotypes. Consistent with the effects on the NFκB pathway, LPS-induced cytokine/chemokine production was broadly inhibited in the cells from $GRK5^{-/-}$ mice. To examine the *in vivo* relevance of these findings, we injected $GRK5^{-/-}$ and $GRK5^{+/+}$ mice with LPS and measured plasma cytokine levels at various times after injection. Confirming the *in vitro* data, LPS-induced cytokines/chemokines were significantly inhibited *in vivo*, in the $GRK5^{-/-}$ mice. Associated with these effects LPS-induced liver injury is also decreased in the $GRK5^{-/-}$ mice. Taken together, our findings demonstrate that GRK5 acts as a positive regulator of LPS-induced inflammatory signaling and further suggest that these findings could have potential implications for drug development in inflammatory diseases.

INTRODUCTION

G-protein coupled receptor kinases (GRKs) are serine-threonine protein kinases that regulate the phosphorylation and desensitization of G-protein coupled receptors [1]. GRK family (seven members identified, GRK1-7) is subdivided into three main groups on the basis of sequence homology viz. rhodopsin kinase (GRK1 and GRK7), β-adrenergic receptor kinase (GRK2 and GRK3), and the GRK4 kinase (GRK4, GRK5 and GRK6) subfamilies. Although these seven members share certain characteristic features, they are distinct enzymes with specific properties. GRK5 is the best characterized member of the GRK4 subfamily of GRKs which is expressed ubiquitously in all mammalian tissues. It is a membrane associated protein which has been shown to be selectively required for muscarinic receptor desensitization [2]. Recent studies have also shown a nuclear role for the GRK4 family of kinases, owing to the presence of a "Nuclear localization signal" in their sequence[1, 3].

Apart from their role in receptor desensitization, recent studies showed that GRKs also perform other cellular functions by phosphorylating non-receptor substrates such as tubulin, and synucleins [4, 5]. GRKs also interact with a variety of other cellular proteins such as caveolin, calmodulin and actin [6-8]. Along the same lines, recently, GRK5 was also found to interact with members of the IκB (Inhibitor of κB) family. In this regard, GRK5 was shown to interact with and phosphorylate NFκB1 p105 as well as IκBα [9, 10]. The functional outcomes of these studies were primarily studied using macrophage cell line. Role of GRK5 in regulating NFκB pathway in primary macrophages and the functional relevance of this regulation *in vivo,* however, is presently not known.

NF-κB family of transcription factors (NFκB1p50, NFκB2p52, RelA(p65), RelB, c-Rel) play an essential role in regulating both innate and adaptive immunity. These proteins are held in the cytoplasm under unstimulated conditions in association with an inhibitory protein family called IκB (α, β, ε, p100, p105, Bcl-3) {reviewed in [11]}. In the canonical NFκB pathway, stimulation with inducers such as LPS or TNFα activates IκB kinase complex, which, phosphorylates IκBα as well as other IκBs including p105. Phosphorylated IκBα undergoes proteasomal degradation thus allowing NFκB factors to translocate to the nucleus and bind onto their cognate DNA binding sites thus initiating the transcription of a wide array of genes including those of cytokines and chemokines. NFκB, although essential for regulating both innate and adaptive immune responses, its constitutive activation is often associated with several inflammatory diseases such as sepsis, rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, asthma and many more. Because of this role in inflammatory diseases, IKKβ has been targeted for drug development, but has faced some pitfalls because inhibition of IKKβ not only prevents the deleterious effects of NFκB pathway, it also blocks the protective effects of the NFκB pathway, thereby tipping the balance between inflammatory and antiinflammatory effects of the NFκB pathway towards the harmful outcomes. Thus further understanding of the regulators of the NFκB pathway is an important area being actively pursued by many research laboratories.

Based on our previous studies, we hypothesized that deficiency of GRK5 in mice would result in inhibition of NFκB pathway in response to inflammatory stimuli and that would be associated with inhibition of several cytokines and chemokines both

from macrophages and *in vivo* in mice. We demonstrate here that GRK5 is a critical mediator of TLR4-induced NFκB pathway in primary macrophages and *in vivo*. Importantly, our results also demonstrate a crucial role for GRK5 in inflammation induced by TLR4 activation *in vivo* in mice.

MATERIALS AND METHODS

Materials

Protease inhibitor cocktail tablets were from Roche Diagnostics (Indianapolis, IN), Phospho ERK, Phospho p38, Phospho JNK, JNK, NFκB1 Phospho P105, phospho-IκBα antibodies were from Cell Signaling Technology (Boston, MA). ERK, NFκB1 P105 were from Santa Cruz Biotechnology. Tubulin antibody was from sigma. Monoclonal GRK5 antibody was from Upstate Biotechnology. E. coli LPS (0111:B4) from Sigma was used for mice injections. Ultra pure LPS from Invivogen was used for *in vitro* peritoneal macrophage stimulation.

Animals

Heterozygous GRK5 mice (backcrossed to C57BL6 background for at least 5 generations) were purchased from Jackson labs. Heterozygous mice were bred to obtain wild type and homozygous GRK5 knockout mice. The litter mate wild types and knockouts were further bred and the F1 and F2 wild type and knockout mice were used for the experiments. Animals were housed four to five mice per cage at 22–24°C in rooms with 50% humidity and a 12-h light–dark cycle. All animals were given mouse chow and water *ad libitum*. All animal procedures were approved by the Michigan State University Institutional Animal Care and Use Committee and conformed to NIH guidelines. Tail tips were used for isolating genomic DNA and genotyping performed by PCR. All experiments were performed on female mice, 6-8 weeks of age.

Peritoneal Macrophage isolation

To isolate peritoneal macrophages, mice were injected by intra-peritoneal injection with 1ml of 4% thioglycollate. Peritoneal macrophages were collected by performing a peritoneal cavity lavage after 4 days of thioglycollate injection in Dulbecco's phosphate buffered saline (DPBS). Cells were washed atleast three times and then counted and plated on cell culture plates in RPMI 1640 media supplemented with 10% fetal bovine serum (Invitrogen) and penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37° C in 5% CO_2. After around 18 hours of plating, cells were serum starved for ~ 3-4 hours and stimulated with LPS (1μg/ml) for the indicated time points.

Cytokine analysis

A mouse 23-plex multiplex based assay was used to determine the cytokine/chemokine concentrations according to manufacturer's instructions via Luminex 100 technology as described previously [12]. Plasma from LPS injected mice collected at different time intervals and supernatants from peritoneal macrophages stimulated with LPS for different time points were used to assess the cytokine/chemokine levels.

Western blot analysis

Cells were lysed in lysis buffer (20mM Tris-HCl (pH 7.4), 1mM EDTA, 150mM NaCl) containing 1% Triton X-100 with protease inhibitors. Lysed cells were then centrifuged at a maximum speed (13,000 X g) for 10 min at 4°C and protein concentration of the supernatants determined by Bradford assay. Western blotting was performed as described previously [9]. Briefly, equal amounts of protein were run on polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes

were then blocked in Licor blocking buffer (Licor Biosciences) or 5% w/v skimmed milk for 1 hour after which the membranes were incubated in primary antibodies overnight. Secondary antibodies used were either fluorescently tagged or HRP-conjugated. Blots were developed either on Licor odyssey or using chemiluminescence.

Statistical analysis

All values are represented as mean ± SEM. Data were analyzed and statistics performed using GRAPHPAD PRISM software (San Diego, California). The student's t-test was used to compare mean values between two experimental groups and Analysis of Variance (ANOVA) with Bonferroni post test was used to compare more than two groups. P value of less than 0.05 was considered significant.

RESULTS

Reduced NFκB activation in GRK5 knockout macrophages

Stimulation of cells with LPS and other cytokines particularly TNFα and IL-1β is known to activate IκBα-NFκB pathway, which plays an essential role in controlling both innate and adaptive immune responses by regulating the transcription of a large number of genes including cytokines and chemokines. As described before, under resting cellular conditions, NFκB is retained in the cytosol by the inhibitory protein IκBα. However, stimulation with LPS triggers the phosphorylation of IκBα by IκB kinase-β (IKKβ). Phosphorylated IκBα then undergoes proteasomal degradation releasing NFκB subunits p50 and p65, which now translocate into the nucleus, bind to its cognate DNA sequence and evoke gene transcription. Previous studies from our laboratory have shown that IκBα interacts with GRK5 and that GRK5 phosphorylates IκBα at least at one of the sites (serine32) phosphorylated by IKKβ. In Raw264.7 macrophages cell line, we demonstrated that depletion of GRK5 levels significantly blocks TNFα-induced IκBα phosphorylation [9]. Based on these results, we proposed that GRK5 interaction with and phosphorylation of IκBα mediates TNFα-induced NFκB activation in this cell line model. Sorriento et al, at the same time published that GRK5 interaction with IκBα in endothelial cells, negatively regulates LPS-induced IκBα phosphorylation. These studies found that this effect of GRK5 was independent of its kinase activity [13]. In previous studies, LPS-induced p105 phosphorylation was shown to be negatively regulated by GRK5 in Raw264.7 macrophages cell line [10]. Because of these various observations, we set out to examine the role of GRK5 in NFκB signaling in physiologically relevant cells. For that, we obtained thioglycollate-

elicited primary peritoneal macrophages from $GRK5^{+/+}$ and $GRK5^{-/-}$ mice and initially tested the effect of LPS on NFκB signaling. We first determined phosphorylation of IκBα in $GRK5^{+/+}$ and $GRK5^{-/-}$ macrophages in response to LPS. IκBα was not phosphorylated either in $GRK5^{+/+}$ and $GRK5^{-/-}$ macrophages under basal conditions. Treatment with LPS however caused a marked increase in phosphorylation of IκBα, which peaked at 60 min post stimulation in $GRK5^{+/+}$ cells. Consistent with our previous findings, in $GRK5^{-/-}$ cells, IκBα phosphorylation was significantly inhibited (WT:100±0.000%; KO:49.742±10.907%) ($p < 0.001$) as compared to $GRK5^{+/+}$ control cells (**Fig. 4.1A**). To test the significance of this in terms of NFκB activation, we examined the nuclear translocation of NFκB subunit p65 in response to LPS in $GRK5^{+/+}$ and $GRK5^{-/-}$ macrophages. Confirming our finding on IκBα phosphorylation, nuclear translocation of p65 was significantly inhibited in the knockout cells compared to the wild type macrophages (**Fig. 4.1B**). Furthermore, electrophoretic mobility shift assays demonstrate greatly attenuated NFκB binding to its consensus sequence in $GRK5^{-/-}$ cells compared to the $GRK5^{+/+}$ macrophages (**Fig. 4.1C**). Taken together, these results suggest that GRK5 positively regulates LPS induced NFκB signaling. Interestingly, LPS-induced phosphorylation of NFκB p105 (another IκB protein) was ~20% higher at all time points in the GRK5 knockout macrophages compared to the wild type cells, but was not statistically significant (**Fig. 4.1D**).

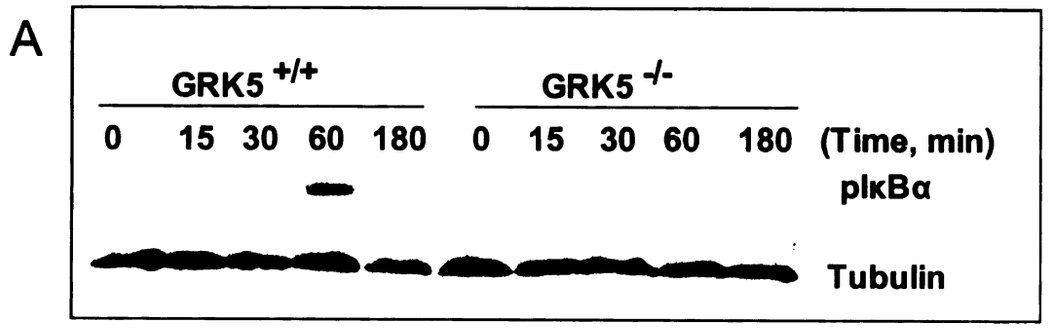
Figure 4.1 GRK5 null peritoneal macrophages show reduced NFκB activation

A. Thioglycollate elicited peritoneal macrophages from both $GRK5^{+/+}$ and $GRK5^{-/-}$ mice were stimulated with LPS (1μg/ml) for various time points as indicated. Cell lysates were extracted and separated by SDS/PAGE, transferred to nitrocellulose membrane and immunoblotted with primary antibodies against phospho-IκBα and tubulin. Secondary antibodies were either HRP labeled or fluorescent tagged and the blots were developed by chemiluminescence or using LI-COR Biosciences Odyssey system respectively. Representative blots for PIκBα and tubulin and quantification is shown in **A.** ***$p<0.001$, N=4.

B. Peritoneal macrophages were stimulated with LPS as above and nuclear extracts were prepared and immunoblotted for NFκB p65 (Rel A) as shown. Actin is shown as a loading control. A representative blot from three such experiments is shown.

C. Nuclear extracts were also incubated with IRDye® 700-labelled NFκB oligonucleotide probes and an EMSA was performed as described in the materials and methods section. Gels were then scanned on a LI-COR Biosciences Odyssey system to detect binding. A representative gel from three such experiments is shown.

D. Lysates from peritoneal macrophages stimulated with LPS (1μg/ml) were also immunoblotted using primary antibody against NFκB1 p105 (another IκB protein). Representative blot and quantification is shown. Tubulin was used as a loading control.


A
GRK5 +/+
GRK5 -/-
0 15 30 60 180 0 15 30 60 180 (Time, min)
pIκBα
Tubulin

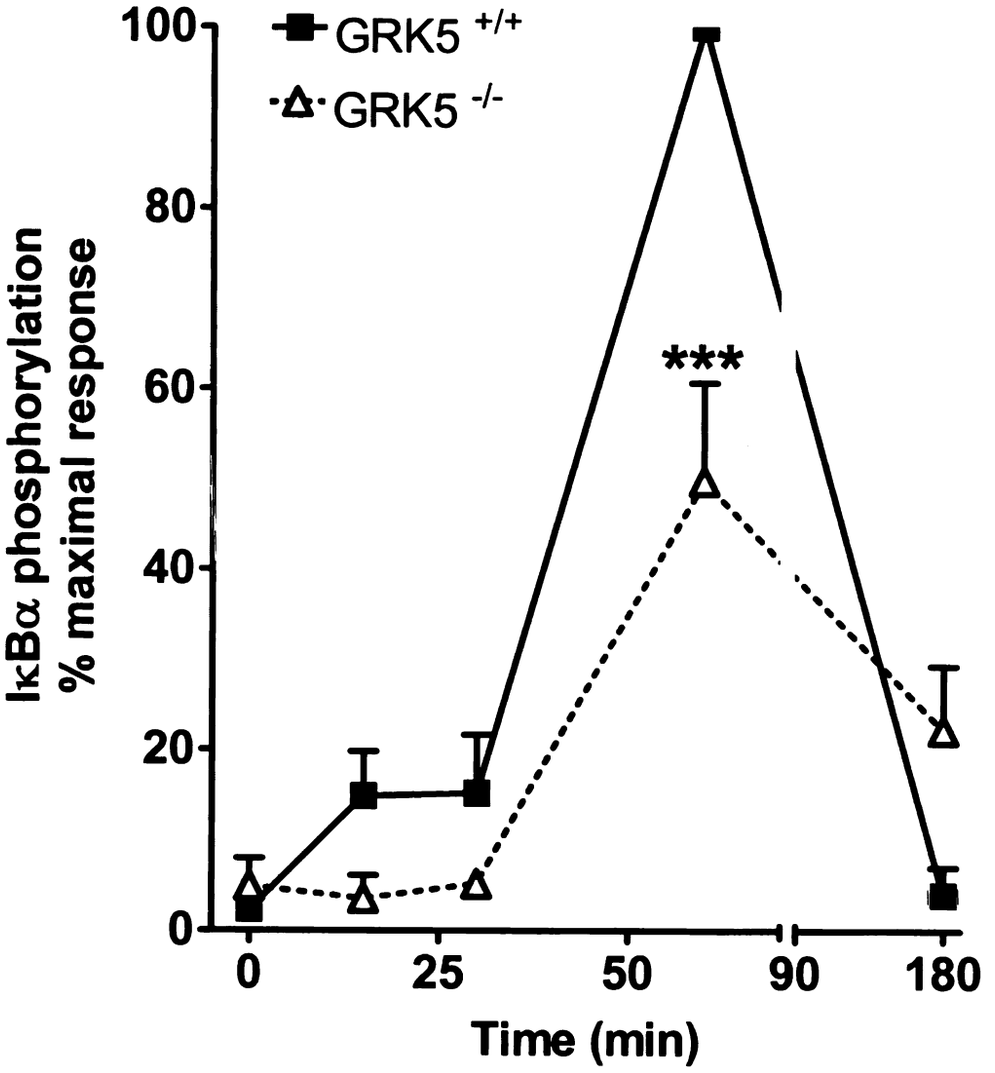

GRK5 +/+
GRK5 -/-
IκBα phosphorylation
% maximal response
100
80
60
40
20
0
***
0
25
50
90
180
Time (min)

Figure 4.1. Continued....

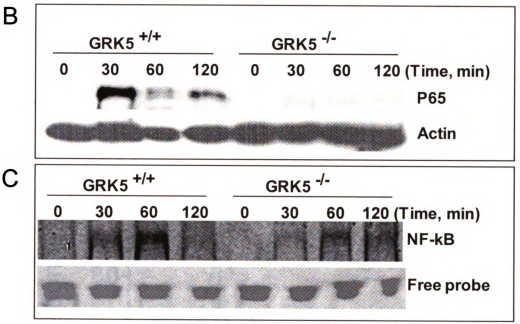
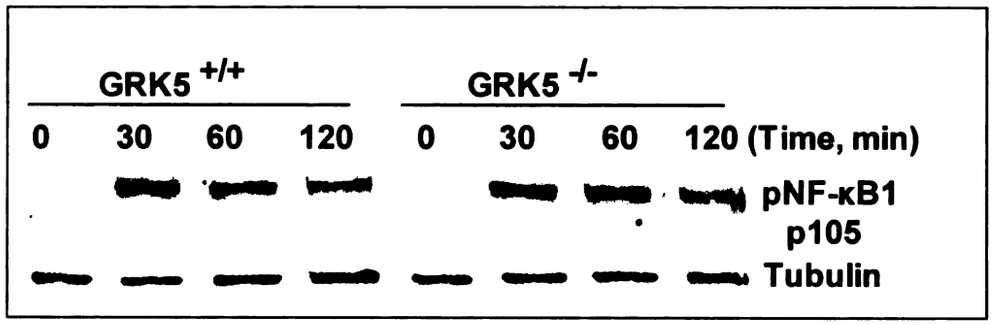
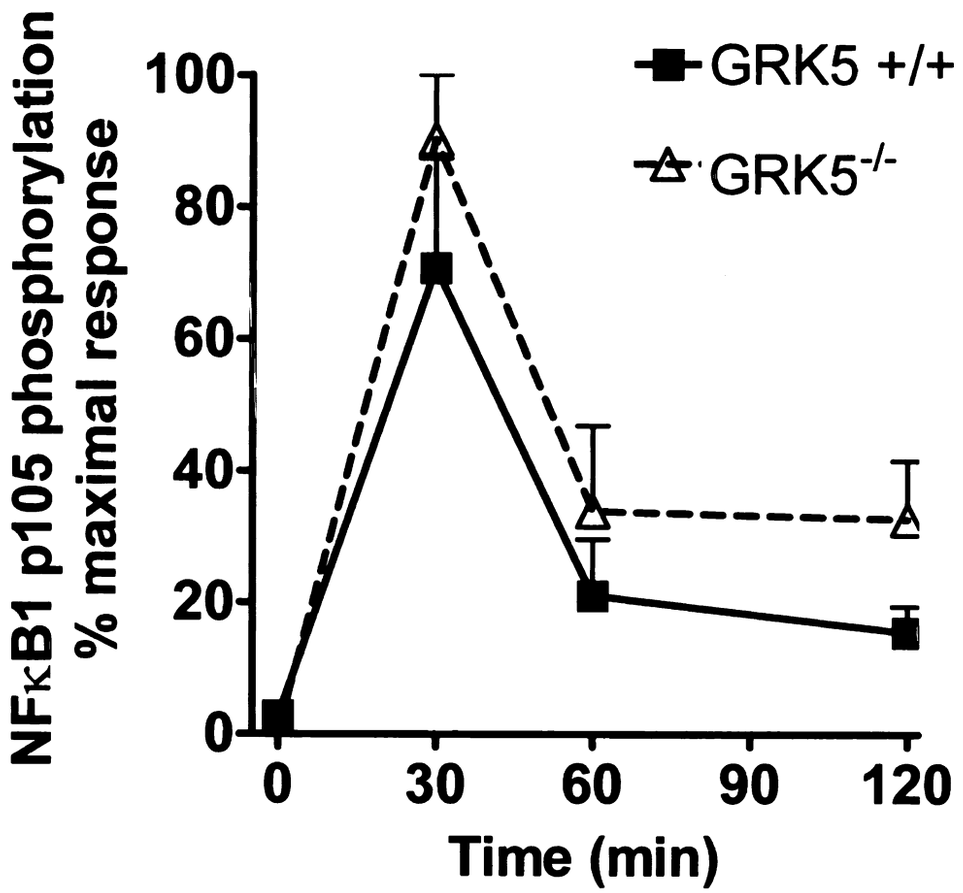


Figure 4.1. Continued....

D





GRK5 does not affect TLR4-induced MAPK activation

LPS stimulation of TLR4 also leads to the activation of MAP kinases in addition to NFκB. Because MAPKs (ERK, JNK and p38) are also important regulators of inflammatory response and to further determine whether our observed findings on GRK5 regulation of NFκB was specific for that pathway, we sought to examine the LPS-induced activation status of the three important MAPKs in primary macrophages from $GRK5^{-/-}$ mice and $GRK5^{+/+}$ control mice. Peritoneal macrophages were stimulated with LPS (1μg/ml) for various time points and immunoblotting performed to determine the phosphorylation of MAPKs at different time points. Immunoblot analysis however revealed no difference in the kinetics or magnitude of phosphorylation of ERK, JNK and p38 between wild type and GRK5 deficient macrophages suggesting that the GRK5 signaling in response to LPS is selective for IκB-NFκB pathway (**Fig. 4.2A, 4.2B, 4.2C**).

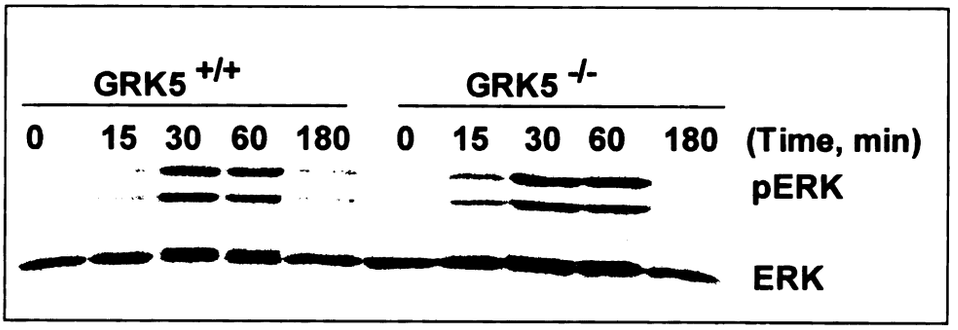
Impaired LPS-induced cytokine and chemokine production in $GRK5^{-/-}$ peritoneal macrophages

To further understand the physiological relevance of our findings, we examined the role of GRK5 in LPS-induced inflammatory cytokine/chemokine production. We hypothesized that because NFκB regulates a number of inflammatory genes, deficiency of GRK5 would broadly inhibit several cytokines/chemokines induced by LPS. For this, we treated primary macrophages from $GRK5^{+/+}$ and GRK $5^{-/-}$ mice with LPS for various time points and collected the cell culture supernatants. Cytokine/chemokine levels in the supernatants were measured using 23-plex Biorad's

Figure 4.2 GRK5 has no effect on TLR4-induced MAPK activation

Thioglycollate elicited peritoneal macrophages from both $GRK5^{+/+}$ and $GRK5^{-/-}$ mice were stimulated with LPS (1μg/ml) for various time points as indicated. Cell lysates were extracted and separated by SDS/PAGE, transferred to nitrocellulose membrane and immunoblotted with primary antibodies against phospho-ERK, ERK, phospho-JNK, JNK, phospho-p38 and tubulin. Secondary antibodies were fluorescently labeled and the blots were developed using LI-COR Biosciences Odyssey system. (**A**) P-ERK, ERK and its quantitation is shown. (**B**) shows a representative blot and quantitation for p-JNK, JNK. (**C**) show a representative blot and quantitation for p38 and tubulin. N=4.

A


GRK5 +/+
GRK5 -/-
0 15 30 60 180 0 15 30 60 180 (Time, min)
pERK
ERK

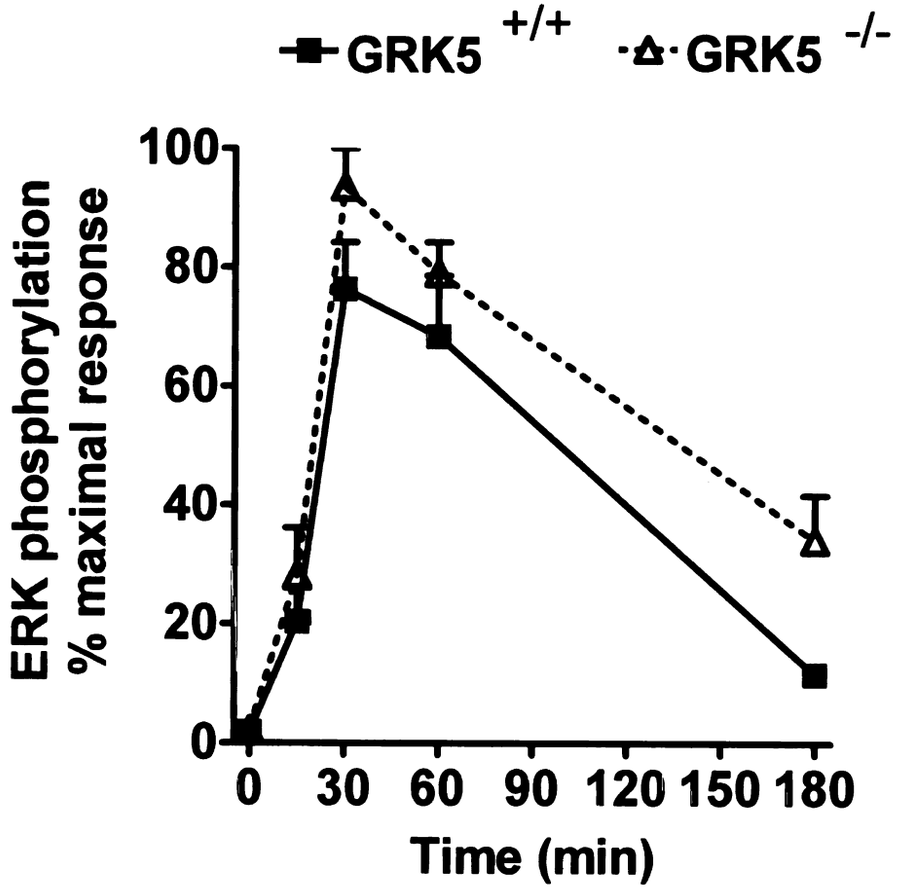
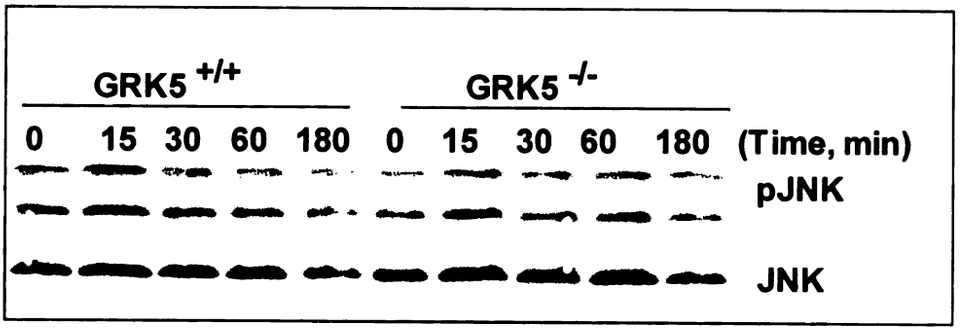

GRK5 +/+
GRK5 -/-
ERK phosphorylation
% maximal response
100
80
60
40
20
0
0 30 60 90 120 150 180
Time (min)

Figure 4.2. Continued....

B



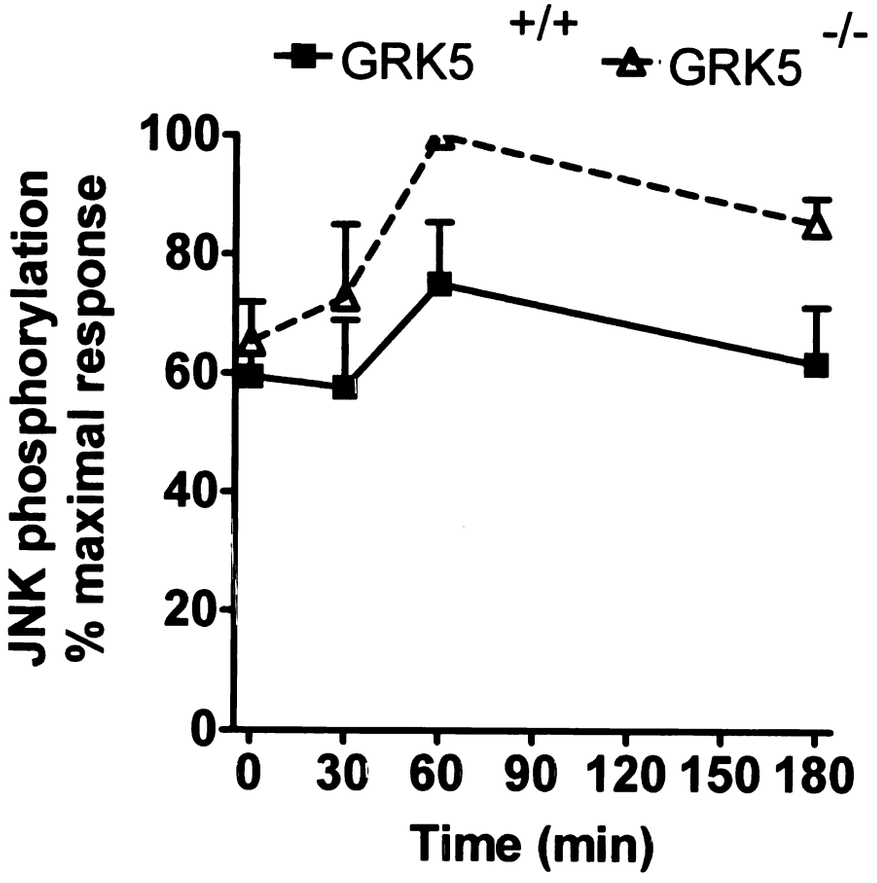
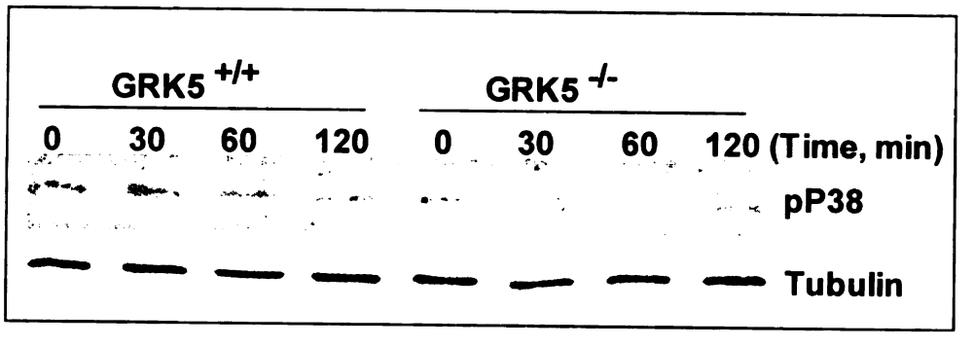
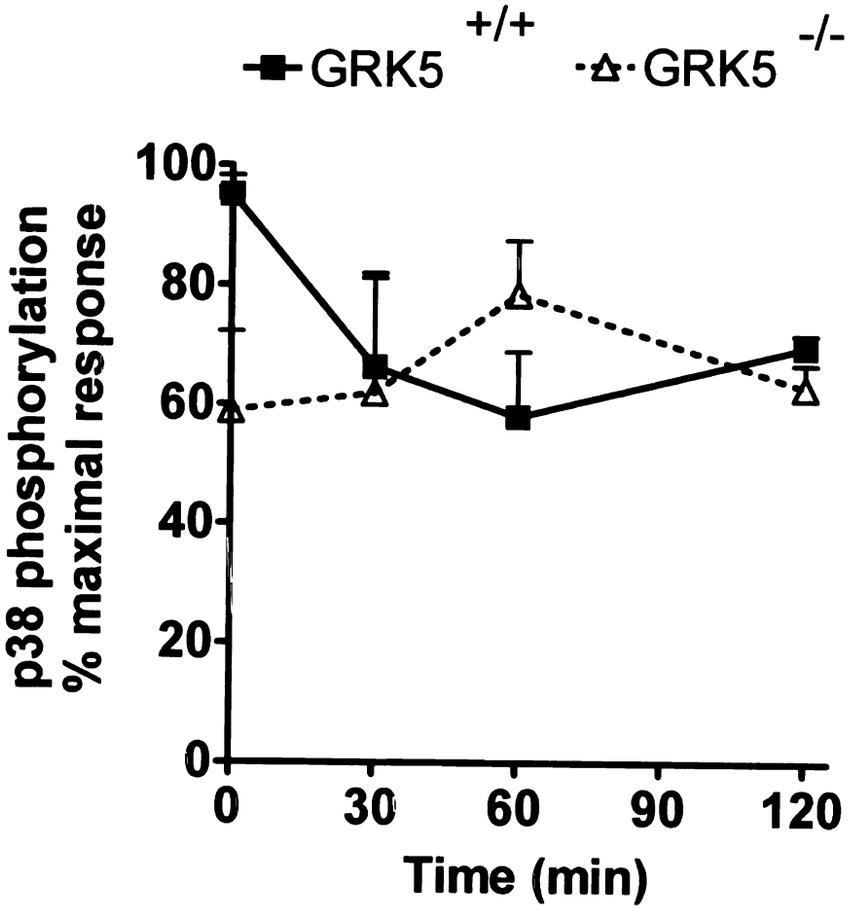


Figure 4.2. Continued....

C





bioplex assay. As predicted, our results indicate that LPS-induced production of several inflammatory cytokines/chemokines is inhibited in the GRK5 knockout mice macrophages. In particular, after 12 hours of LPS treatment the following cytokines/chemokines were markedly inhibited: IL-2 (**Fig. 4.3a**, WT: 3.975±0.727pg; KO: 2.285±0.189pg), IL-3 (**Fig. 4.3b**, WT: 1.680±0.370pg; KO: 0.934±0.136pg), IL-4 (**Fig. 4.3c**, WT: 5.654±1.233pg; KO: 3.226±0.419pg), IL-5 (**Fig. 4.3d**, WT: 6.216±1.120pg; KO: 3.559±0.344pg), IL-12p70 (**Fig. 4.3e**, WT: 19.521±3.439pg; KO: 11.656±1.014pg), IL-12p40 (**Fig. 4.3f**, WT: 195.308±30.828pg; KO: 105.707±8.911pg), IL-17 (**Fig. 4.3g**, WT: 8.630±2.152pg; KO: 3.129±0.425pg), MCP1 (**Fig. 4.3h**, WT: 691.623±115.893pg; KO: 447.905±44.838pg), IFNγ (**Fig. 4.3i**, WT: 12.667±2.670pg; KO: 6.710±0.927pg), KC (**Fig. 4.3j**, WT: 211.496±40.370pg; KO: 96.345±6.589pg), GM-CSF (**Fig. 4.3k**, WT: 11.271±2.138pg; KO: 6.378±0.570pg), Eotaxin (**Fig. 4.3l**, WT: 91.907±15.865pg; KO: 57.302±5.426pg). An important point to note is that some cytokines/chemokines were similar between the wild type and KO suggesting that GRK5 does not regulate all cytokine/chemokine production *en masse*. These included TNFα, IL-1β, IL-6, IL-9, IL-13, MIP1β, IL-1α, IL-10, GCSF, MIP1α, RANTES (data not shown).

Impaired LPS signaling *in vivo* in GRK5 knockout mice

The secretion of cytokines and chemokines into the plasma is an essential step which orchestrates the anti-infection process. Cytokines and chemokines contribute by enhancing the microbicidal activities of phagocytosing cells such as monocytes and macrophages as well as by recruiting leukocytes to the site of infection. Although contributing towards the anti-infectious process, their excessive production can cause

Figure 4.3 LPS induction of cytokines and chemokines in peritoneal macrophages from WT ($GRK5^{+/+}$) and GRK5 null ($GRK5^{-/-}$) mice

Thioglycollate elicited peritoneal macrophages were stimulated with 1μg/ml LPS in cell culture plates. Cell culture supernatants were then collected at various time intervals and secretary levels of cytokines / chemokines were determined using Biorad's 23 plex Luminex based assay. Cells were lysed and protein concentration was determined by Bradford assay. Levels of cytokines / chemokines were normalized to total protein concentration and shown in picograms. Cytokines and chemokines shown include IL-2 (**a**), IL-3 (**b**), IL-4, (**c**), IL-5 (**d**), IL-12p70 (**e**), IL-12p40 (**f**), IL-17 (**g**), MCP1 (**h**), IFNγ (**i**), KC (**j**), GM-CSF (**k**) and Eotaxin (**l**). Data was analyzed by Two way ANOVA followed by a Bonferroni posttest for each group. N=6. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

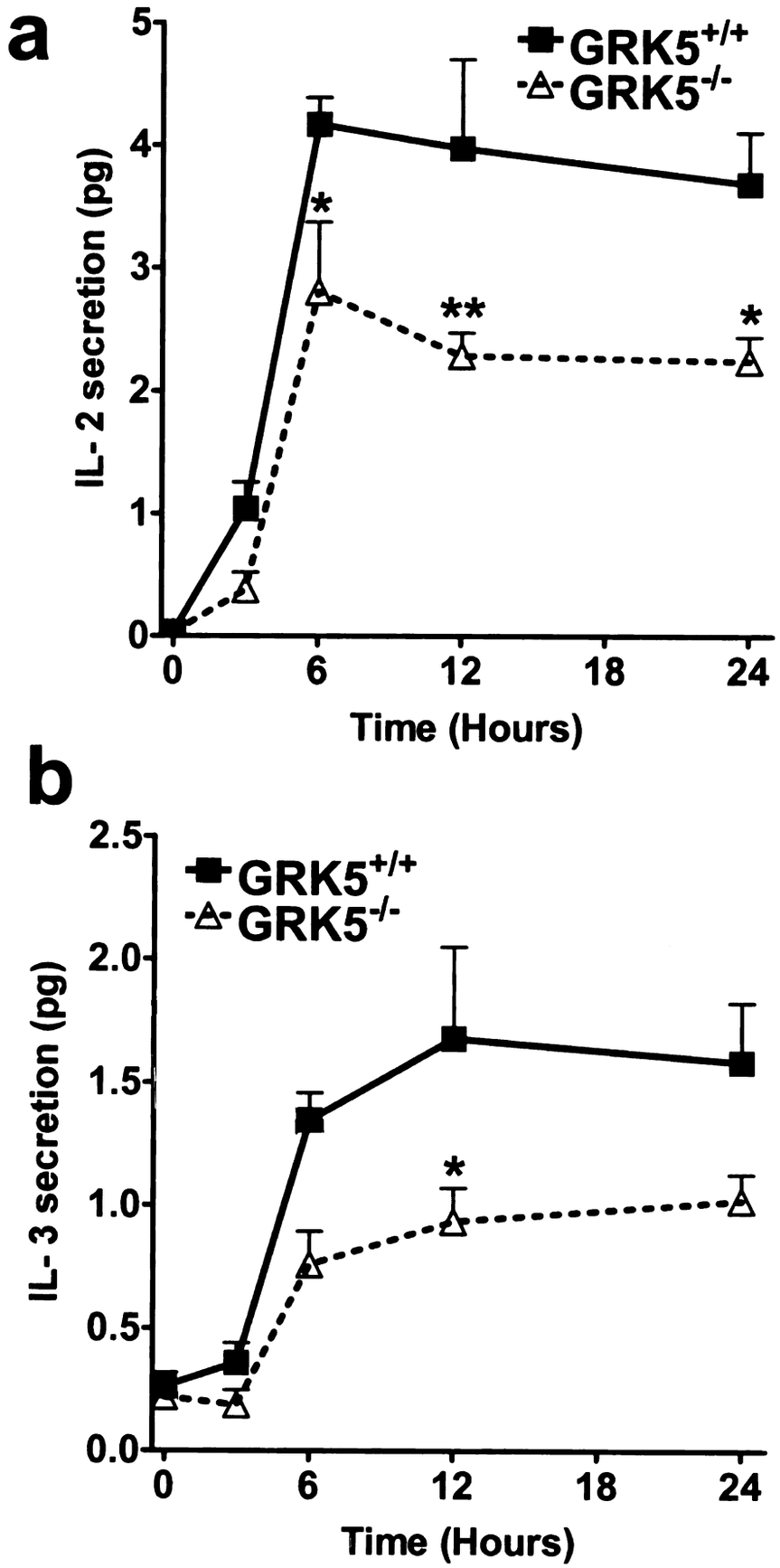

a
GRK5+/+
GRK5-/-
IL-2 secretion (pg)
0
1
2
3
4
5
*
**
*
0
6
12
18
24
Time (Hours)
b
GRK5+/+
GRK5-/-
IL-3 secretion (pg)
0.0
0.5
1.0
1.5
2.0
2.5
*
0
6
12
18
24
Time (Hours)

Figure 4.3. Continued....

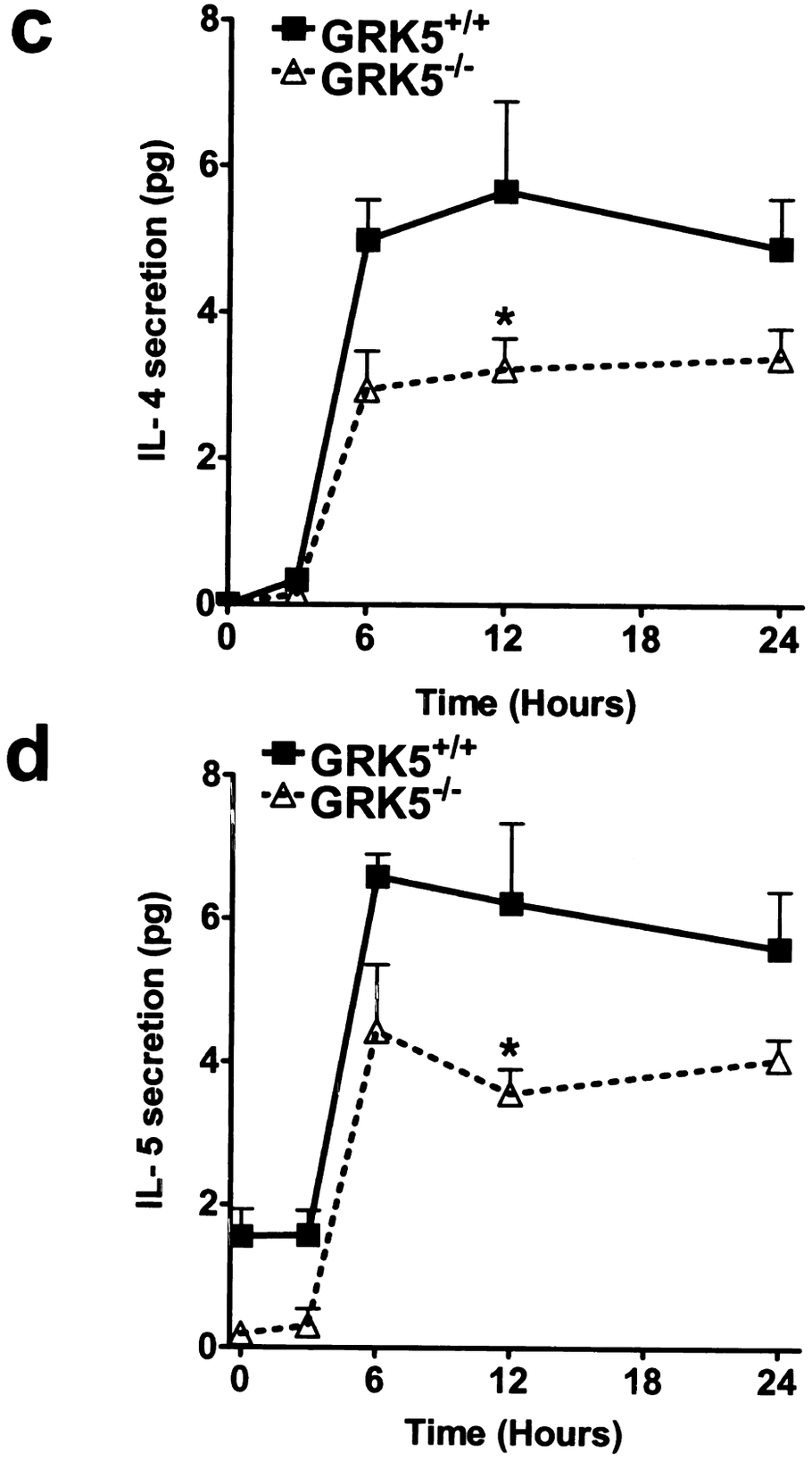
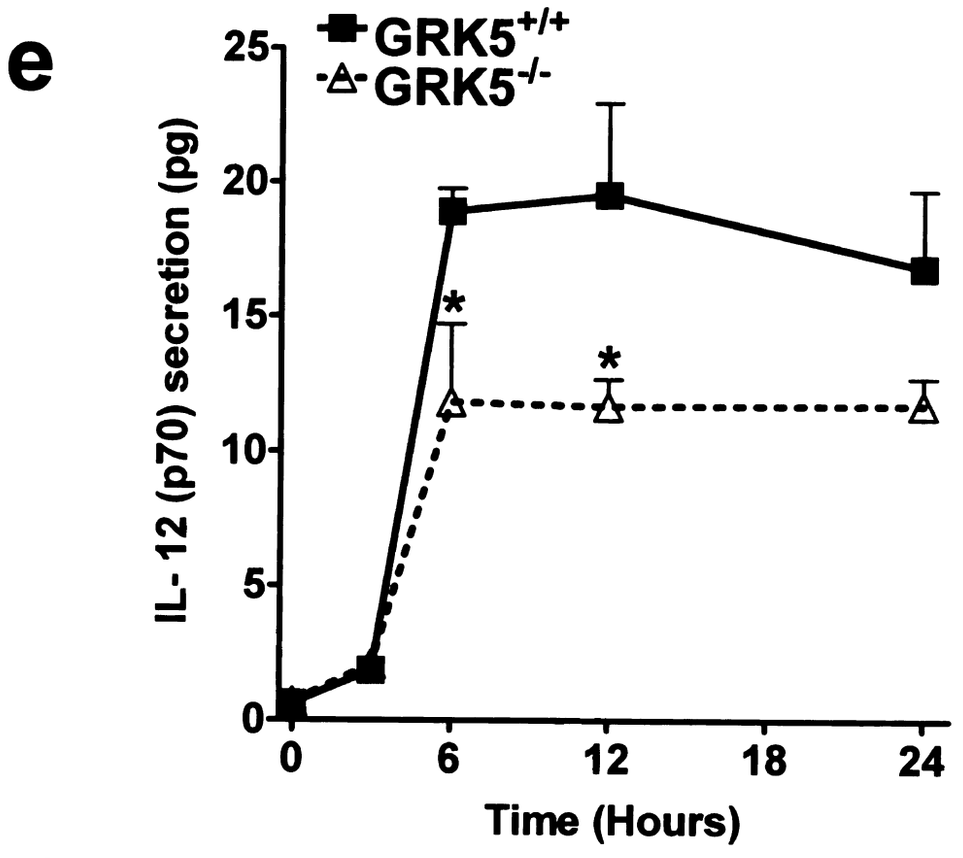


Figure 4.3. Continued....



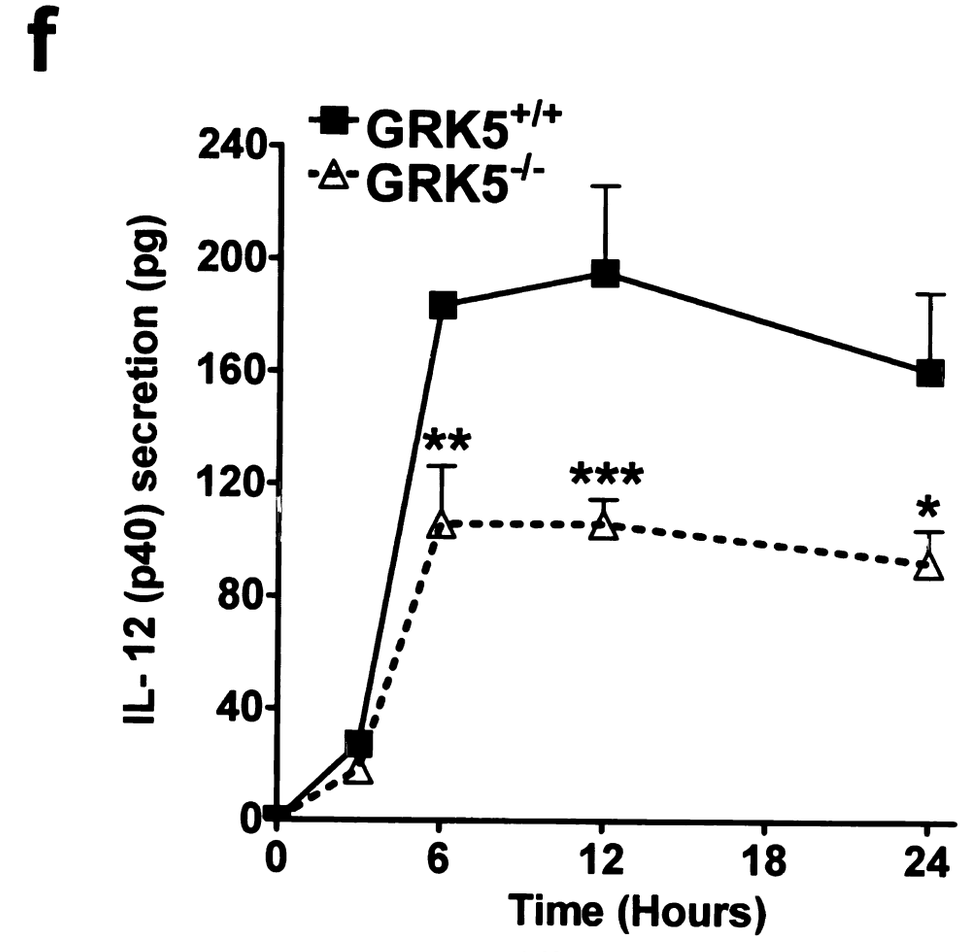


Figure 4.3. Continued....

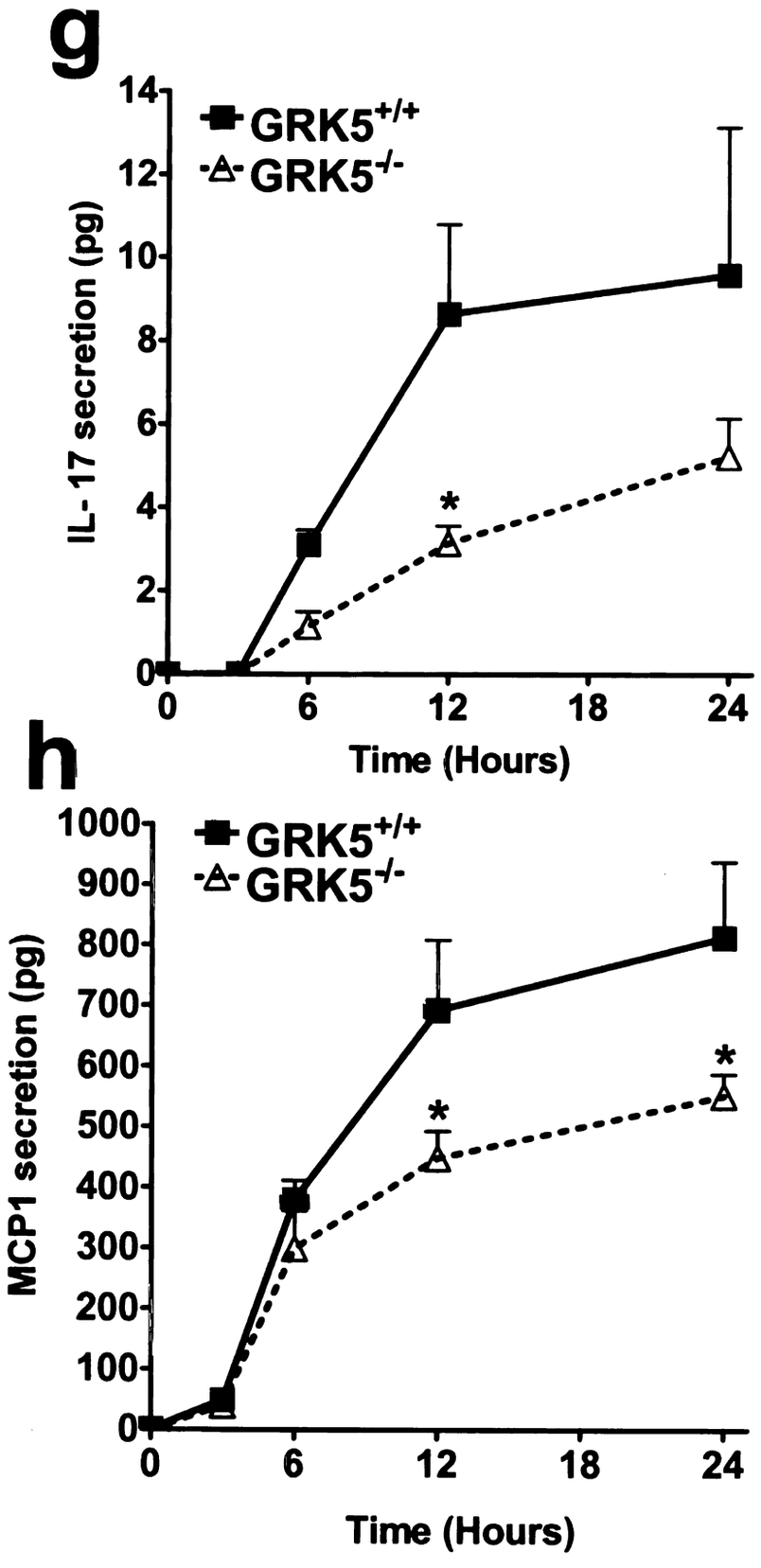
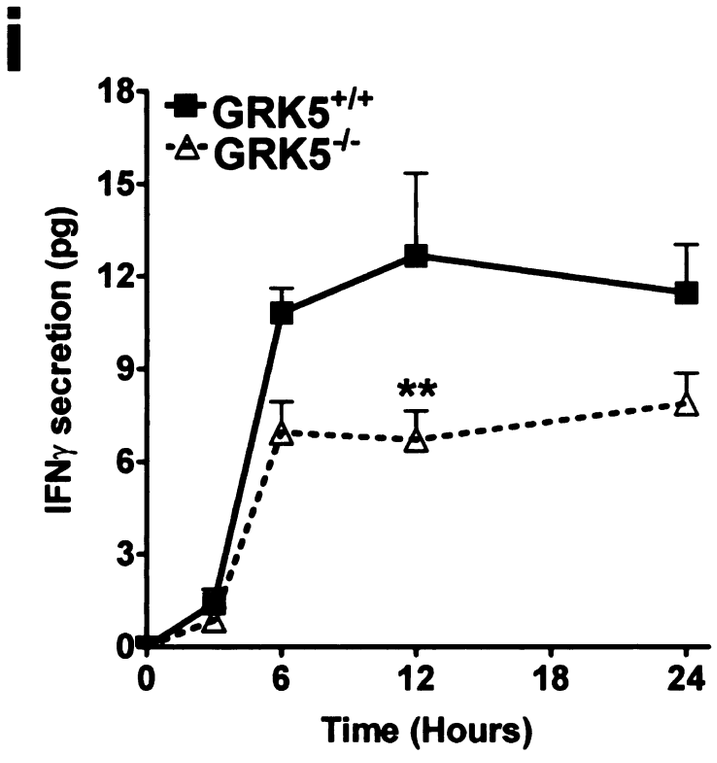


Figure 4.3. Continued....



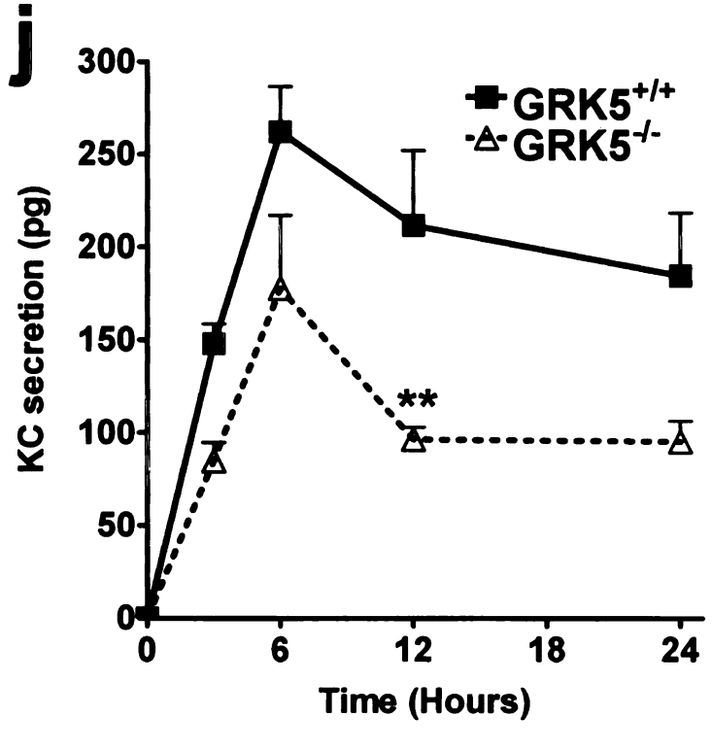
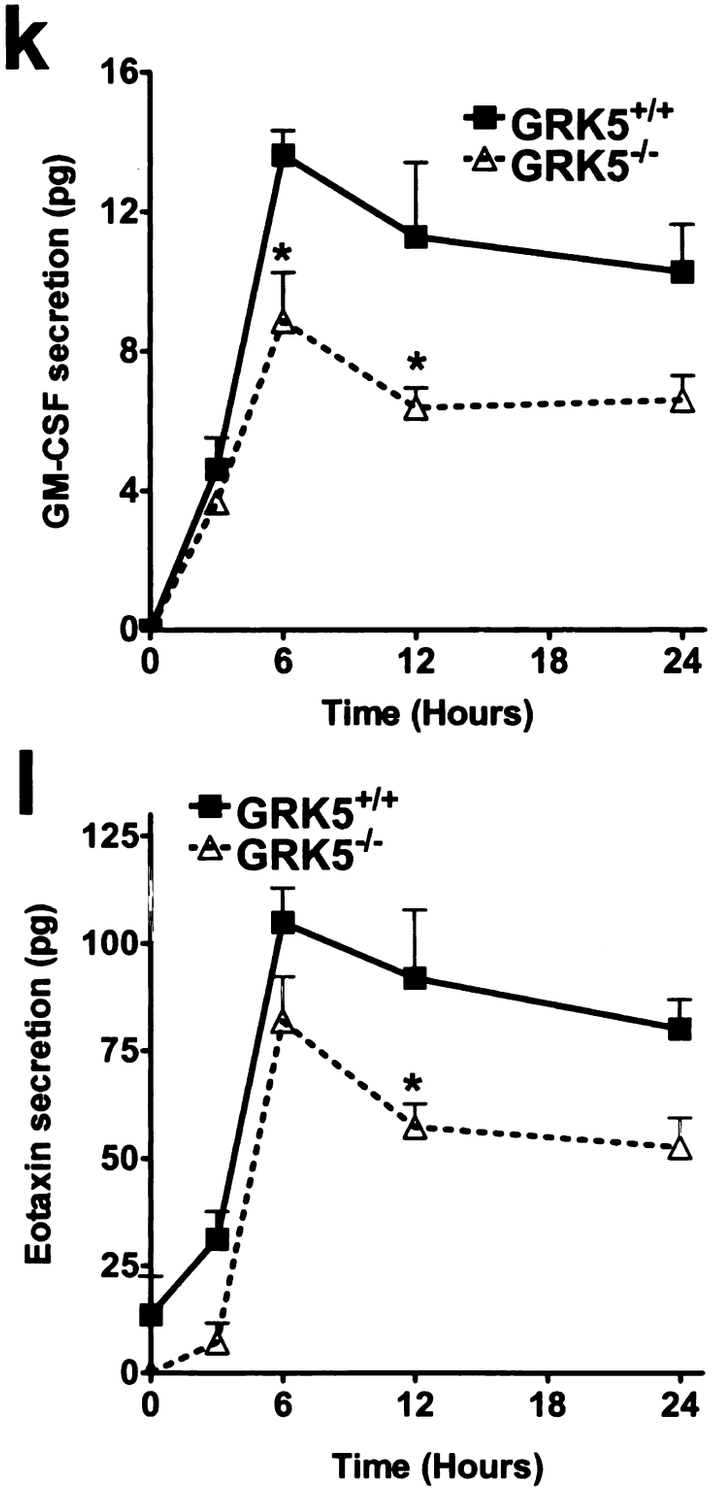


Figure 4.3. Continued....



severe side effects. Detectable levels of these inflammatory mediators i.e. cytokines and chemokines in the blood stream are indicative of their exacerbated production. Plasma is one of the major sources which can be utilized to measure the levels of these inflammatory mediators. Moreover, evaluation of plasma cytokines over a time course can provide a better understanding of the nature and severity of the disease process. To investigate the role of GRK5 in TLR4 signaling *in vivo*, we sought to determine the LPS induced production of cytokines and chemokines in mice plasma. For this purpose, $GRK5^{+/+}$ and $GRK5^{-/-}$ mice were intra-peritoneally injected with LPS (30μg/gm body weight) and blood collected at 1, 3 and 12 hours after injection. Using Biorad's 23-plex cytokine assay, we determined the plasma levels of various cytokines and chemokines after LPS injection. We rationalized that, because GRK5 mediates a broad spectrum of cytokine/chemokines in the primary macrophages *in vitro*, LPS injection *in vivo* in mice would result in diminished cytokine responses in the $GRK5^{-/-}$ mice. Consistent with this prediction, LPS-induced cytokine/chemokine levels were broadly inhibited in the $GRK5^{-/-}$ mice compared to $GRK5^{+/+}$. Again, as is the case with the primary macrophages, not all cytokine/chemokines were affected in this manner. Only a subset of cytokines/chemokines were affected by GRK5 deficiency suggesting selective regulation of these cytokines/chemokines by GRK5. Cytokines that were inhibited in the $GRK5^{-/-}$ mice included TNFα (**Fig. 4.4a**, WT: 218576±35294pg/ml; KO: 119970±23211pg/ml at 1 h time point), IL-1β (**Fig. 4.4b**, WT: 2237.927±648.049pg/ml; KO: 1052.435±108.982pg/ml at 12 h time point), IL-2 (**Fig. 4.4c**, WT: 54.615±2.745pg/ml; KO: 36.817±2.053pg/ml at 12 h time point), IL-3 (**Fig. 4.4d**, WT: 37.070±6.248pg/ml; KO: 22.973±2.274pg/ml at 12 h time point), IL-4 (**Fig.**

Figure 4.4 Profile of plasma cytokines after LPS challenge in WT ($GRK5^{+/+}$) and GRK5 null ($GRK5^{-/-}$) mice

Plasma cytokines TNFα (**a**), IL-1β (**b**), IL-2 (**c**), IL-3 (**d**), IL-4 (**e**), IL-5 (**f**), IL-10 (**g**), IL-12 (p40) (**h**), IL-12 (p70) (**i**), IL-13 (**j**), IL-17 (**k**), MCP1 (**l**), Rantes (**m**), Eotaxin (**n**) were determined in WT and $GRK5^{-/-}$ mice after an intra-peritoneal injection of 30μg/g body weight LPS at specified times viz. 1 hr, 3 hrs and 12 hrs (N=6 per group). Data was analyzed by Two way ANOVA followed by a Bonferroni posttest for each animal group. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

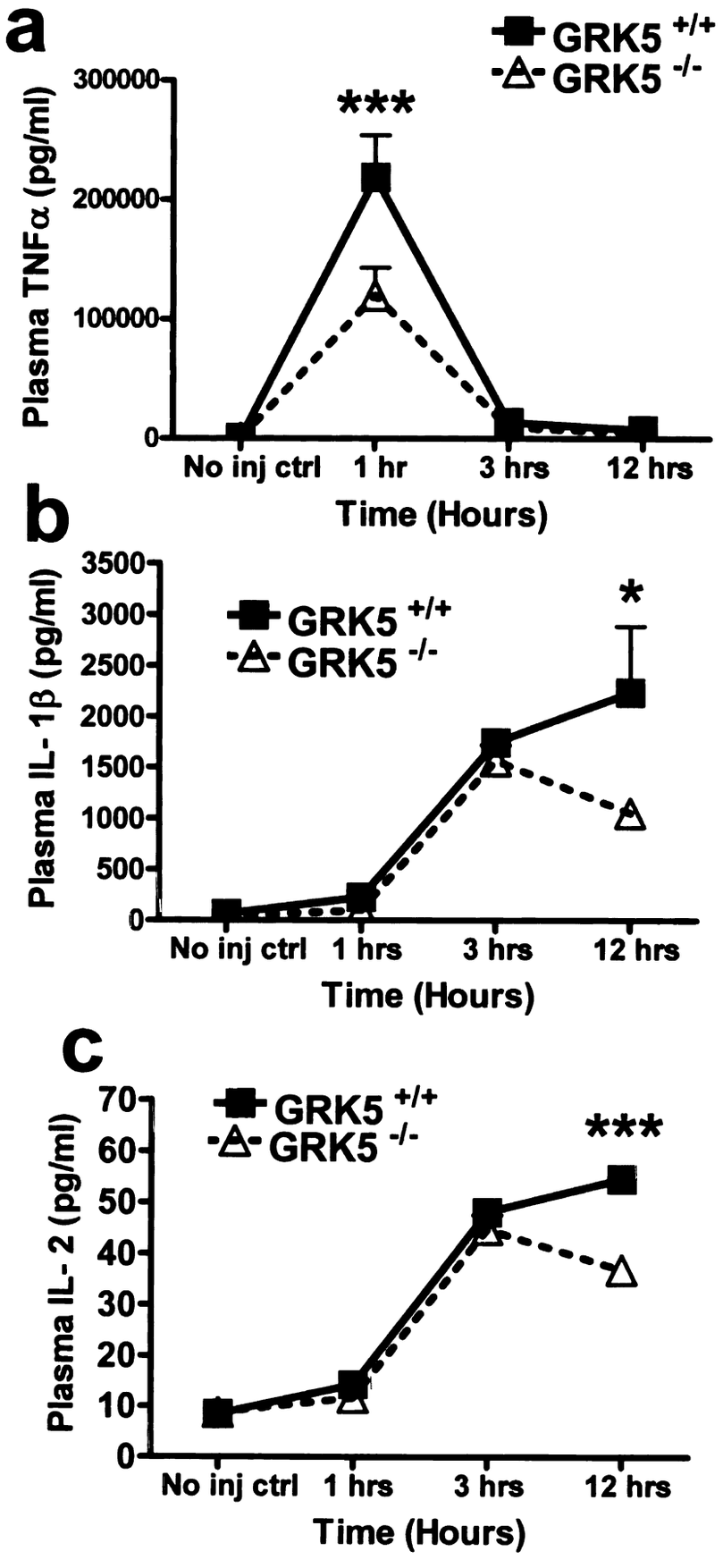

a
GRK5 +/+
GRK5 -/-
300000
200000
100000
0
***
Plasma TNFα (pg/ml)
No inj ctrl
1 hr
3 hrs
12 hrs
Time (Hours)
b
3500
3000
2500
2000
1500
1000
500
0
GRK5 +/+
GRK5 -/-
*
Plasma IL-1β (pg/ml)
No inj ctrl
1 hrs
3 hrs
12 hrs
Time (Hours)
c
70
60
50
40
30
20
10
0
GRK5 +/+
GRK5 -/-
***
Plasma IL-2 (pg/ml)
No inj ctrl
1 hrs
3 hrs
12 hrs
Time (Hours)

Figure 4.4. Continued....

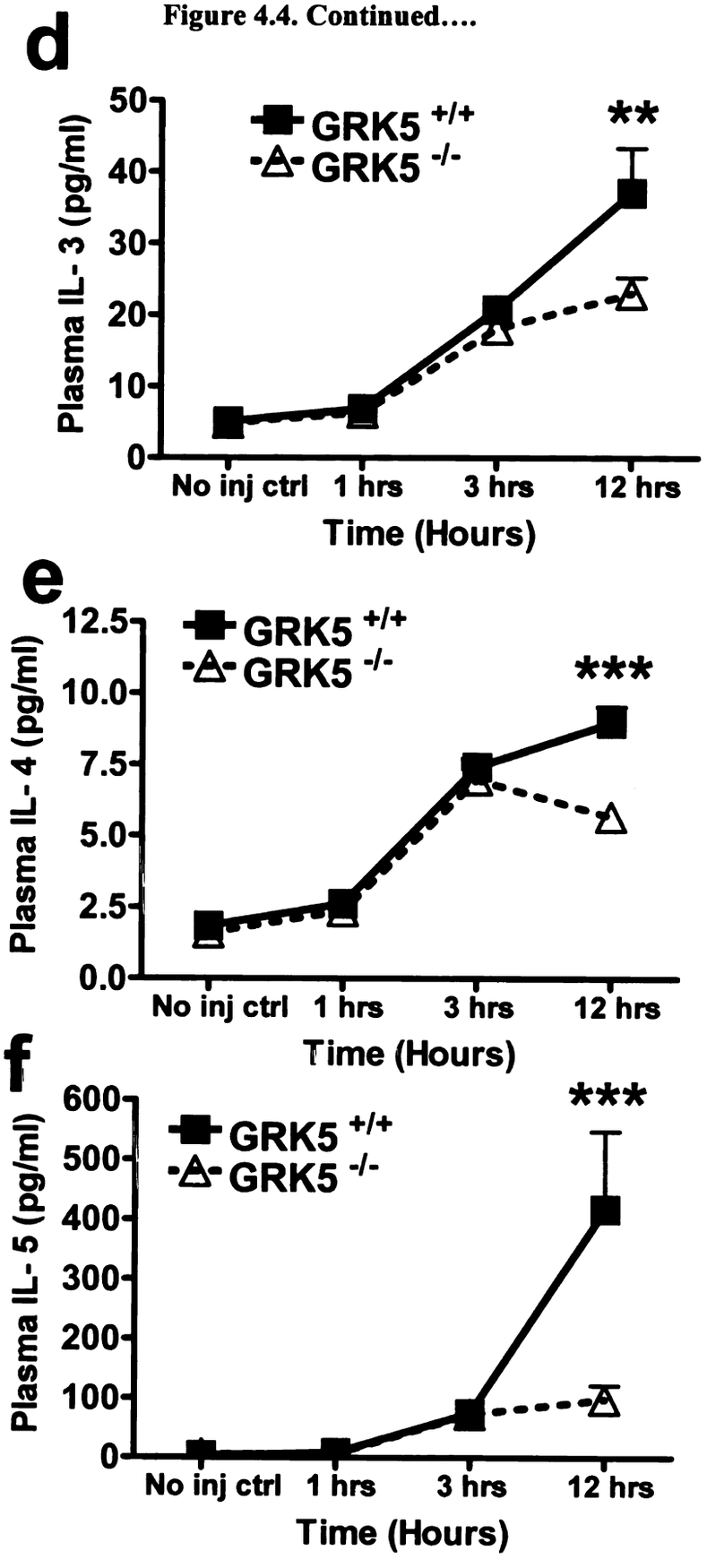
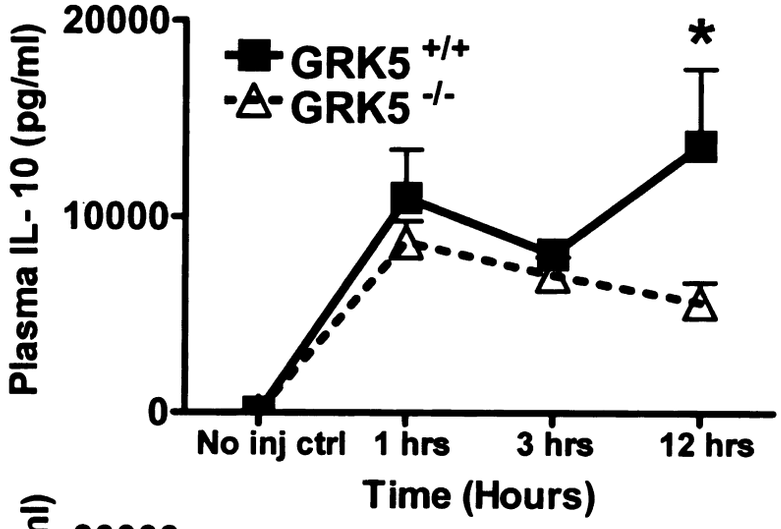


Figure 4.4. Continued....

g



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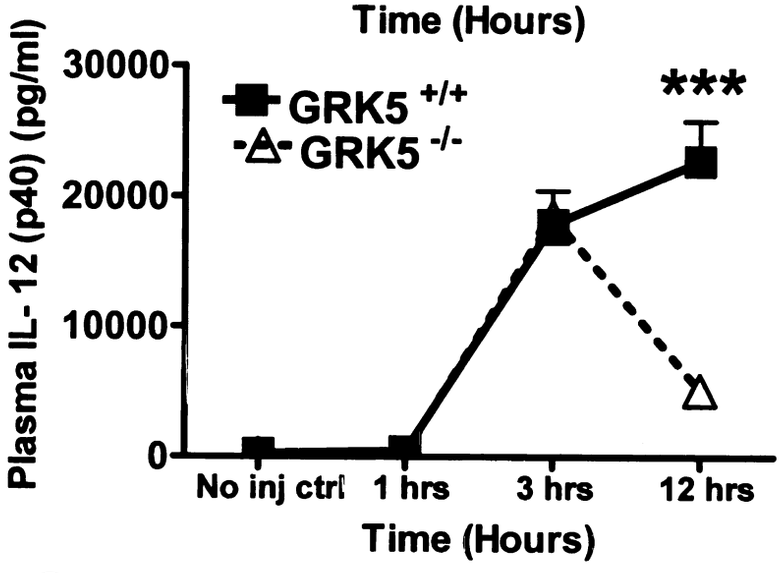


i

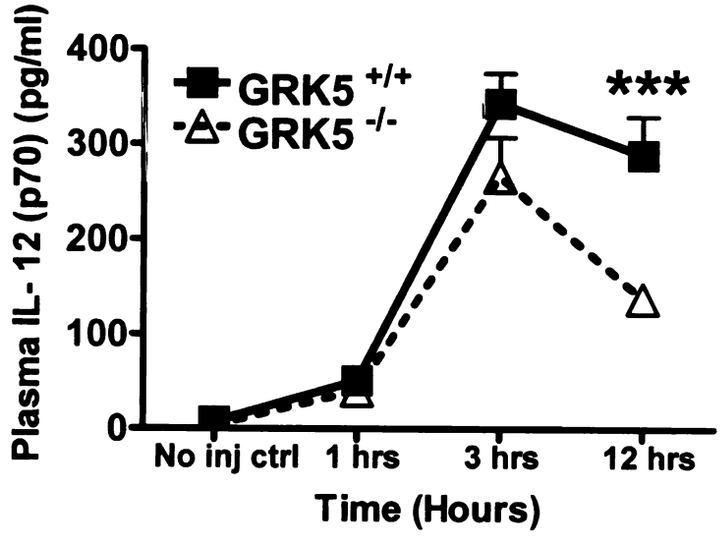


Figure 4.4. Continued....

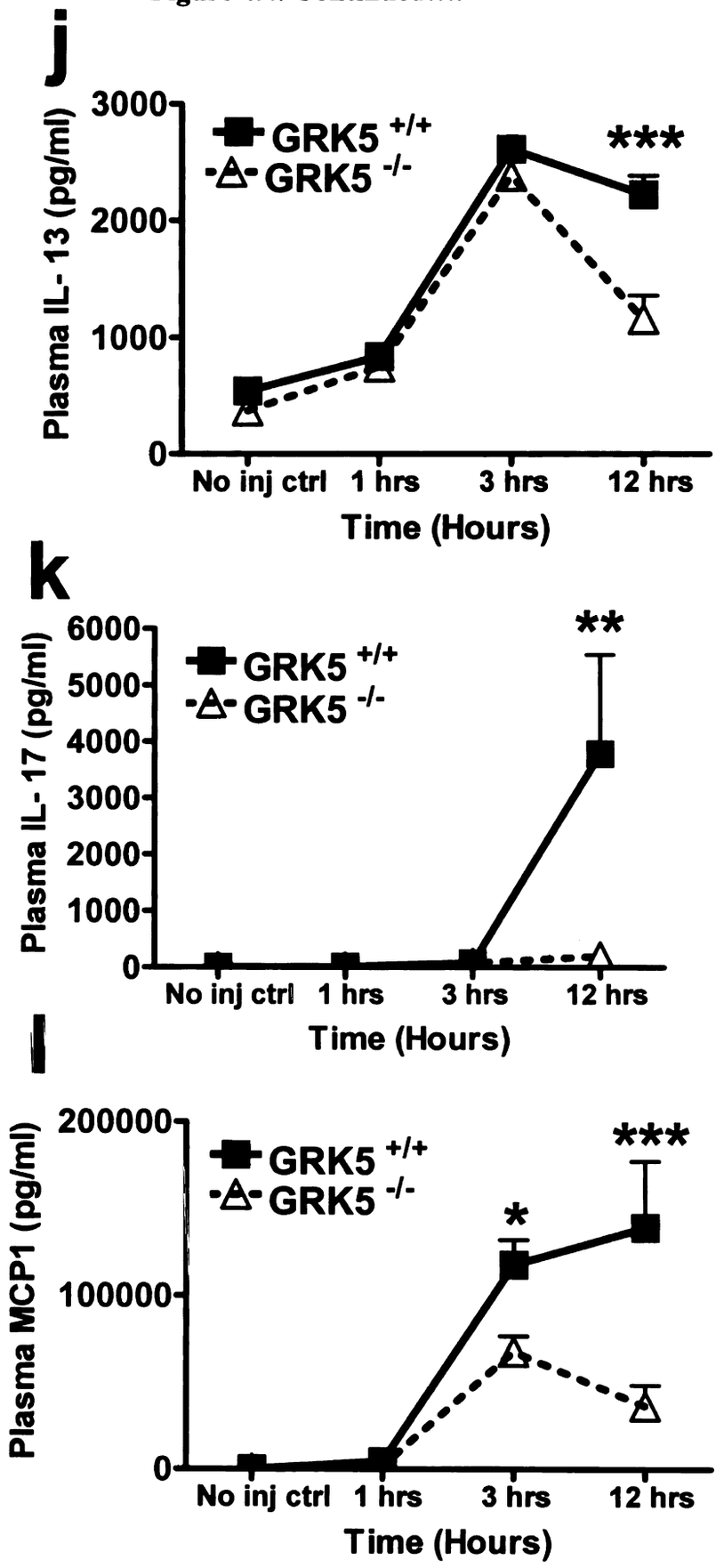
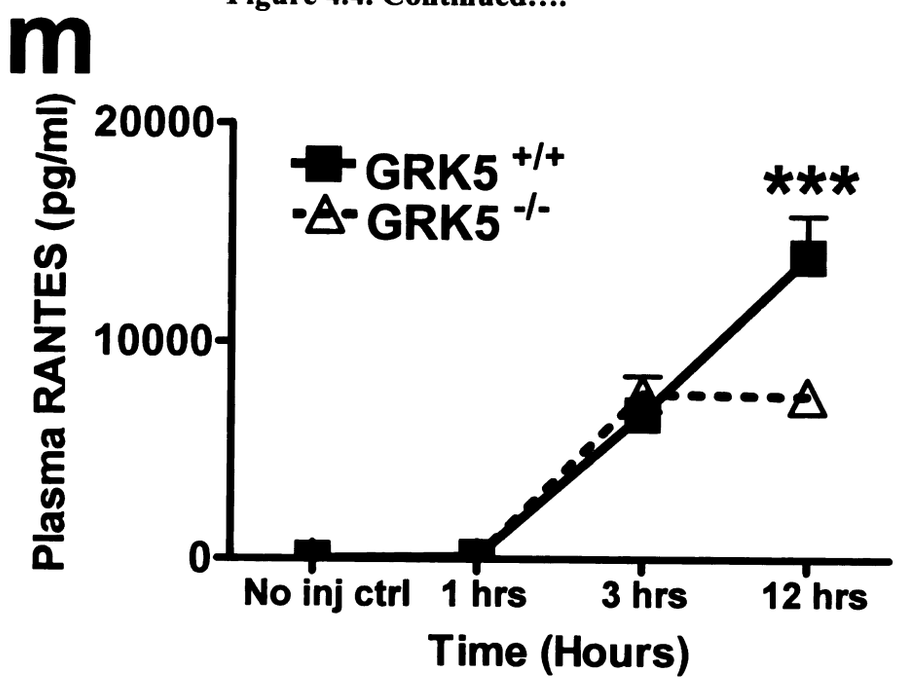
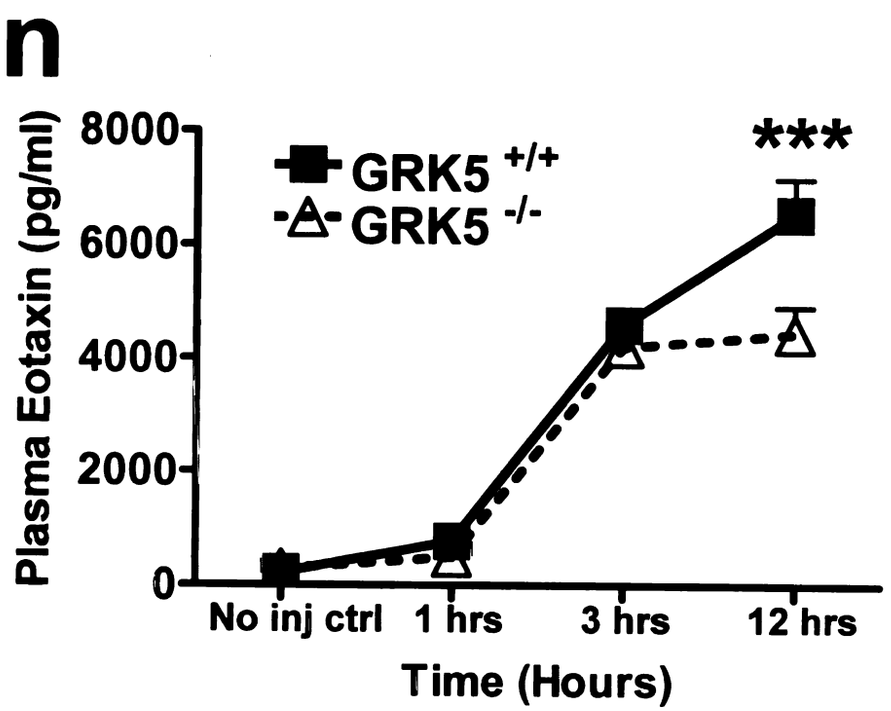


Figure 4.4. Continued....





4.4e, WT: 8.970±0.571pg/ml; KO: 5.672±0.375pg/ml at 12 h time point), IL-5 (**Fig. 4.4f**, WT: 417.993±129.706pg/ml; KO: 98.120±24.171pg/ml at 12 h time point), IL-10 (**Fig. 4.4g**, WT: 13648.600±3891.250pg/ml; KO: 5645.438±1033.724pg/ml at 12 h time point), IL-12p40 (**Fig. 4.4h**, WT: 22694±3038pg/ml; KO: 5101±1129pg/ml at 12 h time point), IL-12p70 (**Fig. 4.4i**, WT: 288±40pg/ml; KO: 137±13pg/ml at 12 h time point) IL-13 (**Fig. 4.4j**, WT: 2220±163pg/ml; KO: 1158±201pg/ml at 12 h time point), IL-17 (**Fig. 4.4k**, WT: 3780±1756pg/ml; KO: 195±61pg/ml at 12 h time point), MCP1 (**Fig. 4.4l**, WT: 139016±38025pg/ml; KO: 36490±12175pg/ml at 12 h time point), Rantes (**Fig. 4.4m**, WT: 13920±1855pg/ml; KO: 7510±651pg/ml at 12 h time point), Eotaxin (**Fig. 4.4n**, WT: 6548±615pg/ml; KO: 4443±471pg/ml at 12 h time point). Cytokines that were not affected by GRK5 deficiency included GCSF, GM-CSF, IFNγ, IL-1α, IL-6, IL-9, KC, MIP1α, MIP1β (data not shown).

Diminished liver injury in GRK5$^{-/-}$ mice

Due to the fact that a major group of "pro-inflammatory" cytokines and chemokines were markedly decreased in GRK5$^{-/-}$ mice, we hypothesized that tissue injury seen as a result of LPS-induced endotoxemia would be reduced in GRK5$^{-/-}$ mice. To this end, we assessed the extent of liver damage by determining the plasma concentrations of liver injury marker alanine transaminase (ALT) at 12 hours after LPS challenge, both in control GRK5$^{+/+}$ and GRK5 deficient i.e. GRK5$^{-/-}$ mice. Our results show that GRK5$^{-/-}$ mice display significantly reduced levels of plasma ALT as compared to GRK5$^{+/+}$ mice (50±4 U/L in GRK5$^{-/-}$ mice verses 84±10 U/L in GRK5$^{+/+}$ mice) demonstrating that GRK5 deficiency results in reduced LPS dependent

Figure 4.5 GRK5$^{-/-}$ mice show reduced LPS-induced liver injury

GRK5$^{+/+}$ and GRK5$^{-/-}$ mice were injected with LPS and plasma levels of liver injury marker ALT were measured after 12 hours. Data was analyzed by unpaired t test; N=6. *$p<0.05$


GRK5 +/+
GRK5 -/-
ALT level (U/L)
100
75
50
25
0
*

production of inflammatory mediators which translates into a decreased liver injury (**Fig. 4.5**).

Taken together, these results suggest that GRK5 plays an important role in LPS mediated signaling pathway affecting crucial transcription factors responsible for the production of a wide array of pro- as well as anti-inflammatory cytokines, not only in primary macrophages *in vitro*, but also *in vivo*.

DISCUSSION

LPS binding onto TLR4 induces the activation of NFκB as well MAPK signaling pathways. In this study we show that GRK5, a GPCR kinase is a regulator of LPS-induced NFκB signaling pathway. GRK5 null primary macrophages show a significant reduction in LPS-induced phosphorylation of IκBα as well as nuclear translocation of p65, a subunit of NFκB. Furthermore, the binding of NFκB onto its consensus DNA oligonucleotide was also found to be significantly inhibited in GRK5$^{-/-}$ macrophages. These findings are consistent with our recent report that siRNA mediated knockdown of GRK5 in Raw264.7 macrophage cell line significantly blocks TNFα-induced NFκB signaling. In this study we not only confirm our previous findings, we also provide evidence that GRK5, by mediating NFκB signaling, has biologically relevant role, in the production of inflammatory cytokines/chemokines in both primary macrophages *in vitro* and in mice *in vivo*.

Our studies further show that the MAPKs including ERK1/2, JNK and p38 are not regulated by GRK5. LPS-induced P105-ERK pathway was found to be negatively regulated by GRK5 in Raw264.7 macrophage cell line using RNAi against GRK5 [10]. Although we observed ~20% enhancement of pP105 and pERK levels in the GRK5$^{-/-}$ macrophages, it did not reach statistical significance. Thus, Raw264.7 macrophages are clearly different from primary macrophages with regard to the role of GRK5 in ERK activation. It is also quite possible that the different results obtained may be related to the mechanism by which GRK5 was depleted. Germline deletion of a gene could have different consequences when compared to RNAi knockdown. In particular other compensatory mechanisms can come into play when germline deletion is used.

Interestingly, with regard to GRK5 regulation of IκBα, it appears that both methods lead to similar effects suggesting that GRK5 is an important if not the only regulator of IκBα-NFκB signaling. It is important to point out that other kinases including IKKα, casein kinase and ribosomal S6K have also been shown to regulate IκBα-NFκB signaling by phosphorylating IκBα [14-16]. It is clear in the GRK5 knockout that other kinases (especially IKKβ) are also phosphorylating IκBα because phosphorylation in the absence of GRK5 was not completely blocked, but was inhibited ~50%.

LPS binding onto TLR4 receptor leads to the activation of both NFκB and MAPK signaling that eventually induces the expression and production of several cytokines and chemokines. We found that the secretion of several inflammatory cytokines and chemokines is regulated by GRK5 in peritoneal macrophages as well as under *in vivo* conditions. LPS induction of TNFα, IL-1β, IL-2, 3, 4, 5, 10, IL-12p40, IL-12p70, IL-13, IL-17, MCP1, Rantes and eotaxin were found to be inhibited in $GRK5^{-/-}$ mouse plasma.

Cytokines are soluble mediators that coordinate inflammation by being secreted from one cell and activate receptors on other cells. TNFα is the first cytokine that appears in the blood of experimental animal models after LPS injection as well as in human volunteers receiving LPS [17]. TNFα causes the induction of a large number of inflammatory mediators by acting through a signaling cascade as well as by autocrine mechanisms [18, 19]. This has been shown by blocking TNFα which caused a decrease in the levels of other cytokines. For e.g. in baboons infected with *E.coli*, blocking TNFα significantly reduced the levels of IL-1α, IL-6 and IL-8 [20, 21].

Concentration of TNFα observed in sepsis patients correlates with the severity and outcome of the disease. For eg., concentrations of TNFα as well as IL-1β and IL-2 in plasma were found to be higher in patients of septic shock as compared to patients of sepsis alone [22]. In our studies, LPS injection resulted in the secretion of TNFα in the plasma of both $GRK5^{+/+}$ and $GRK5^{-/-}$ mice at 1 hour post injection. The levels observed were, however, significantly less in $GRK5^{-/-}$ mice.

IL-1 family of cytokines, in particular IL-1β is a key pro-inflammatory mediator. IL-1β is released in response to an insult and initiates a host defense response by up-regulating other cytokines, acute phase proteins as well as by acting as a potent pyrogen [23, 24]. Furthermore, it has been shown to be required for the clearing of bacterial infections [25]. IL-1β does so by activating MAPK and NFκB pathways [26]. Excessive activation of IL-1β however, results in multi organ failure which is commonly observed in sepsis [27]. We found sustained plasma levels of IL-1β up to 12 hours of LPS injection in $GRK5^{+/+}$ mice. However, IL-1β levels reach a peak by 3 hours and thereby start declining in $GRK5^{-/-}$ mice. At 12 hours, the levels observed in $GRK5^{-/-}$ mice plasma were significantly less as compared to those observed in $GRK5^{+/+}$ mice. Initial increase in IL-1β is important to fight against infection, however, a sustained activation might lead to endotoxemia. We did not see a significant difference in the levels of IL-1β in the cell culture supernatants of peritoneal macrophages however, suggesting that GRK5 might regulate the production

of IL-1β in cells other than macrophages. It is also possible that the integrative pathophysiology present *in vivo* may not be reproducible *in vitro*.

IL-12p40 is produced by activated macrophages, neutrophils, microglia and dendritic cells. IL-12 has been shown to be important for the production of IFNγ and leads to lethality in LPS induced septic shock model of mice [28]. IL-17 is secreted primarily by T lymphocytes and is involved in recruiting neutrophils and acts together with other cytokines such as TNFα and IL-1β, to further enhance inflammation [29]. Eotaxin and MCP1 act as chemoattractants for eosinphils and leucocytes respectively.

IL-13, IL-4 and IL-10 are anti-inflammatory cytokines which act by inhibiting the production of pro-inflammatory cytokines. For instance, IL-13 has been shown to act by inhibiting the production of IL-1β and TNFα which are induced by LPS. Since some of the cytokines which were found to be reduced in GRK5$^{-/-}$ mice are primarily T-cell cytokines, future studies will look into the role of these cell type specific cytokines in inducing inflammation.

Although secretion of pro-inflammatory cytokines serves as an essential prerequisite for initiating an effective innate immune response to fight infection, they are also associated with deleterious effects leading to multi organ failure and ultimately death. Similarly, anti-inflammatory cytokines despite important for controlling an exaggerated inflammatory response, also lead to a suppression of the immune system which is required for a proper functioning of the system. Systemic inflammatory response syndrome (SIRS) is a consequence of an imbalance between pro and anti-inflammatory cytokines. Our studies show that absence of GRK5 reduces the production of several pro as well as anti-inflammatory cytokines. These cytokines

are still produced to some extent which is crucial for inducing an effective immune response. However, since exaggerated production of these inflammatory mediators is modulated by GRK5, we speculate that our results would have important consequences in inflammatory diseases. In fact, our observations on reduced ALT levels in the $GRK5^{-/-}$ mice compared to the $GRK5^{+/+}$ mice suggests that inhibition of GRK5 in sepsis might mitigate organ injury. Future studies will address whether GRK5 would be a viable therapeutic target for inflammatory diseases.

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CHAPTER 5

Myeloid specific G-protein coupled receptor kinase 2 (GRK2) is a negative regulator of NFκB1p105-ERK pathway and limits endotoxemic shock in mice

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ABSTRACT

G-protein coupled receptor kinase 2 (GRK2) is a serine-threonine kinase which plays an important role in phosphorylation and desensitization of G-protein coupled receptors (GPCRs). It is highly expressed in myeloid cells particularly in macrophages and its expression levels are altered in a number of inflammatory disorders including sepsis. Using myeloid cell-specific GRK2 knockout mice ($GRK2^{\Delta mye}$) we demonstrate that GRK2 acts as a negative regulator of LPS-induced inflammatory cytokine production *in vivo*. Consistent with this effect, LPS-mediated lung and liver injury as well as lethality are enhanced in the $GRK2^{\Delta mye}$ mice compared to the wild type litter-mates ($GRK2^{fl/fl}$). Similar to the plasma cytokine levels, peritoneal macrophages from the knockout mice show enhanced secretion of pro-inflammatory cytokines in response to LPS stimulation. To elucidate the biochemical and signaling mechanisms mediating these pathophysiological effects of GRK2, we examined the activation of various signaling pathways in response to LPS in $GRK2^{fl/fl}$ and $GRK2^{\Delta mye}$ mice. Our results indicate that disruption of GRK2 in the macrophages selectively enhances the activation of NFκB1 P105-ERK1/2 pathway in response to LPS stimulation. Importantly, inhibition of the ERK pathway limits the enhanced production of some LPS-induced cytokines/chemokines in the GRK2 knockout mice, demonstrating the role of the enhanced ERK activation in the pro-inflammatory phenotype of the GRK2 knockout macrophages. Taken together, our studies reveal previously undescribed role for GRK2 in LPS-induced p105-ERK pathway and the consequent pro-inflammatory cytokine production and endotoxemia in mice.

INTRODUCTION

G-protein coupled receptor kinases (GRKs) are serine-threonine kinases that recognize and phosphorylate activated G-protein coupled receptors (GPCRs) thus causing a cessation of G-protein-dependent signaling. GRK2 is one of seven members of GRKs and is expressed at high levels in immune cells [1, 2]. Furthermore, expression levels of GRK2 has been found to be altered in immune cells from human patients with a variety of immunological disorders as well as in a number of animal disease models [3-7]. In this regard, recent studies have shown that the expression levels of GRK2 is increased in polymorphonuclear (PMNs) cells from septic patients [8]. In addition, GRK2 levels were found to be increased in human PMNs in response to lipopolysaccharide. This increase in GRK2 levels has been postulated to be important in limiting chemokine receptor (a GPCR)-induced chemotaxis of immune cells. In fact, neutrophils from human septic patients showed significantly attenuated chemotaxis of neutrophils [8]. Furthermore, Toll-like receptor-2 induced polymicrobial sepsis was shown to mediate downregulation of CXCL2 receptor (a GPCR), as well as CXCL2-induced chemotaxis of neutrophils and this was proposed to be mediated via upregulation of GRK2 expression in these immune cells. Several other studies have also determined the role of GRK2 in immune cell chemotaxis owing to the fact that chemokine receptors belong to the GPCR family and that the observations are remarkably in-tune with the known classic role for GRK2, that is, GPCR desensitization. In spite of these seminal advances in GRK2 biology, role of GRK2 in macrophages particularly in response to non-GPCRs is not well understood.

More importantly, role of myeloid cell-specific GRK2 in lipopolysaccharide-induced inflammation and endotoxemia *in vivo* is not known.

Lipopolysacchrides (LPS) activates a class of innate immune receptors called the Toll-like receptors (TLRs) which act as the first line of host defense against bacterial infections. Toll like receptors (TLRs) recognize a wide variety of microbial products known as pathogen associated molecular patterns (PAMPs) from bacteria, virus and other pathogens. Among the TLRs, LPS specifically activates TLR4 and triggers an inflammatory response that is necessary to fight against infection. Under endotoxemic conditions, however, this system is over-stimulated and therefore the cytokine response elicited by the host is harmful to the host resulting in endotoxemic shock and eventual death. Activation of TLR4 by LPS triggers the recruitment of adapter proteins such as TRIF and Myd88 as well as other TIR domain containing proteins that eventually activates the inhibitor of κB kinase (IKK) complex [9]. The activated IKK complex then phosphorylates IκBα (an inhibitor of NFκB) thereby targeting it for ubiquitination and proteasomal degradation. IκBα degradation then enables the release and nuclear translocation of NFκB which then regulates the expression of genes involved in inflammation as well as innate and adaptive immune responses. In macrophages, activation of IKK complex also phosphorylates NFκB1 p105 (another IκB protein), which under unstimulated conditions is stoichiometrically bound to a MAP3K called TPL2. LPS stimulation and phosphorylation of p105 leads to eventual partial degradation of p105 and subsequent release of TPL2. P105-free TPL2 then activates MEK1/2, which then activates the ERK1/2 pathway. In addition to these pathways, LPS also mediates the activation of p38, JNK, Akt signaling

pathways. TLR4-induced activation of these signaling pathways and the subsequent activation of transcription factors such as NFκB, AP-1 and EGR-1 mediates the pathogenesis of inflammation and endotoxemia and shock.

Although chemotaxis of immune cells play a pivotal role in the pathogenesis of endotoxemia and sepsis, production of inflammatory cytokines and chemokines also play a requisite role in this pathogenesis. Because cells of the myeloid lineage, particularly the macrophages express high levels of GRK2 and that TLR4 activation enhances GRK2 levels significantly in these cells, we hypothesized that myeloid cell-selective knockout of GRK2 will significantly affect the pathogenesis of TLR4-induced inflammation and endotoxemia in mice. In this study, we generated a myeloid-cell-specific GRK2 knockout and demonstrate that GRK2 negatively regulates TLR4-induced NFκB1 p105-ERK pathway, thereby limiting the pathogenesis of endotoxemic shock in mice.

MATERIALS AND METHODS

Materials

Protease inhibitor cocktail tablets were from Roche Diagnostics (Indianapolis, IN), Phospho ERK, Phospho p38, Phospho JNK, JNK, NFκB1 Phospho P105, Phospho AKT, AKT, iNOS, phospho-IκBα and IκBα antibodies were from Cell Signaling Technology (Boston, MA). ERK, NFκB1 P105 and HRP (Horseradish Peroxidase)-conjugated anti-actin antibody were from Santa Cruz Biotechnology. Tubulin antibody was from sigma. Monoclonal GRK2 antibody was from Upstate Biotechnology. E. coli LPS (0111:B4) from Sigma was used for mice injections. Ultra pure LPS from Invivogen was used for *in vitro* peritoneal macrophage stimulation.

Animals

Animals were housed four to five mice per cage at 22–24°C in rooms with 50% humidity and a 12-h light–dark cycle. All animals were given mouse chow and water *ad libitum*. All animal procedures were approved by the Michigan State University Institutional Animal Care and Use Committee and conformed to NIH guidelines. Tail tips were used for isolating genomic DNA and genotyping performed by PCR. All experiments were performed on male mice, 6-8 weeks of age.

Generation of GRK2$^{\Delta mye}$ mice

GRK2$^{fl/fl}$ mice in which exons 3-6 of GRK2 are flanked by LoxP sites were crossed with LysMCre mice to generate GRK2$^{fl/fl\ LysMCre}$ mice. A breeding colony was maintained by mating GRK2$^{fl/fl}$ with GRK2$^{fl/fl\ LysMCre}$. The mice were generated on a mixed C57BL6/129sv background. GRK2$^{fl/fl\ LysMCre}$ were used in

experiments and compared only to littermate $GRK2^{fl/fl}$ controls. LysMCre and $GRK2^{fl/fl}$ mice were genotyped as described previously respectively [10, 11]. Myeloid cell GRK2 deleted mice ($GRK2^{\Delta mye}$) will be referred to as GRK2 deficient and littermate control mice ($GRK2^{fl/fl}$) will be referred to as control mice in the entire manuscript.

Peritoneal Macrophage isolation

To isolate peritoneal macrophages, mice were injected by intra-peritoneal injection with 1ml of 4% thioglycollate. Peritoneal macrophages were collected by performing a peritoneal cavity lavage after 4 days of thioglycollate injection in Dulbecco's phosphate buffered saline (DPBS). Cells were washed atleast three times and then counted and plated on cell culture plates in RPMI 1640 media supplemented with 10% fetal bovine serum (Invitrogen) and penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37° C in 5% CO_2. After around 18 hours of plating, cells were serum starved for ~ 3-4 hours and stimulated with LPS (1μg/ml) for the indicated time points.

Cytokine analysis

A mouse 23-plex multiplex based assay was used to determine the cytokine/chemokine concentrations according to manufacturer's instructions via Luminex 100 technology as described previously [12]. Plasma from LPS injected mice collected at different time intervals and supernatants from peritoneal macrophages stimulated with LPS for different time points were used to assess the cytokine/chemokine levels.

Bronchioalveolar lavage fluid (BALF)

Bronchioalveolar lavage fluid was collected at different intervals after LPS injection (using 2ml of 0.9% Normal saline). In each mice, around 90% of the total injected volume was consistently recovered. The BALF was centrifuged at 450g for 10 min. The supernatants were used to determine the concentration of total proteins using Bradford assay with bovine serum albumin (BSA) as a standard.

Western blot analysis

Cells were lysed in lysis buffer (20mM Tris-HCl (pH 7.4), 1mM EDTA, 150mM NaCl) containing 1% Triton X-100 with protease inhibitors. Lysed cells were then centrifuged at a maximum speed (13,000 X g) for 10 min at 4°C and protein concentration of the supernatants determined by Bradford assay. Western blotting was performed as described previously [13]. Briefly, equal amounts of protein were run on polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were then blocked in Licor blocking buffer (Licor Biosciences) or 5% w/v skimmed milk for 1 hour after which the membranes were incubated in primary antibodies overnight. Secondary antibodies used were either fluorescently tagged or HRP-conjugated. Blots were developed either on Licor odyssey or using chemiluminescence.

Morphological assessment of liver injury:

Livers from control as well as GRK2 deficient mice, both LPS injected as well as PBS (vehicle control) injected for 12 hours were fixed in 10% buffered formalin solution for histological examination after sectioning of tissues and staining with Hematoxylin and Eosin.

Survival study

Six to eight week old mice were challenged via an intra-peritoneal injection of LPS (20μg/gm body weight) from Escherichia Coli (serotype 0111:B4; Sigma-Aldrich, St. Louis, MO). The mice were monitored for LPS induced lethality every 6 hours for a period of 48 hours. Differences in survival were analyzed using Kaplan-Meier test using Prism 5 software (GraphPad Software, La Jolla, CA). A value of less than 0.05 was considered as statistically significant.

Statistical analysis

All values are represented as mean ± SEM. Data were analyzed and statistics performed using GRAPHPAD PRISM software (San Diego, California). The student's t-test was used to compare mean values between two experimental groups and Analysis of Variance (ANOVA) with Bonferroni posttest was used to compare more than two groups. P value of less than 0.05 was considered significant.

RESULTS

Generation and characterization of myeloid cell deficient GRK2 mice

Generation of myeloid cell specific GRK2 deficient mice was accomplished by first breeding mice expressing Cre recombinase under the control of Lysozyme-M (LysM) promoter (LysM-Cre mice express Cre-recombinase specifically in myeloid cells) with mice homozygous for Lox-P flanked GRK2 alleles ($GRK2^{fl/fl}$). Double heterozygous mice obtained from this breeding ($GRK2^{fl/-}$ $LysMCre^{+/-}$) were further intercrossed to obtain $GRK2^{fl/fl}$ $LysMCre^{+/-}$ mice (myeloid -specific GRK2 deficient: $GRK2^{\Delta mye}$) and $GRK2^{fl/fl}$ (control mice). A breeding colony was then maintained to breed $GRK2^{fl/fl}$ $LysMCre^{+/-}$ with $GRK2^{fl/fl}$ to generate both $GRK2^{fl/fl}$ $LysMCre^{+/-}$ as well as $GRK2^{fl/fl}$ mice. $GRK2^{\Delta mye}$ and $GRK2^{fl/fl}$ littermates were used throughout this study. $GRK2^{\Delta mye}$ were born at the expected Mendelian ratios. Furthermore, immunoblot analysis of peritoneal macrophages and bone marrow derived macrophages showed the absence of GRK2 protein only in macrophages from $GRK2^{\Delta mye}$ mice but not in $GRK2^{fl/fl}$ mice (**Figure 5.1C and 5.1D**). However, the expression of GRK2 was not affected in whole organs such as lungs and spleen as shown in **Figure 5.1E**.

Myeloid lineage GRK2 deficiency does not affect the development or maintenance of myeloid cells

Since GRK2 has been shown to be essential for embryonic development of mice, we examined the total numbers of myeloid lineage cells in both GRK2 deficient

and control mice. For this purpose, we performed a complete blood count (CBC) on both GRK2 deficient and control mice. As shown in **Figure 5.2**, we did not observe any difference in the total numbers of RBC's, platelets, WBCs, neutrophils, lymphocytes, monocytes, suggesting no phenotypic difference in terms of development of blood cells in myeloid cell-specific deficiency of GRK2. LPS treatment however, did cause a significant increase in the number of RBCs as well as hemoglobin content in both control and GRK2 deficient mice (**Figure 5.2A and 5.2B**). Furthermore, there was a significant increase in the number of segmented neutrophils and a marked decrease in the number of lymphocytes after LPS treatment in both control and GRK2 deficient mice (**Figure 5.2C**). This however, was also not different between the two genotypes. Interestingly, there was a significant increase in the number of eosinophils in LPS treated GRK2 deficient mice compared to the corresponding controls (**Figure 5.2D**). While the significance of this is not clear in endotoxic shock, it is possible that tissue factor (TF) expression by eosinophils may play a role in hypercoagulation observed in endotoxic mice and this may be exaggerated in the GRK2 deficient mice [14].

However, we did not observe any difference in the plasma prothrombin time or partial thromboplastin time in LPS treated control mice and GRK2 deficient mice (data not shown). Even though plasma coagulability is not affected in GRK2 knockout, it is possible that increased eosinophils in LPS treated GRK2 deficient mice might lead to an increased TF which could then cause increased deposition of fibrin clots in small blood vessels, thus impairing tissue perfusion and ultimately causing an organ failure.

Figure 5.1 Generation and characterization of GRK2$^{\Delta mye}$ mice

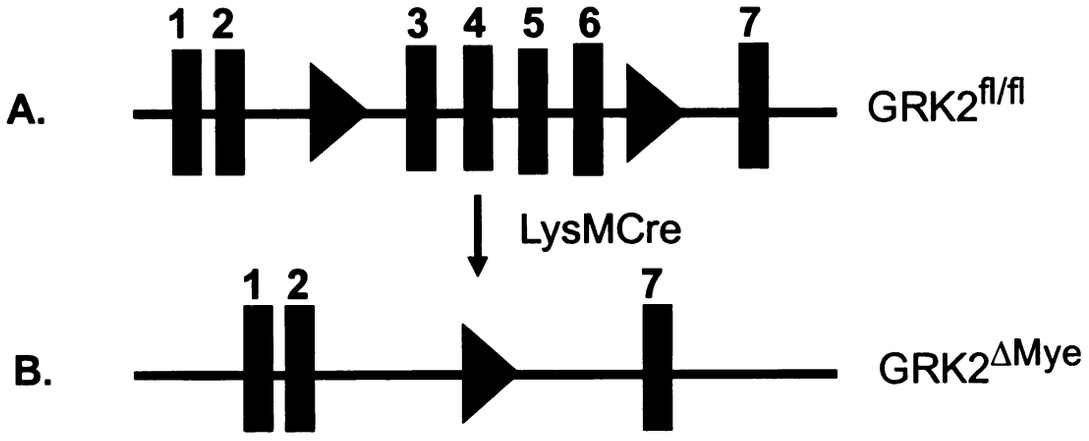
A. Schematic representation of the presence of loxP sites surrounding exons 3-6 that were targeted for deletion with Cre recombinase

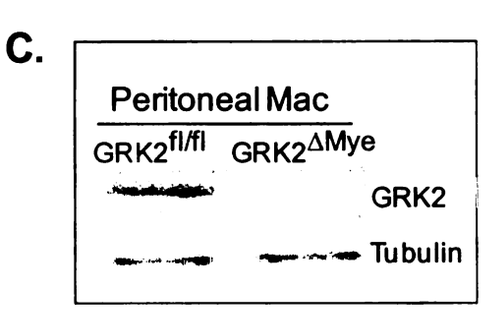
B. Cre mediated recombination leads to the deletion of exons 3-6 of GRK2 gene

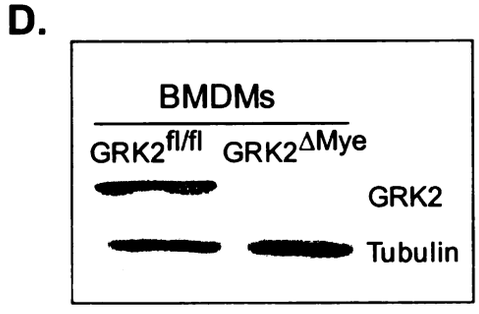
C. Western blot showing the absence of GRK2 protein in thioglycollate elicited peritoneal macrophages from GRK2$^{\Delta mye}$ (GRK2 deficient mice) mice as compared to GRK2$^{fl/fl}$ (littermate control) mice. Tubulin is shown as a loading control.

D. Western blot showing the absence of GRK2 protein in Bone marrow derived macrophages from GRK2$^{\Delta mye}$ mice as compared to GRK2$^{fl/fl}$ mice. Tubulin is shown as a loading control.

E. Western blot showing that GRK2 is normally expressed in organs such as lungs and spleen in both GRK2$^{\Delta mye}$ as well as GRK2$^{fl/fl}$ mice


A.
1 2 3 4 5 6 7
GRK2fl/fl
LysMCre
B.
1 2 7
GRK2ΔMye


C.
Peritoneal Mac
GRK2fl/fl
GRK2ΔMye
GRK2
Tubulin


D.
BMDMs
GRK2fl/fl
GRK2ΔMye
GRK2
Tubulin

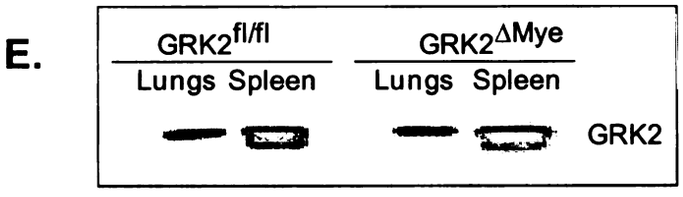

E.
GRK2fl/fl
Lungs Spleen
GRK2ΔMye
Lungs Spleen
GRK2

Figure 5.2 Complete blood count of cells from GRK2$^{\Delta mye}$ verses GRK2$^{fl/fl}$ mice

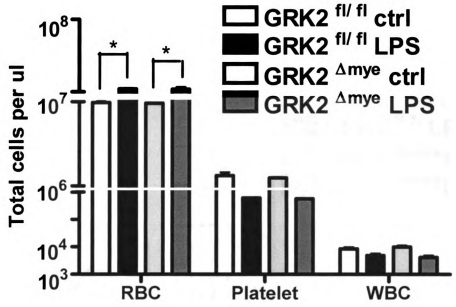
A. RBC, platelet and WBC counts were performed 12 hours after injection of LPS. Blood was collected using sodium citrate anticoagulant and the counts were performed. Cells are represented as per ul.

B. Graph showing the total protein and Hemoglobin content in g/dl in the blood of both GRK2$^{fl/fl}$ and GRK2$^{\Delta mye}$ control mice (injected with PBS) verses LPS injected mice.

C. Graph showing the percent segmented neutrophils, lymphocytes and monocytes in the blood of both GRK2$^{fl/fl}$ and GRK2$^{\Delta mye}$ control mice (injected with PBS) verses LPS injected mice.

D. Graph showing the percent eosinophils in the blood of both GRK2$^{fl/fl}$ and GRK2$^{\Delta mye}$ control mice (injected with PBS) verses LPS injected mice.

A.


GRK2 fl/ fl ctrl
GRK2 fl/ fl LPS
GRK2 Δmye ctrl
GRK2 Δmye LPS
Total cells per ul
10^8
10^7
10^6
10^4
10^3
*
*
RBC
Platelet
WBC

B.

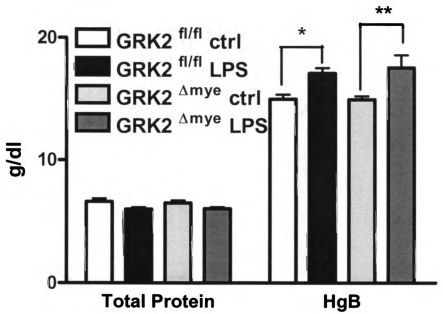
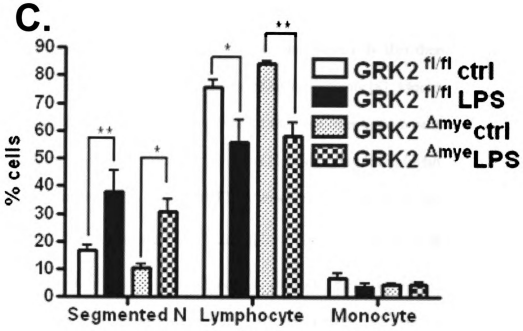
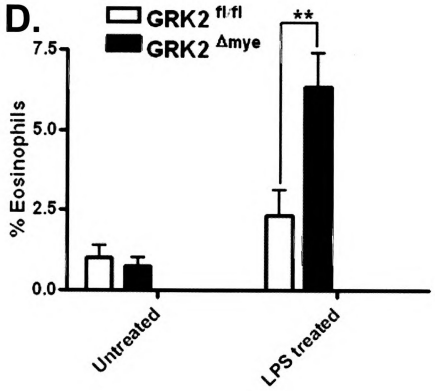

GRK2 fl/fl ctrl
GRK2 fl/fl LPS
GRK2 Δmye ctrl
GRK2 Δmye LPS
g/dl
20
10
0
*
**
Total Protein
HgB

Fig 5.2 continued……





Deficiency of GRK2 in myeloid cell lineage causes an increased secretion of cytokines and chemokines in plasma in response to LPS injection

GRK2 is highly expressed in myeloid cells particularly in neutrophils and macrophages. Furthermore, it has been shown previously that there is an increased expression of GRK2 in the neutrophils of sepsis patients [8]. Moreover, we have also found that the expression levels of GRK2 increase over time in primary macrophages from mice in response to LPS stimulation [15]. Keeping these findings in mind and to further characterize the role of GRK2 in sepsis, we injected LPS (30μg/g body weight) in GRK2 deficient ($GRK2^{\Delta mye}$) and their littermate controls ($GRK2^{fl/fl}$) by intra-peritoneal route and collected blood at different time intervals. Plasma levels of cytokines and chemokines were determined using Biorad's 23-plex assay. Our results show that IL-12 (p40), MIP1α and IL-10 are significantly enhanced in the GRK2 deficient mice compared to controls. Although plasma IL-12 (p40) levels peaked at 3 hours in the control mice, in GRK2 deficient mice these levels were significantly elevated at 12 hours post-injection (31363±8069 pg/ml in GRK2 deficient compared to 14547±3071 in control mice). Similar to IL-12 (p40), IL-10 levels were also significantly enhanced in the GRK2 deficient mice at 12 hours post-injection (17300±5938 pg/ml in GRK2 deficient compared to 6146±2046 in control mice). Compared to IL-12 (p40) and IL-10, MIP1α levels were markedly enhanced in the GRK2 deficient mice at 1 and 3 hours post-LPS injection. At one hour, MIP1α levels were 1695±149 pg/ml in GRK2 deficient compared to 1019±149 pg/ml in control mice. Similarly, at 3 hour post-LPS injection, MIP1α levels 3627±314 pg/ml in GRK2 deficient compared to 2604±330 in control mice. In addition to these three

inflammatory factors, IL-6 (at 3 hours: levels were 143229±40133 pg/ml in GRK2 deficient mice compared to 85806±24085 pg/ml in control mice), MIP1β (at 3 hours: levels were 22888±3736 pg/ml in GRK2 deficient mice as compared to 16646±2952 pg/ml in control mice) and IL-17 (at 12 hours: levels were 1670±621 pg/ml in GRK2 deficient mice as compared to 801±245 pg/ml in control mice) were also enhanced in GRK2 deficient mice, but did not reach statistical significance. Compared to these factors, a similar trend (enhanced in GRK2 deficient mice compared to controls) was observed for IL-1α, IL-1β, eotaxin, RANTES, MCP1 and IL-9. (**Figure 5.3**). Importantly, plasma levels of cytokines/chemokines including IL-2, IL-3, IL-4, IL-5, IL-12 (p70), IL-13, TNFα, IFNγ, GCSF, GMCSF and KC were not different between control and GRK2 deficient mice after LPS injection, suggesting selective regulation of TLR4-induced cytokines/chemokines by myeloid cell-specific GRK2. Taken together, these results suggest that GRK2 somehow acts as a negative regulator of LPS induced inflammatory response and hence the deficiency of GRK2 can augment the inflammatory response to LPS.

Enhanced tissue injury and mortality in GRK2 deficient mice

Lung Injury: Acute lung injury in sepsis is associated strongly with cytokine/chemokine response and is characterized by an initial recruitment of immune cells such as neutrophils and macrophages soon after the onset of infection as well as lung edema with extravasation of plasma proteins due to an increase in vascular permeability. Hence, detection of plasma proteins serves as one useful indicator of

Figure 5.3 LPS injection causes an enhanced secretion of pro and anti-inflammatory cytokines in the plasma of GRK2$^{\Delta mye}$ mice

Mice were injected with LPS (30μg/g body weight) via an intra-peritoneal injection and blood was collected at different time intervals. Secretion of cytokines and chemokines was assessed using a Biorad 23 plex multiplex based assay. Levels of IL-12 (p40), MIP1α, IL-10, MIP1β, IL-6, IL-17, IL-1α, IL-1β, Eotaxin, Rantes, MCP1 and IL-9 are shown in pg/ml. N=11 for 1, 3 and 12 hrs. N=6 for 0, 6 and 18 hrs. *$p<0.05$; **$p<0.01$.

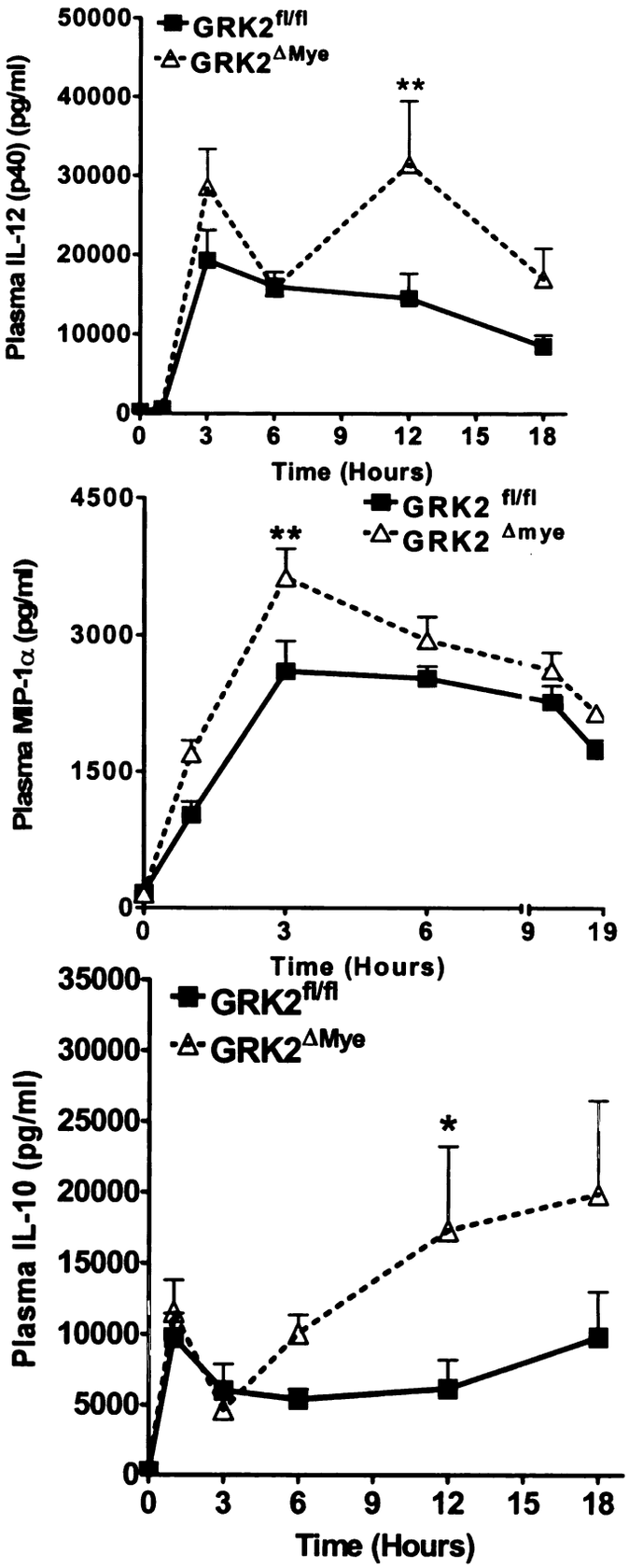

GRK2fl/fl
GRK2ΔMye
Plasma IL-12 (p40) (pg/ml)
50000
40000
30000
20000
10000
0
**
0 3 6 9 12 15 18
Time (Hours)
GRK2 fl/fl
GRK2 Δmye
Plasma MIP-1α (pg/ml)
4500
3000
1500
0
**
0 3 6 9 19
Time (Hours)
GRK2fl/fl
GRK2ΔMye
Plasma IL-10 (pg/ml)
35000
30000
25000
20000
15000
10000
5000
0
*
0 3 6 9 12 15 18
Time (Hours)

Figure 5.3. Continued.

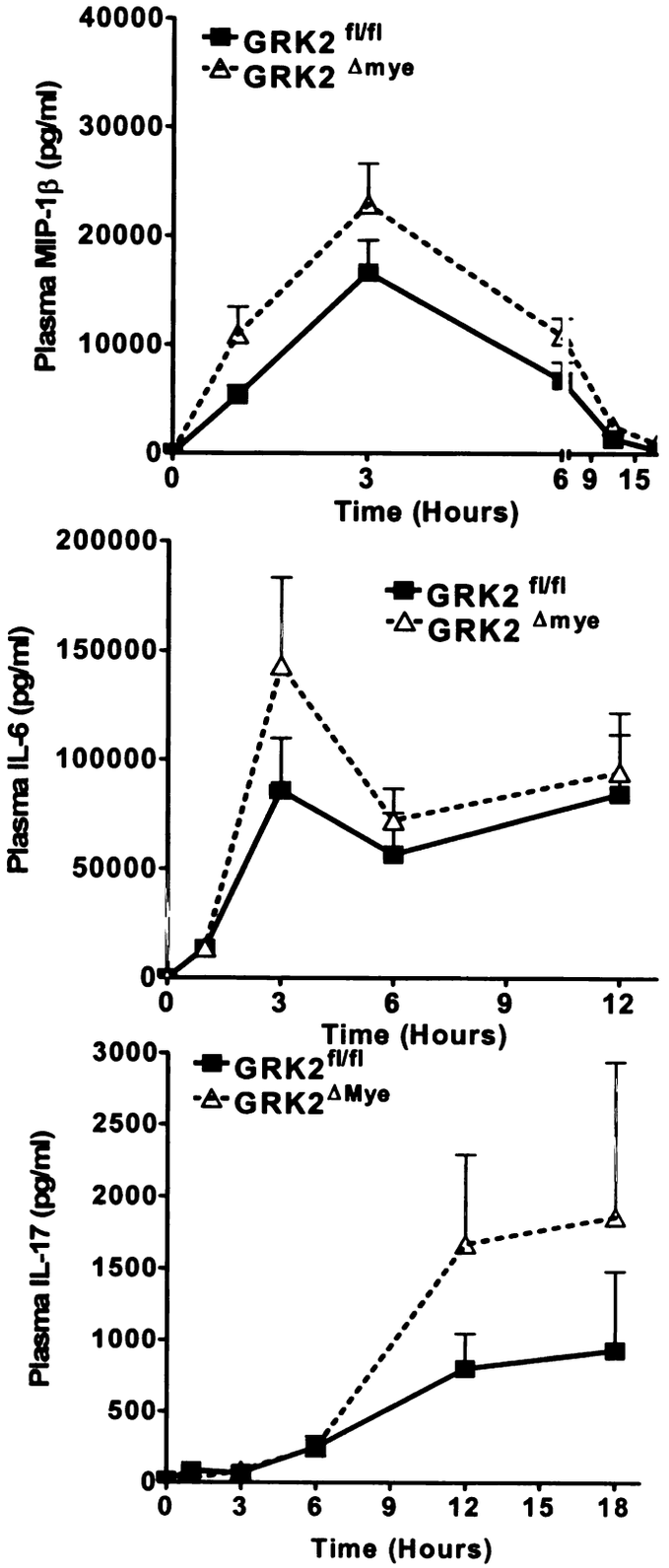


Figure 5.3. Continued.

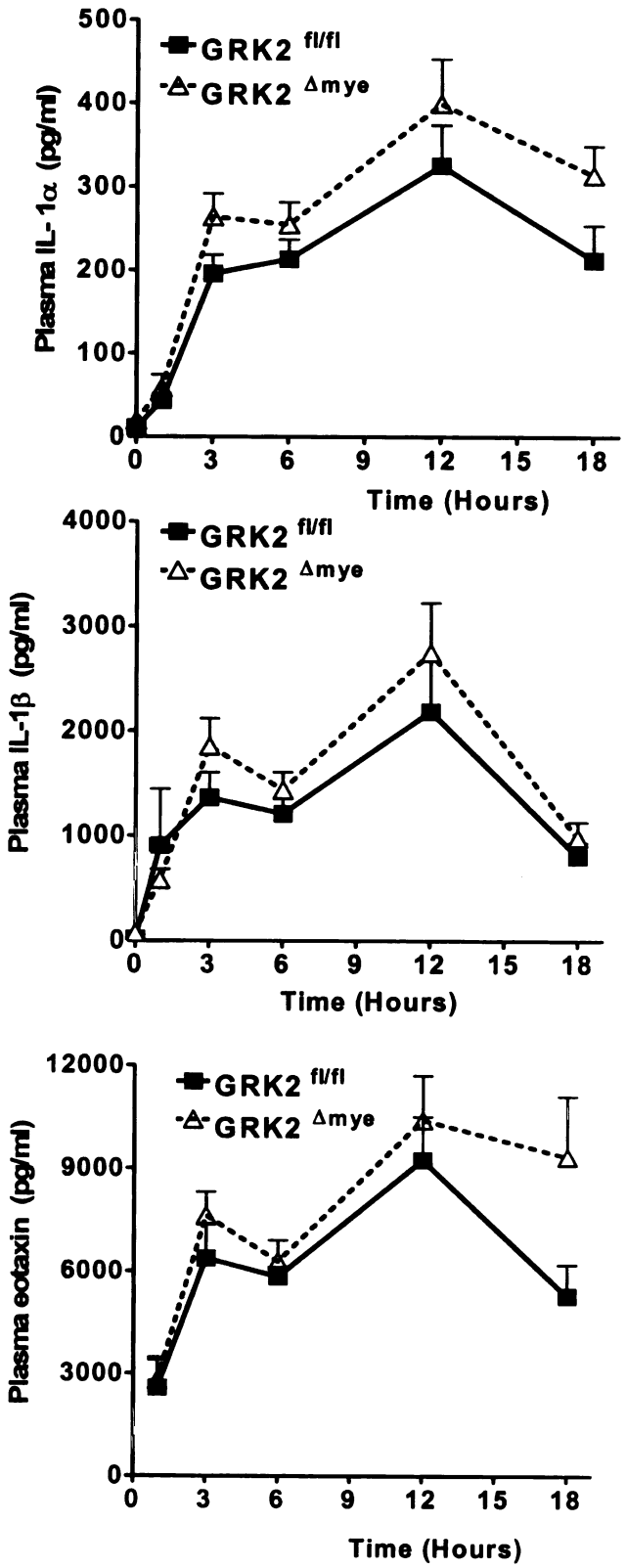
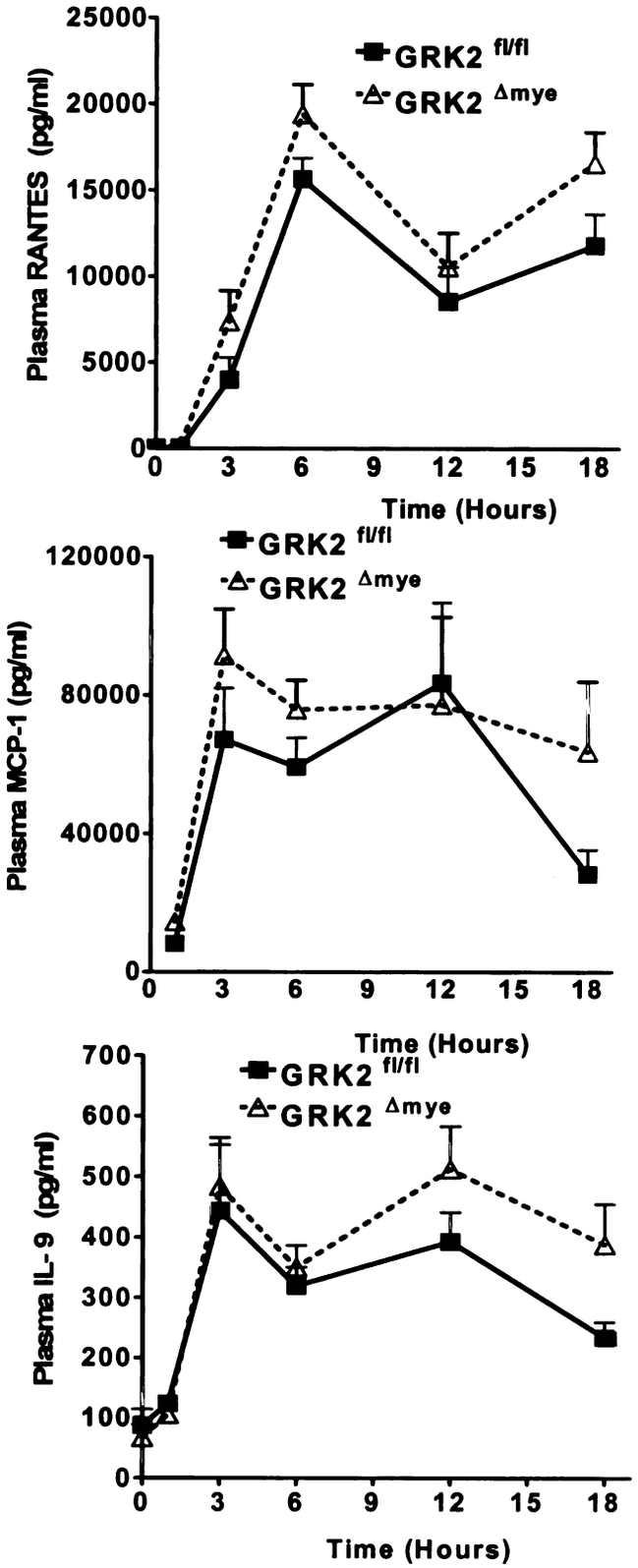


Figure 5.3. Continued.



lung injury. We hypothesized that the enhanced cytokine/chemokine response observed in the GRK2 deficient mice will be associated with exaggerated lung injury and permeability. Increase in lung permeability in response to LPS administration was determined by measuring the leakage of total protein into bronchoalveolar lavage (BAL) fluid. As shown in **Figure 5.4A** there was a significantly enhanced protein content in LPS injected GRK2 deficient mice as compared to their littermate controls at 12 hours post injection (0.060±.004 μg/μl protein in control compared to 0.093±.009 μg/μl protein in the GRK2 deficient mice), suggesting that the lung injury in response to LPS is worse in the GRK2 deficient mice compared to the control mice. These results suggest that the enhanced cytokine response observed *in vivo* may be associated with the exaggerated lung injury observed in the GRK2 deficient mice in response to LPS injection.

Liver injury: In addition to the lungs, liver injury is another significant consequence of endotoxemia. To examine if the enhanced cytokine response also affected liver pathology, we performed a histopathological analysis of the livers from 12-hour LPS-injected control and GRK2 deficient mice. As predicted, the inflammatory response to LPS in the GRK2 deficient mice was found to be considerably enhanced as evidenced by much more intense accumulation of inflammatory cells and marked edema with fibrin strands in prominent periportal areas (**Figure 5.4B**). This suggests that the inflammatory response to systemic LPS administration develops much more excessively in mice deficient in myeloid cell GRK2 when compared to control mice.

Endotoxic mortality: Organ failure in response to endotoxemia eventually leads to mortality in mice. Because lung and liver injury were exaggerated in the GRK2

deficient mice compared to controls, we hypothesized that the mortality of GRK2 deficient mice would be higher than the control mice. As predicted, we found that 50% of the GRK2 deficient mice died within the first 24 hours of LPS injection as compared to only 20% in the control group. Our results further show that whereas 100% of the GRK2 deficient mice subjected to LPS induced sepsis died within 42 hours of injection, the GRK2 control mice showed a significantly enhanced survival rate with 30% surviving at 48 hours (**Figure 5.4C**). These results suggest that myeloid cell GRK2 plays a protective role in LPS induced endotoxemic death.

Enhanced LPS-induced cytokine response in GRK2 deficient primary macrophages

Results presented above suggest that the deficiency of GRK2 in myeloid cells can affect the cytokine/chemokine response in plasma. Although myeloid cells, in particular monocytes, neutrophils and macrophages are the major cells involved in the secretion of cytokines and chemokines, other cells such as lymphoid cells (T cells and B cells) as well as fibroblasts are also involved in this process. To further investigate if myeloid cells are the major producers of this response *in vivo*, we determined the effect of LPS stimulation on the secretion of cytokines and chemokines in cell culture supernatants. For this purpose, we isolated primary peritoneal macrophages from both GRK2 deficient as well as control mice and stimulated them with LPS (1μg/ml) under *in vitro* conditions. As was observed *in vivo* in the GRK2 deficient mice, LPS-induced cytokine/chemokine responses were significantly enhanced in primary macrophages *in vitro*. As shown in **Figure 5.5,** we observed a markedly enhanced secretion of pro-

Figure 5.4 $GRK2^{\Delta mye}$ show enhanced tissue injury and mortality as compared to $GRK2^{fl/fl}$ mice

A. Mice were injected with LPS (30μg/g body weight) via intra-peritoneal route and broncho-alveolar lavage fluid was collected after 12 hours. The cells were pelleted by centrifuging at 450g for 10min and the supernatant was used to measure the total protein using Bradford assay. Protein is shown in μg/μl.

B. Morphological changes in mouse liver sections of LPS-injected myeloid cell GRK2 deficient verses control mice. **(A)** Control mice liver **(B)** GRK2 deficient mice liver. Arrows show neutrophils, arrowheads show fibrin in venous spaces. (H&E, original magnification X 10). **(C)** Control mice liver. Neutrophils and minimal edema present in periportal areas. **(D)** GRK2 deficient mice liver. Numerous neutrophils; star shows edema; arrowhead shows fibrin stands in prominent periportal areas (H&E, original magnification, X 20).

C. GRK2 deficient mice ($GRK2^{\Delta mye}$) and littermate control mice ($GRK2^{fl/fl}$) were injected intra-peritoneally with 20μg/gm LPS and their survival was monitored over a period of 48 hours. Black line shows the survival rate for control mice (N=10) and dashed line shows the survival rate of GRK2 deficient mice (N=10). Data were analyzed with the Kaplan-Meier test.

A.

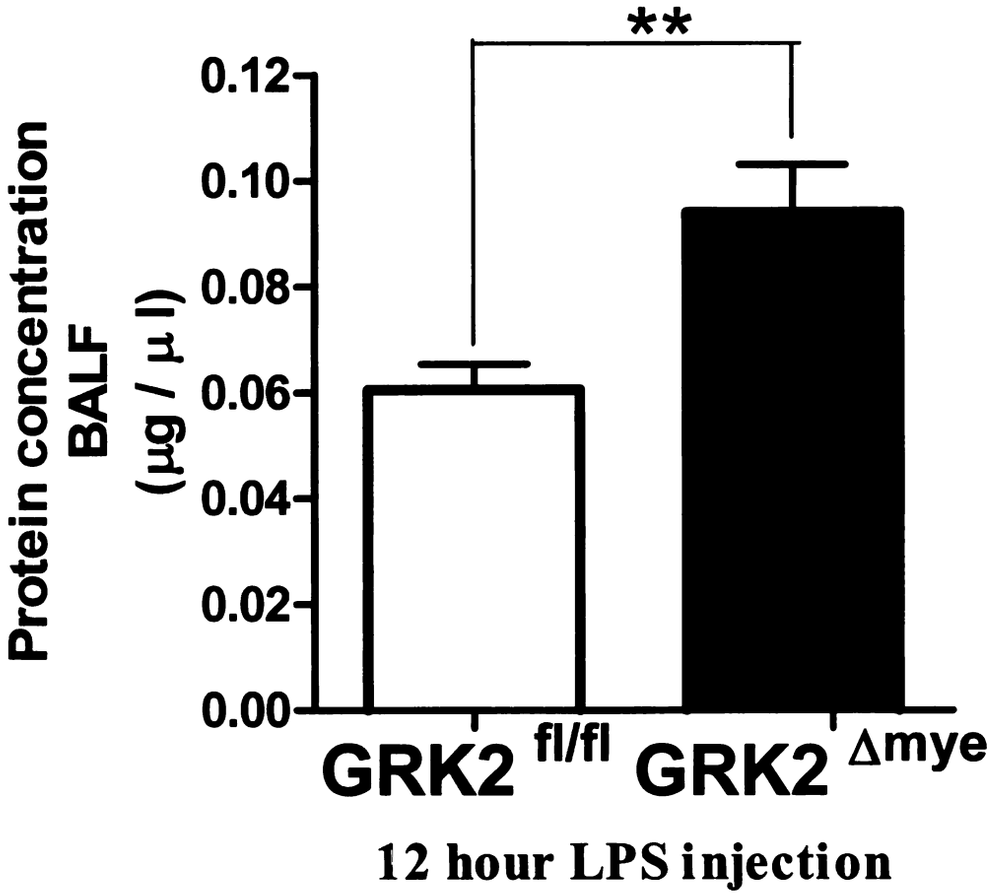

**
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Protein concentration
BALF
(µg / µ l)
GRK2 fl/fl
GRK2 Δmye
12 hour LPS injection

Figure 5.4. Continued....

B.

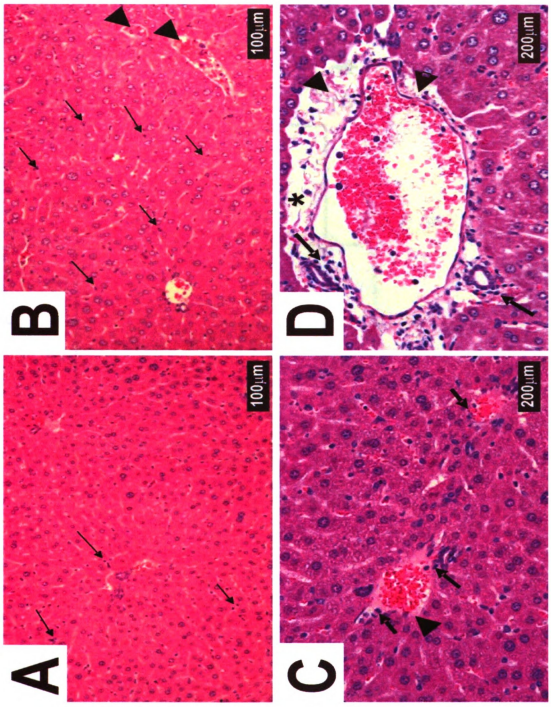
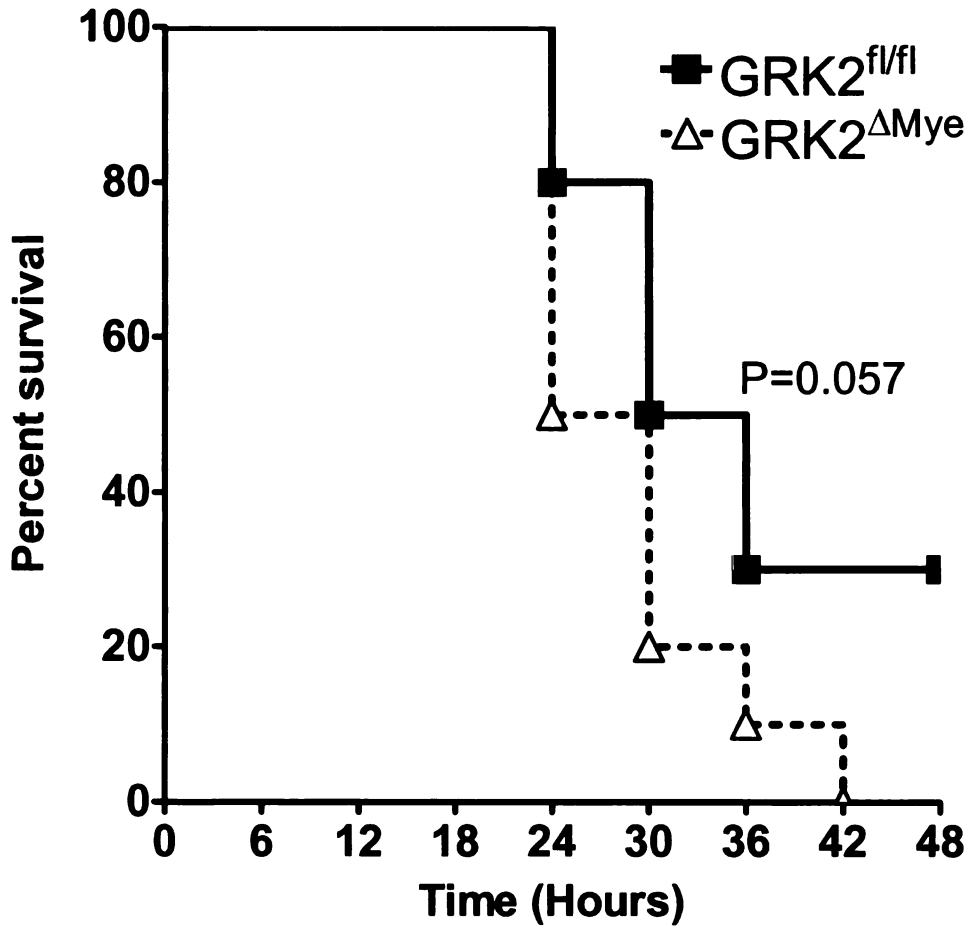


Figure 5.4. Continued....

C.



inflammatory cytokines viz. TNFα (592±47pg in $GRK2^{\Delta mye}$; 363±60pg in $GRK2^{fl/fl}$ at 6 h), IL-6 (185±26pg in $GRK2^{\Delta mye}$; 80±19pg in $GRK2^{fl/fl}$ at 24 h), IL-1α (5.8±1.1pg in $GRK2^{\Delta mye}$; 3.2±1.1pg in $GRK2^{fl/fl}$ at 24 h), IL-1β (55.2±5.3pg in $GRK2^{\Delta mye}$; 27.5±4.7pg in $GRK2^{fl/fl}$ at 24 h), IL-9 (24±2pg in $GRK2^{\Delta mye}$; 11±2pg in $GRK2^{fl/fl}$ at 12 h) as well as chemokines viz. MCP1 (1552± 82pg in $GRK2^{\Delta mye}$; 710±111pg in $GRK2^{fl/fl}$ at 12 h), MIP1α (1640±178pg in $GRK2^{\Delta mye}$; 815±136pg in $GRK2^{fl/fl}$ at 24h) and GCSF (117±22pg in $GRK2^{\Delta mye}$; 47±10pg in $GRK2^{fl/fl}$ at 24 h) in the GRK2 deficient mice macrophages compared to control cells. Importantly, cytokines including IL-2, IL-12p40, IFNγ and IL-17 did not differ between control and GRK2 deficient mice macrophages, suggesting selective regulation of TLR4-induced cytokine responses by GRK2. To determine the differences between macrophages and neutrophils in terms of the cytokine regulation, we also assessed the levels of cytokines and chemokines in neutrophils (treated *in vitro*) from these mice. Compared to the results obtained with macrophages we observed that neutrophils from GRK2 deficient mice show an enhanced secretion of only G-CSF and KC compared to control mice (**Figure 5.6**). Other cytokines such as IL-1α, IL-1β, IL-6, IL-12 (p70), GMCSF, MIP1α, and MIP1β showed a similar trend although these were not statistically significant. Furthermore, some cytokine/chemokines (IL-12p40, RANTES, IL-17) were not different between control and GRK2 deficient mice suggesting specific regulation of some cytokines by GRK2 in the neutrophils.

GRK2 deficiency augments LPS-stimulated ERK phosphorylation but not P38 and JNK phosphorylation in primary macrophages

Figure 5.5 Peritoneal macrophages from GRK2$^{\Delta mye}$ mice secrete enhanced amounts of pro-inflammatory cytokines and chemokines in response to LPS stimulation

Thioglycollate-elicited peritoneal macrophages were stimulated with LPS (1μg/ml) in 12-well cell culture plates and cell culture medium was collected at different time intervals. Levels of inflammatory mediators were determined using biorad 23 plex assay. Cells were lysed to determine the protein concentration using Bradford method. Levels of TNFα, IL-6, IL-1α, IL-1β, IL-9, MCP1, MIP1α and GCSF are shown as pg/μg of total cellular protein. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

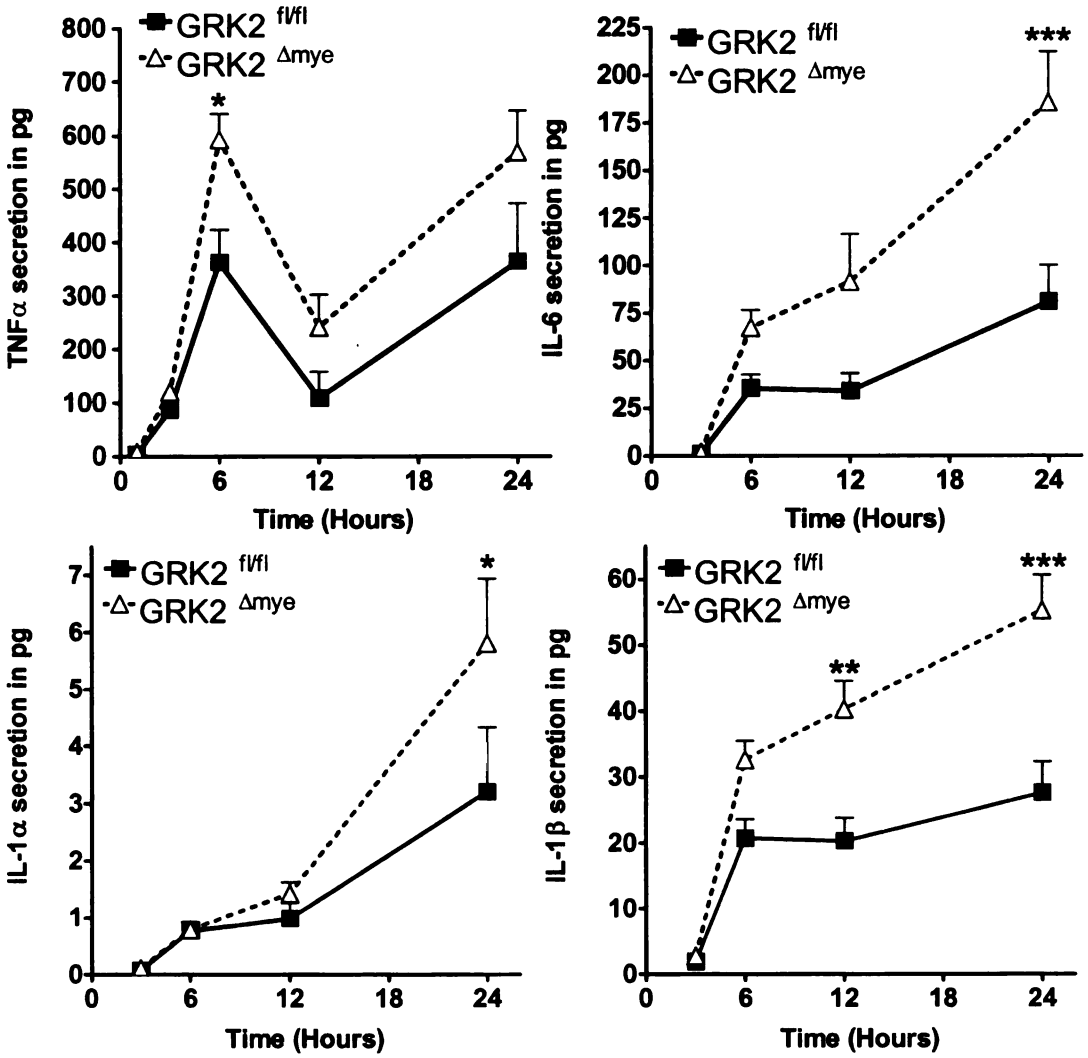

GRK2 fl/fl
GRK2 Δmye
TNFα secretion in pg
IL-6 secretion in pg
IL-1α secretion in pg
IL-1β secretion in pg
Time (Hours)
*
**
***

Figure 5.5 Continued...

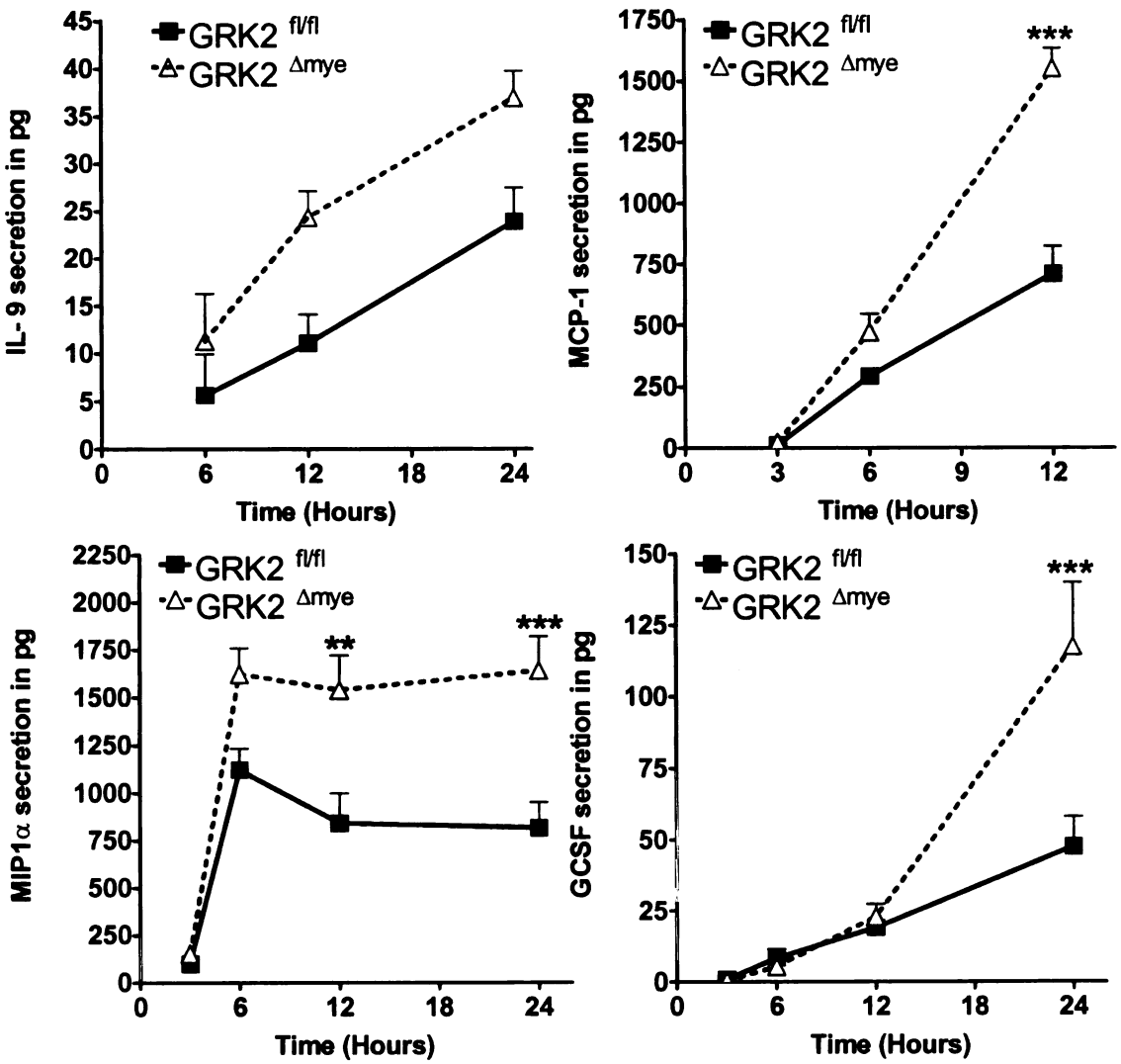


Figure 5.6 Neutrophils from GRK2$^{\Delta mye}$ mice secrete enhanced amounts of pro-inflammatory cytokines and chemokines in response to LPS stimulation

Thioglycollate-elicited neutrophils were stimulated with LPS (1μg/ml) in 12-well cell culture plates and cell culture medium was collected at different time intervals. Levels of inflammatory mediators were determined using biorad 23 plex assay. Cells were lysed to determine the protein concentration using Bradford method. Levels of GCSF, KC, IL-1α, IL-1β, IL-6, IL-12p70, GM-CSF, MIP1α and MIP1β are shown as pg/μg of total cellular protein. **$p<0.01$.

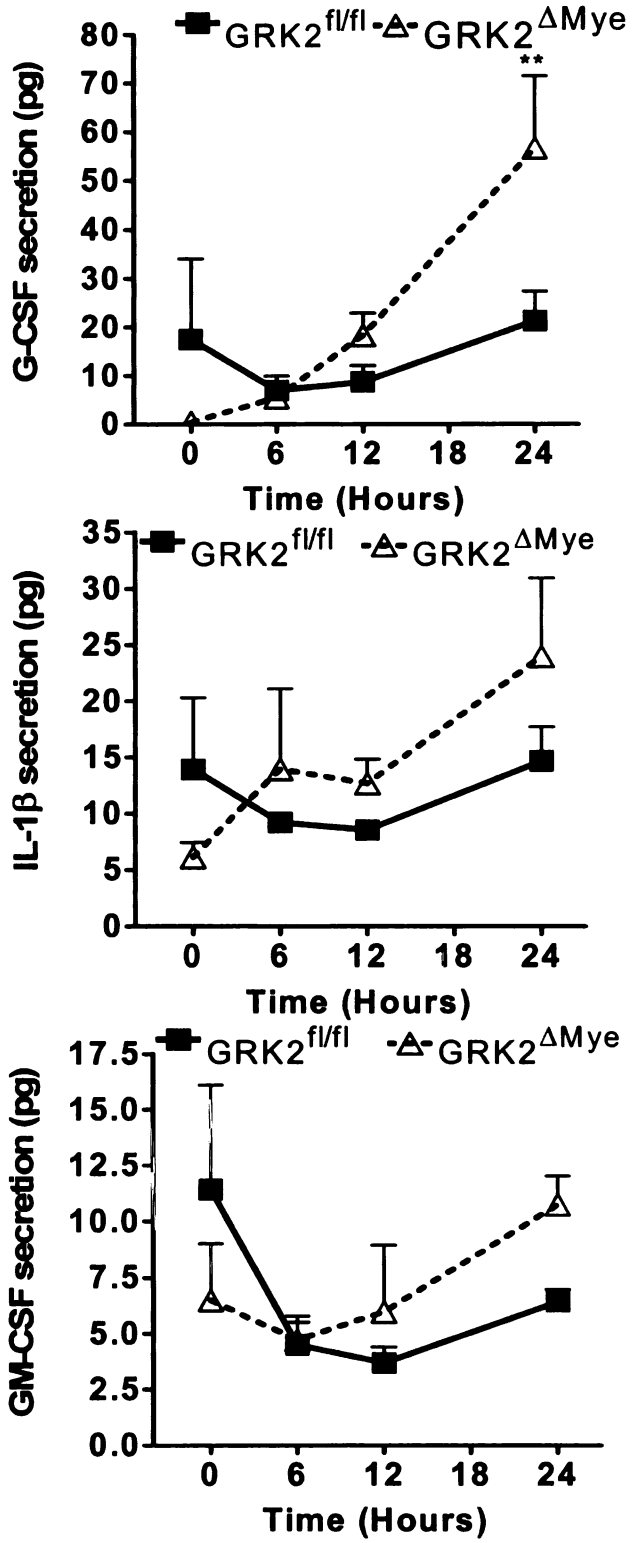

GRK2fl/fl
GRK2ΔMye
G-CSF secretion (pg)
80
70
60
50
40
30
20
10
0
**
0
6
12
18
24
Time (Hours)
GRK2fl/fl
GRK2ΔMye
IL-1β secretion (pg)
35
30
25
20
15
10
5
0
0
6
12
18
24
Time (Hours)
GRK2fl/fl
GRK2ΔMye
GM-CSF secretion (pg)
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
0
6
12
18
24
Time (Hours)

Figure 5.6 Continued....

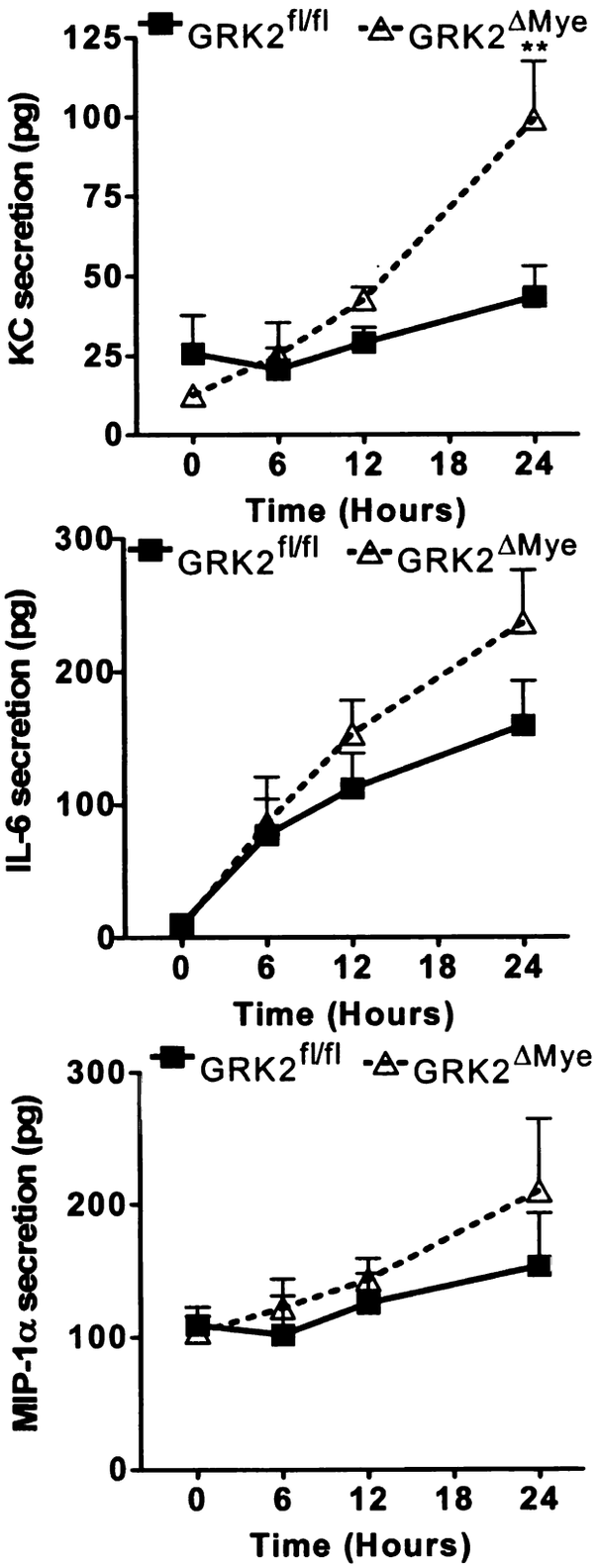
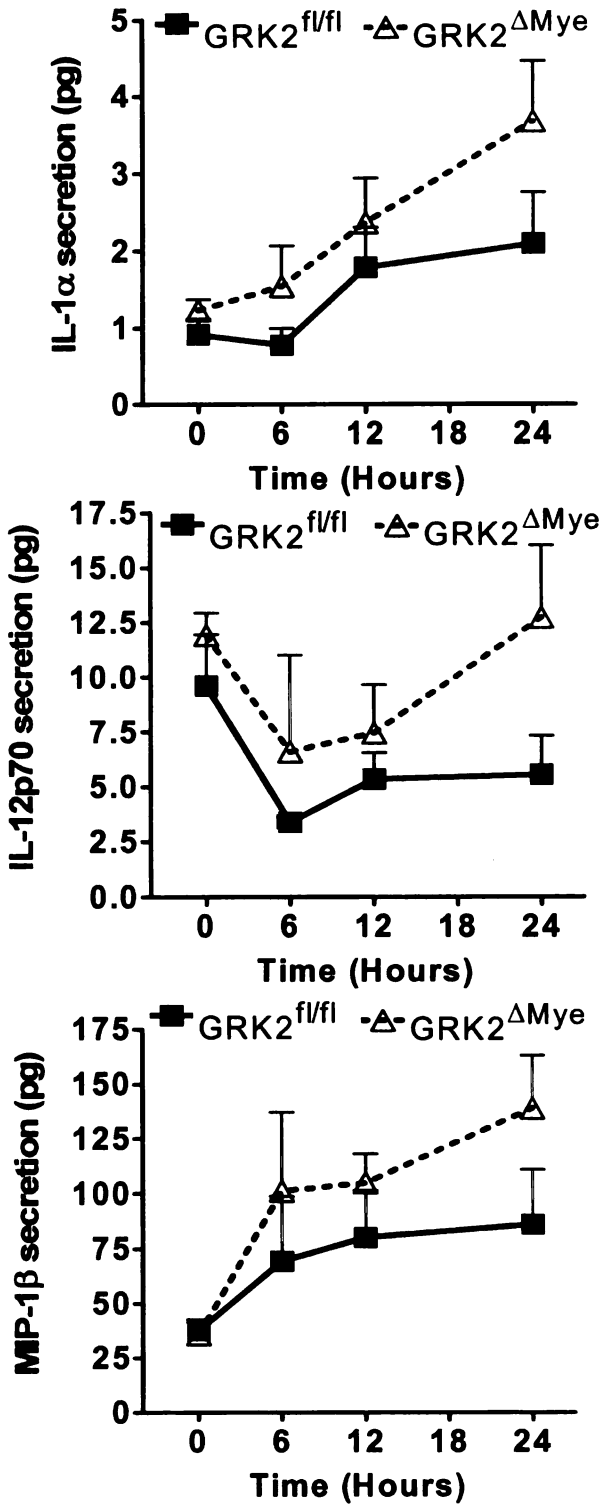


Figure 5.6 Continued….



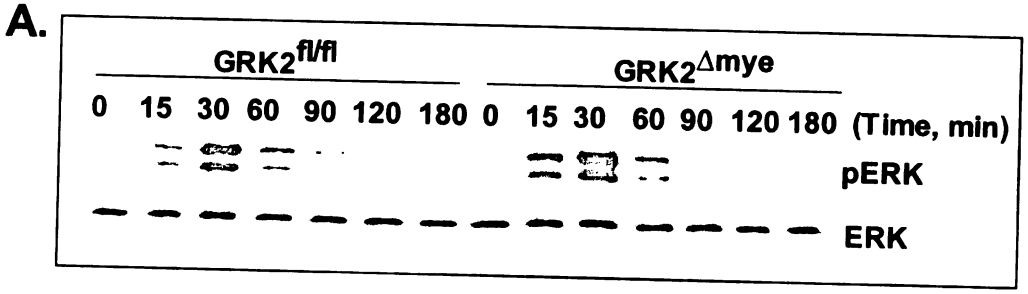
To begin to understand the mechanisms of how GRK2 negatively regulates LPS-induced cytokine/chemokine response *in vivo* and in primary macrophages *in vitro*, we tested the effect of LPS in GRK2 deficient and control cells on various signaling pathways. LPS stimulation of TLR4 signaling leads to the activation of MAPK signaling cascades including ERK, JNK and p38 kinases as well as IκBα-NFκB signaling pathways. These pathways have been shown to be the central regulators of inflammatory responses in endotoxemia. Therefore, we assessed the phosphorylation status of ERK, JNK, p38 and IκBα in the primary macrophages both in control as well as GRK2 deficient macrophages upon exposure to LPS. Peritoneal macrophages from GRK2 deficient and control mice were stimulated with LPS for various time points and immunoblotting performed for pERK1/2, pJNK, pP38 and pIκBα. Our results suggest an interestingly selective role for GRK2 in LPS-induced ERK phosphorylation. Thus, although LPS-stimulated pJNK, pP38 and pIκBα levels were similar between the control and GRK2 deficient macrophages (**Fig. 5.7B**), pERK levels were markedly enhanced in the GRK2 deficient cells (**Figure 5.7A**). Phospho-ERK levels were 36±11% and 95±3% at 15 and 30 min respectively in GRK2 deficient cells, whereas in control cells the levels reached only 13±3% and 51±8% at 15 and 30 min respectively. We also examined the levels of pAkt and pGSK3 (known to be regulated by GRK2) after LPS stimulation and did not find any evidence for their regulation in these cells by GRK2 (**Figure 5.8**).

GRK2 negatively regulates NF-κB1-p105-TPL2-MEK-ERK pathway in primary macrophages

Figure 5.7 GRK2 deficiency causes an enhanced LPS stimulated ERK1/2 activation in peritoneal macrophages

A. $GRK2^{\Delta mye}$ and control primary peritoneal macrophages were stimulated with 1μg/ml of LPS for different time points and the phosphorylation of MAPKs and IκBα was determined by western blotting. A representative blot for phospho ERK and ERK and quantification for the same is shown in **(A).** ***$p<0.001$ compared to control. N=6.

B. A representative blot for phospho-p38, actin, phospho-JNK, JNK and phospho-IκBα, tubulin is shown.


A.
GRK2fl/fl
GRK2Δmye
0 15 30 60 90 120 180 0 15 30 60 90 120 180 (Time, min)
pERK
ERK

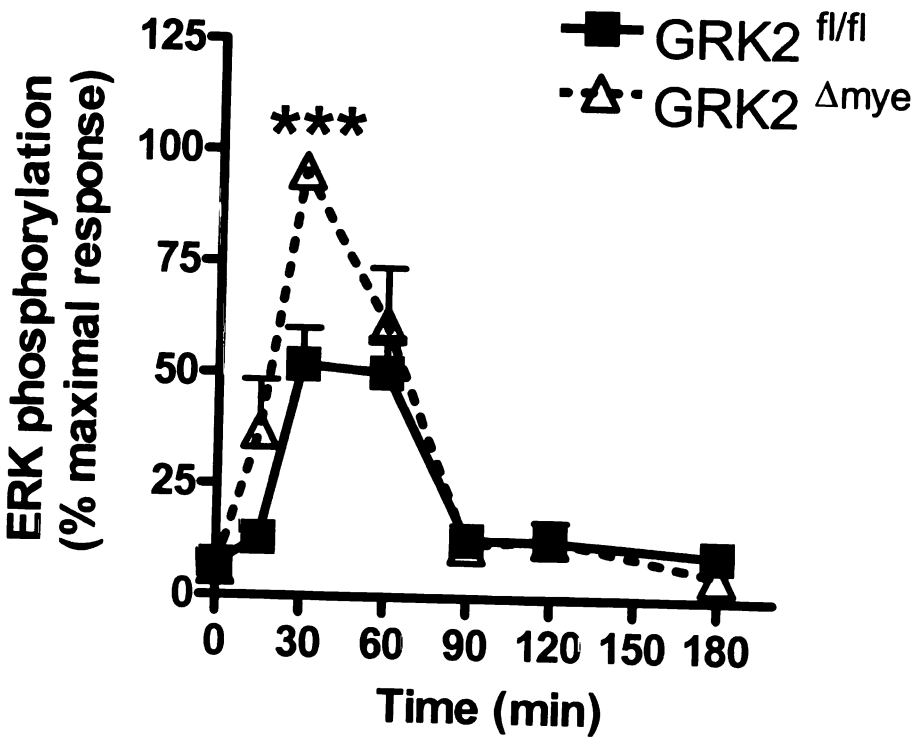

GRK2 fl/fl
GRK2 Δmye
***
ERK phosphorylation
(% maximal response)
125
100
75
50
25
0
0 30 60 90 120 150 180
Time (min)

Figure 5.7. Continued....

B.

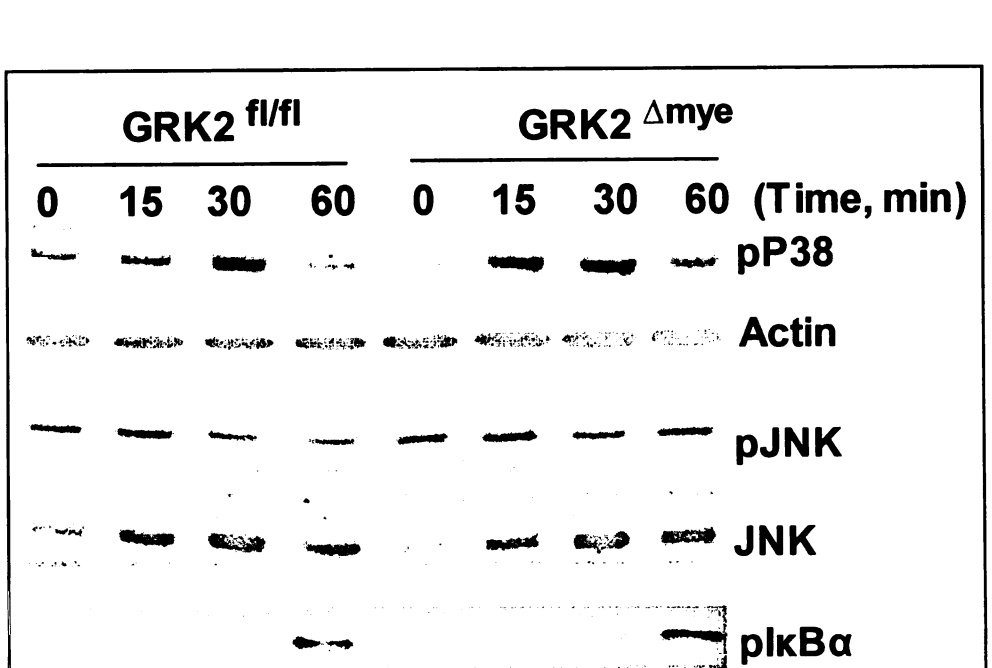
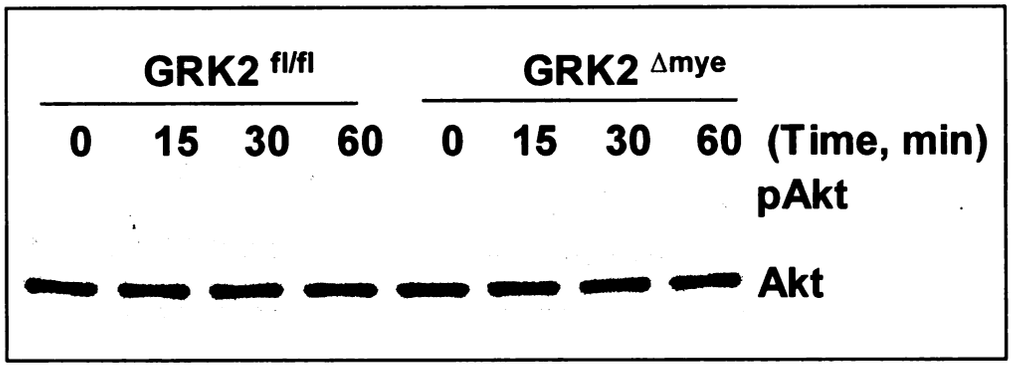
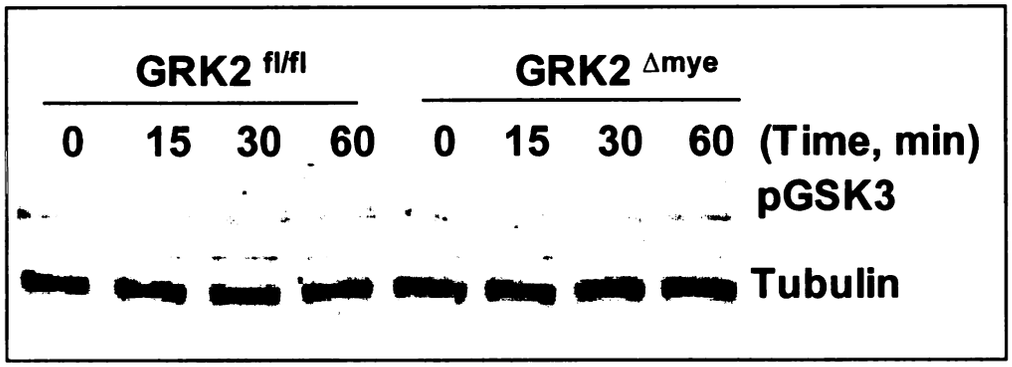


Figure 5.8 GRK2 deficiency does not affect LPS stimulation of Akt and GSK3β activation in peritoneal macrophages

$GRK2^{\Delta mye}$ and control primary peritoneal macrophages were stimulated with 1μg/ml of LPS for different time points and the phosphorylation of Akt and GSK3β was determined by western blotting. A representative blot for phospho-Akt, Akt, phospho-GSK3β and tubulin is shown.

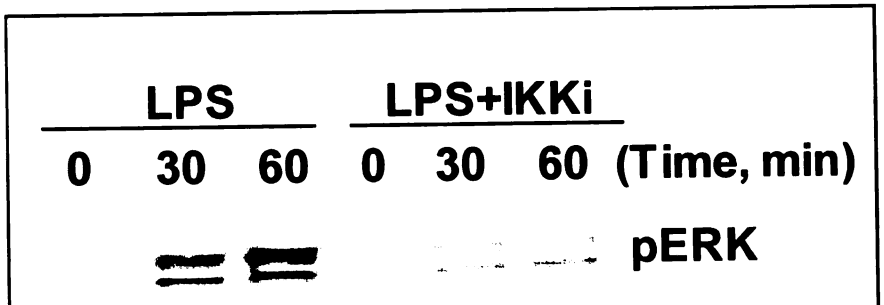

GRK2 fl/fl
GRK2 Δmye
0 15 30 60 0 15 30 60 (Time, min)
pAkt
Akt


GRK2 fl/fl
GRK2 Δmye
0 15 30 60 0 15 30 60 (Time, min)
pGSK3
Tubulin

To further elucidate the biochemical mechanisms of GRK2 regulation of the ERK pathway, we examined the upstream regulators of ERK phosphorylation in primary macrophages. Previous studies have demonstrated that ERK activation in macrophages in uniquely regulated via LPS-stimulated IKKβ-NFκB1 p105 pathway. That is, under unstimulated conditions, p105 (an IκB family member bound to NFκB p50 subunits) is also stoichiometrically bound to TPL2 (a MAP3K). When activated, p105 is phosphorylated by IKKβ, which then undergoes partial degradation releasing NFκB p50 subunits as well as TPL2. TPL2 then phosphorylates and activates MEK1/2, which then activates ERK1/2. We first confirmed the existence of IKKβ mediated ERK signaling pathway in peritoneal macrophages. For this purpose, primary peritoneal macrophages from GRK2 control mice were stimulated with either LPS alone or pretreated with BMS345541 (IKKβ inhibitor) before stimulation with LPS and the phosphorylation of ERK was assessed by immumoblotting. As shown in **Figure 5.9**, we observed that the pharmacological inhibition of IKKβ indeed attenuated ERK phosphorylation confirming the existence of IKKβ-NFκB1 p105 / TPL2-MEK-ERK pathway. To investigate at what level GRK2 regulates this pathway, we examined the phosphorylation of MEK1/2 and p105 after LPS stimulation in control and GRK2 deficient macrophages. Our studies reveal that GRK2 negatively regulates ERK pathway at the level of p105, because LPS-induced MEK1/2 as well as p105 phosphorylation were both enhanced significantly in the GRK2 deficient macrophages compared to control cells (**Figure 5.10**). Phospho-p105 levels were 47±15% and 84±9% at 15 and 30 min respectively after LPS treatment in the GRK2

Figure 5.9 The occurrence of IKKβ-NFκB1p105/Tpl2-MEK-ERK pathway in peritoneal macrophages confirmed by using an IKKβ inhibitor

Peritoneal macrophages from control mice were stimulated with either LPS alone or pretreated with BMS345541 (5μM), a specific inhibitor against IKKβ, 30 minutes prior to stimulation with LPS (1μg/ml) for various time points as shown. Immunoblotting was performed for phospho-ERK and ERK proteins using specific antibodies. A representative blot is shown.


LPS
LPS+IKKi
0
30
60
0
30
60
(Time, min)
pERK
ERK

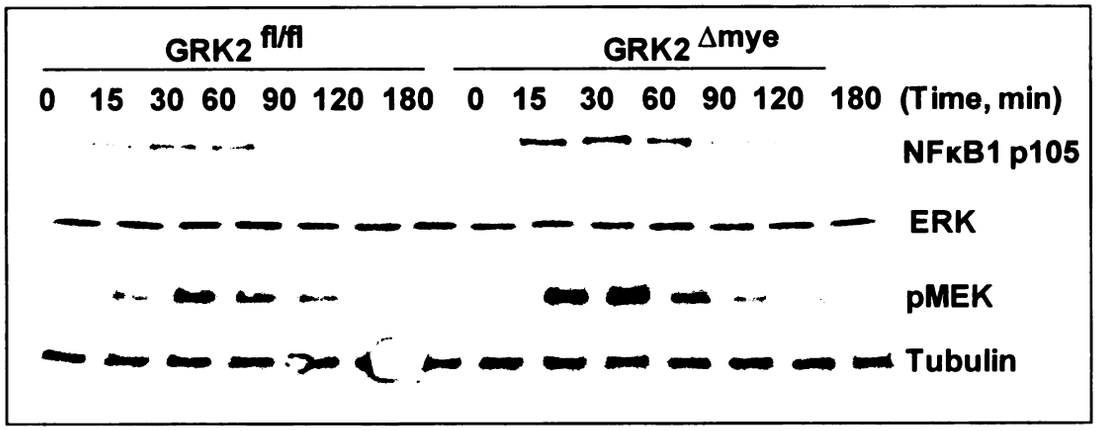
deficient cells compared to 21±5% and 53±10% at 15 and 30 min respectively in control cells. Because LPS-induced IKKβ-mediated IκBα phosphorylation was not affected by GRK2, our results indicate that GRK2 likely regulates at the level of p105. This is further supported by previous studies, which have shown that GRK2 indeed directly interacts with p105, even though these studies did not find any functional significance of this interaction. Taken together our studies indicate that GRK2 interaction with p105 negatively regulates LPS-induced ERK activation, as well as potentially the p50-mediated NFκB activation.

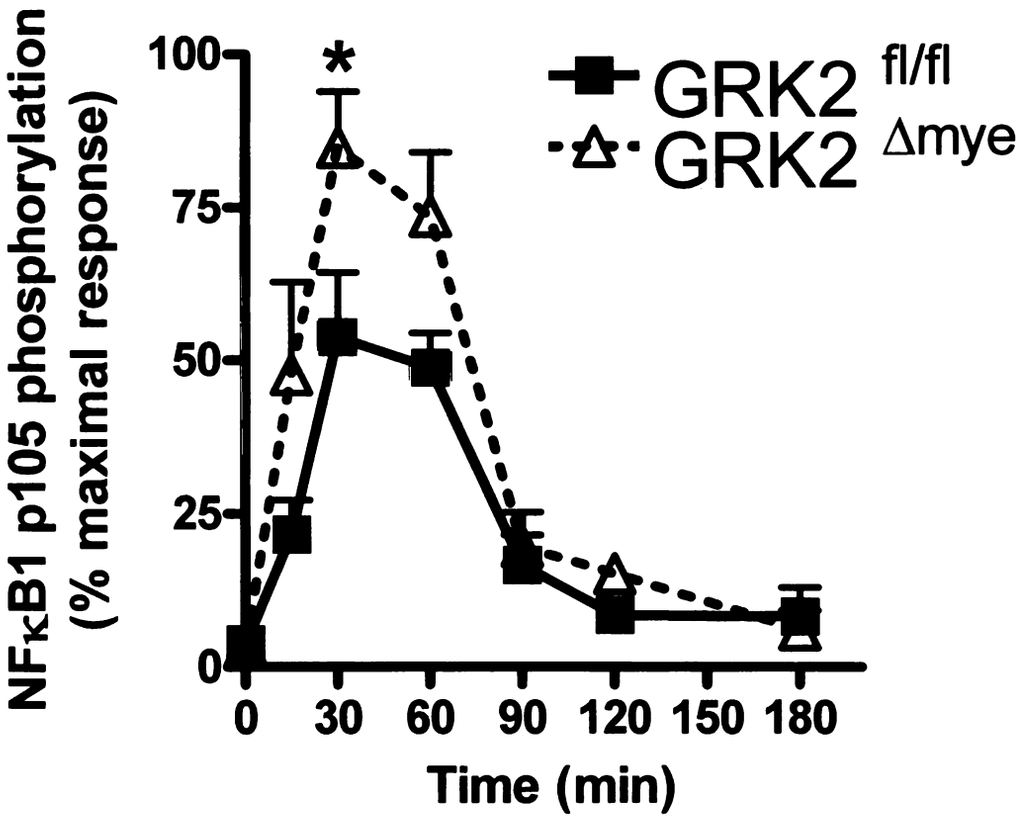
Role of enhanced p105-ERK activation on exaggerated inflammatory response in GRK2 deficient mice

Our results so far suggest that deficiency of GRK2 in primary macrophages results in the enhanced activation of p105-ERK pathway, which is associated with an enhanced inflammatory cytokine response observed in macrophages as well as *in vivo* in mice. To further demonstrate that the effects on the cytokines observed in the GRK2 deficient mice are a result of enhanced p105-ERK activation, we tested the effects of LPS on cytokine/chemokine responses in primary macrophages in presence or absence of an ERK inhibitor. We treated peritoneal macrophages from control and GRK2 deficient mice with LPS in the presence or absence of PD98059 (a MEK inhibitor). Cytokine/chemokine secretion was determined using 23-plex Biorad assay kit. Among the cytokine/chemokines that were enhanced in the GRK2 knockout macrophages, the ERK inhibitor inhibited IL-1α, MIP1α, GCSF and MCP1 in the GRK2 knockout macrophages but not in the control mice macrophages. In the presence of the ERK inhibitor, the levels cytokines/chemokines returned to the levels

Figure 5.10 GRK2 deficiency causes an enhanced LPS stimulated NFκB1 p105 activation in peritoneal macrophages

$GRK2^{\Delta mye}$ and control primary peritoneal macrophages were stimulated with 1μg/ml of LPS for different time points and the activation of NFκB1 p105 and MEK were determined by western blotting. A representative blot for phospho-NFκB1 p105, ERK, phospho-MEK, tubulin and quantification for the same is shown. ERK and tubulin were used as loading controls. *$p<0.05$ compared to control. N=7.


GRK2 fl/fl
GRK2 Δmye
0 15 30 60 90 120 180 0 15 30 60 90 120 180 (Time, min)
NFκB1 p105
ERK
pMEK
Tubulin


NFκB1 p105 phosphorylation
(% maximal response)
100
75
50
25
0
*
GRK2 fl/fl
GRK2 Δmye
0 30 60 90 120 150 180
Time (min)

observe

than the

IL-1α, I

GRK2

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ERK pa

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observed in the control LPS treated cells (except for MIP1α which was slightly higher than the control LPS) (**Fig. 5.11A**). This demonstrates that the enhanced secretion of IL-1α, MIP1α, GCSF and MCP1 result from an enhanced ERK activation observed in GRK2 deficient macrophages. Because IKK-p105 pathway regulates ERK, we examined if the secretion of these inflammatory factors are also affected by inhibition of IKKβ. Except for MCP-1, the other three factors i.e. IL-1α, MIP1α, GCSF were also significantly blocked with the IKKβ inhibitor (BMS345541) (data not shown). Interestingly the enhanced secretion of IL-6, and IL-1β in the GRK2 deficient cells were blocked only by the IKKβ inhibitor and not by ERK inhibitor, suggesting that these two cytokines are regulated by GRK2 exclusively via the IKKβ-p105 pathway likely involving the p50-NFκB activation (**Figure 5.11B**). Although IL-9 was consistently enhanced in the GRK2 deficient cells compared to the control cells, neither BMS345541 nor PD98059 efficiently blocked IL-9 secretion in cells from both genotypes. Thus IL-9 may not be exclusively regulated by IKKβ or ERK.

Taken together, our results demonstrate that GRK2 negatively regulates p105-ERK pathway thereby regulating the production of IL1-α, GCSF, MIP1α and MCP1 in response to LPS.

Figure 5.11 Pharmacological inhibition of ERK and IKKβ cause a significant inhibition of LPS-induced secretion of cytokines and chemokines in peritoneal macrophages from GRK2$^{\Delta mye}$ mice

A. Peritoneal macrophages were either left unstimulated, stimulated with LPS or pretreated with PD98059 (10μM), a MEK inhibitor, 30 minutes prior to stimulation with LPS (1μg/ml) and the cell culture supernatant was collected 24 hours later. The secretion of cytokines and chemokines was assessed as described before using biorad 23 plex assay. N=5. *p<0.05; **p<0.01; ***p<0.001.

B. Peritoneal macrophages were either left unstimulated, stimulated with LPS or pretreated with BMS345541 (5μM), a specific inhibitor against IKKβ, 30 minutes prior to stimulation with LPS (1μg/ml) and the cell culture supernatant was collected 24 hours later. The secretion of cytokines and chemokines was assessed as described before using biorad 23 plex assay. N=5. *p<0.05; **p<0.01; ***p<0.001.

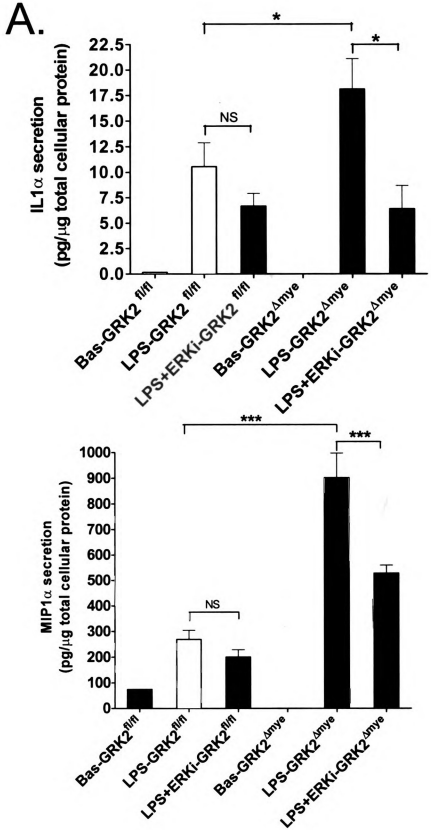

A.
IL1α secretion (pg/μg total cellular protein)
22.5
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
*
*
NS
Bas-GRK2 fl/fl
LPS-GRK2 fl/fl
LPS+ERKi-GRK2 fl/fl
Bas-GRK2 Δmye
LPS-GRK2 Δmye
LPS+ERKi-GRK2 Δmye
MIP1α secretion (pg/μg total cellular protein)
1000
900
800
700
600
500
400
300
200
100
0
***
***
NS
Bas-GRK2fl/fl
LPS-GRK2fl/fl
LPS+ERKi-GRK2fl/fl
Bas-GRK2Δmye
LPS-GRK2Δmye
LPS+ERKi-GRK2Δmye

Figure 5.11. Continued....

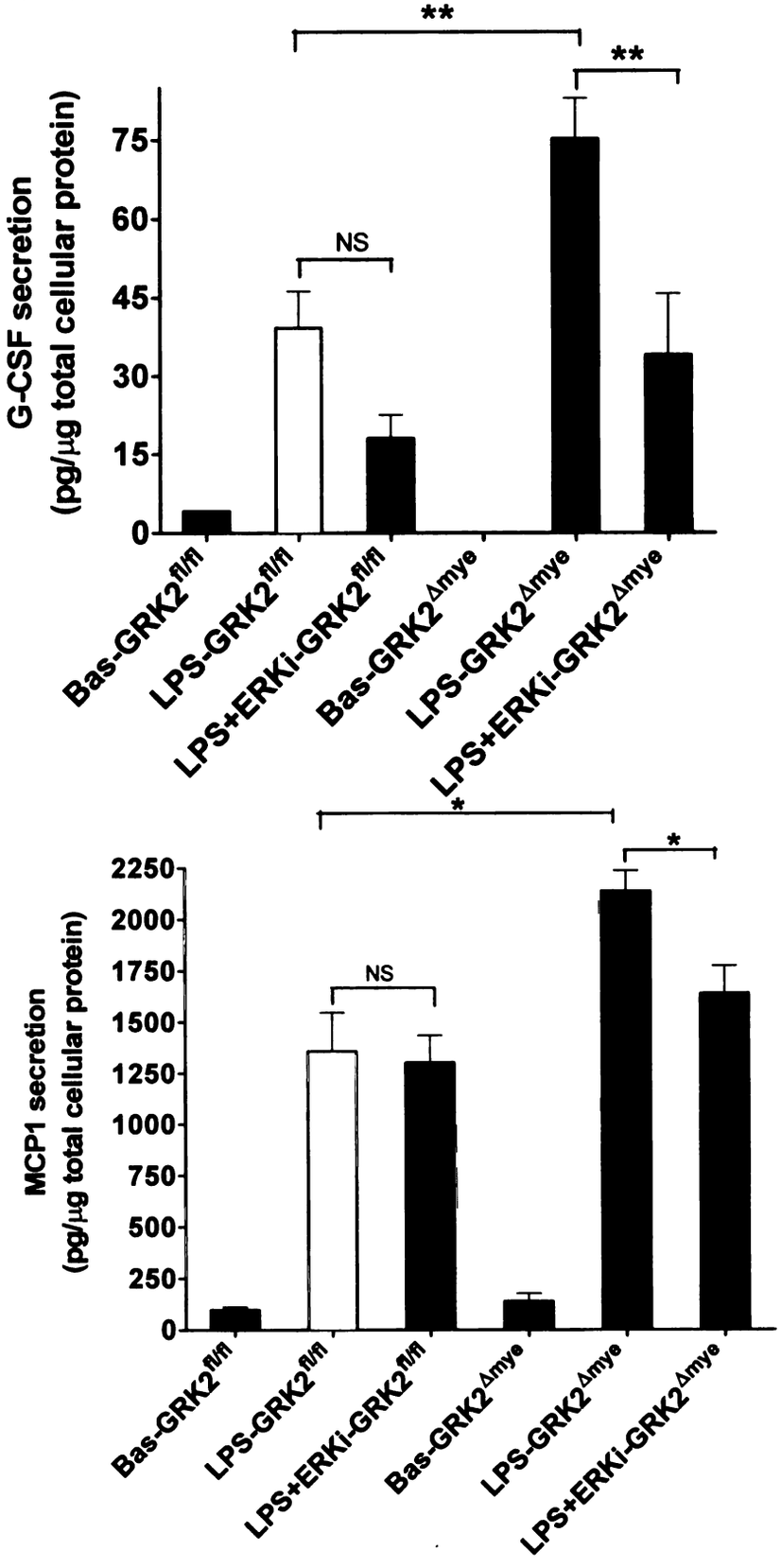
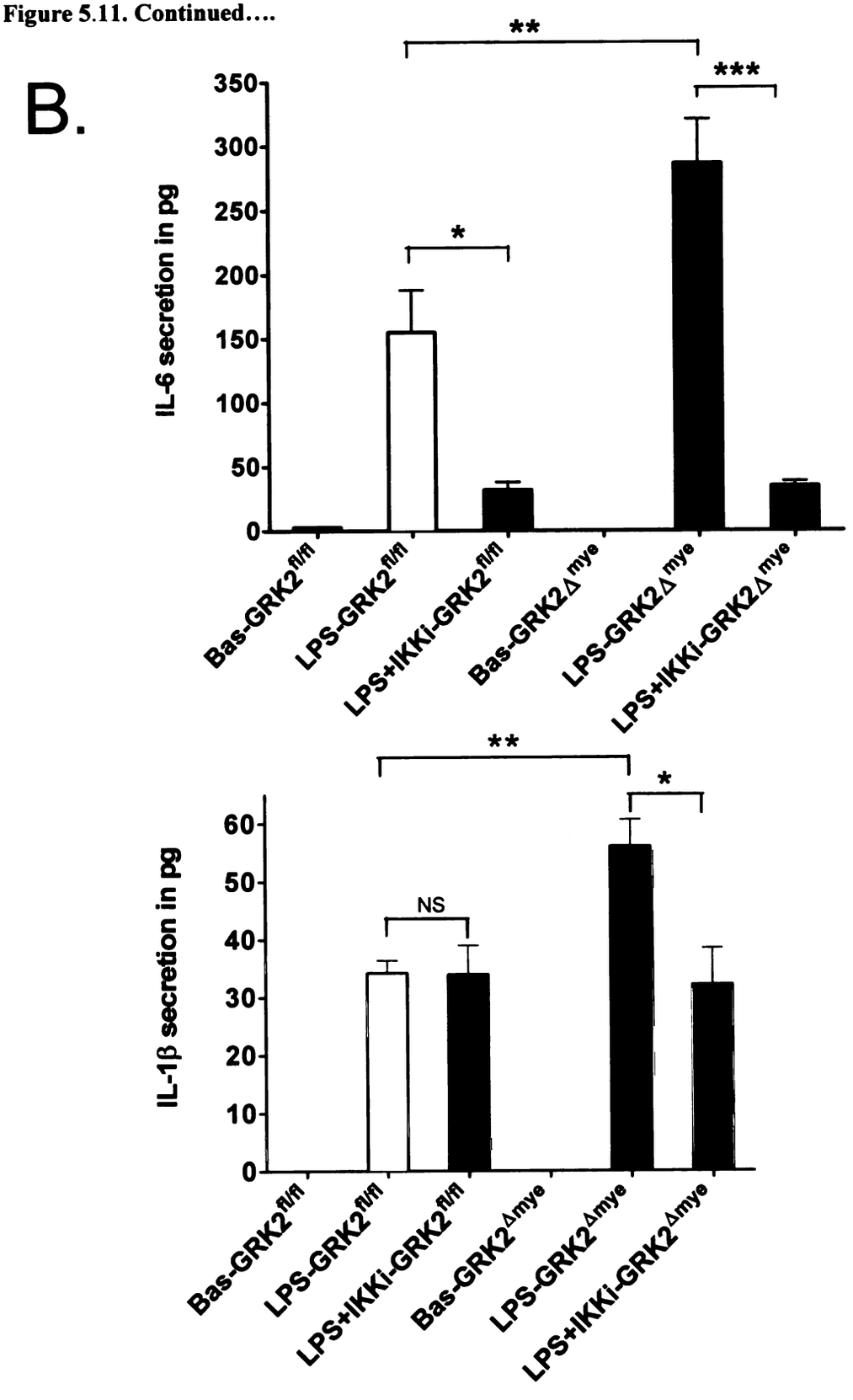


Figure 5.11. Continued....



DISCUSSION

It has been well documented that the expression levels of GRK2 are altered in certain inflammatory disease conditions. For instance, GRK2 levels are significantly reduced in leukocytes from patients of active relapsing-remitting multiple sclerosis (MS) or with secondary progressive MS [3]. Peripheral Lymphocytes of patients with Alzheimers disease have an increased expression both mRNA and protein for GRK2 [16]. GRK expression have also been found to be altered in PBMCs of patients with rheumatoid arthiritis [5]. Although the significance of these changes is not clear particularly in these diseases, studies are just emerging on the pathophysiological role of GRK2 in these diseases in rodent models. For example, in an acute model of arthritis, GRK2 was shown to be a negative regulator of granulocyte chemotaxis to LTB4 (a potent chemoattractant for granulocytes). Heterozygous GRK2 knockout mice showed an increased weight loss as well as development of arthritis was significantly enhanced in these mice [17]. It is, however, not clear what signals regulate the expression levels of GRK2. In a recent study, we demonstrated that TLR4 increases GRK2 levels in primary macrophages [15]. Other studies have demonstrated a similar role for TLR signaling in regulating GRK2 levels in immune cells. Although these studies proposed that the consequence of this increase is in limiting chemokine signaling, our studies presented here demonstrate that GRK2 regulates TLR4 signaling and LPS-induced cytokine/chemokine production and the consequent endotoxic shock.

We show that GRK2 deficient mice show an enhanced liver and lung injury in response to LPS injection and are prone to LPS induced septic shock. This is not surprising in the light of the fact that other studies using heterozygous GRK2 mice

have shown similar results with other disease conditions. For instance, GRK2 +/- mice were found to be more susceptible for arthritis disease severity [17]. GRK2 +/- animals also show an advanced onset of experimental autoimmune encephalomyelitis in association with an increased early cerebral infiltration of inflammatory cells [3]. Recent studies demonstrated that GRK2 +/- mice also show an increased susceptibility to neonatal hypoxic-ischemia brain damage [18]. While this manuscript was in preparation, another study showed the role of cell-type specific GRK2 in regulating hypoxia-ischemia brain damage [19]. Therefore, in general reduced levels of GRK2 increase the susceptibility to several disease conditions and we demonstrate here that myeloid cell GRK2 deficient mice are much more prone to LPS induced septic shock.

The migration of neutrophils to the inflammatory sites although important for host defense, is also in part responsible for tissue damage observed in sepsis. MIP1α, MIP1β, MCP1, Rantes and eotaxin are members of CC chemokine family which serve as major chemoattractants for neutrophils, mononuclear cells as well as eosinophils [20-23]. The secretary levels of all these chemokines were found to be enhanced in GRK2 deficient mice plasma. Out of these however, the levels of MIP1α were found to be significantly increased both in plasma as well as cell culture supernatants. Furthermore, MIP1α was found to be specifically regulated by p105-ERK signaling since ERK inhibitor as well as IKK inhibitor inhibited its increased synthesis. MIP1α is produced by several cell types including lymphocytes, monocytes/macrophages, mast cells, basophils, epithelial cells and fibroblasts and binds to CC chemokine receptor 1 (CCR1 and CCR5) to exert its biological actions. MIP1α plays a crucial role in activation and chemotaxis of several populations of macrophages. It stimulates

the proliferation of mature tissue macrophages and also has been shown to induce the secretion of TNFα, IL-6 and IL-1α from elicited peritoneal macrophages [24]. It is highly expressed in acute as well as chronic lung inflammation [25]. MIP1α alpha has been previously shown to be responsible for LPS induced lung capillary leakage and early mortality in endotoxic shock [23]. We, in our studies also found a substantially increased lung capillary permeability as assessed by the measurement of total protein in the broncho-alveolar lavage fluid suggesting a role for increased MIP1α in enhanced lung injury observed in our studies. Furthermore, there is an increased infiltration of inflammatory cells in the liver resulting in an increased liver damage. Hence, we propose MIP1α to be an important factor responsible for inducing a substantially increased lung and liver injury in GRK2 deficient mice. Another important pro-inflammatory cytokine IL-12p40 is also found to be highly secreted in GRK2 deficient mouse plasma. It is a component of IL-12 cytokine and is an important mediator of cell mediated immunity [26]. Furthermore, it has been shown to play an important role in inflammatory diseases such as experimental autoimmune encephalitis [27]. IL-10, an important anti-inflammatory cytokine was also found to be increased in GRK2 deficient mouse plasma. An increase in an anti-inflammatory cytokine should prevent from leading to endotoxemia in theory. However, studies have illustrated that although increase in IL-10 prevents an over-exuberant immune response, it can also lead to immunosuppression shifting the balance toward lethal consequencies. Since we observe an early lethality in LPS injected GRK2 deficient mice, it is suspected that the balance is shifted towards an increased inflammatory state leading to organ injury and mortality.

Our results demonstrate that the peritoneal macrophages from GRK2 deficient mice have an increased ERK activation in response to LPS treatment as compared to control mice. Inhibitor studies further support our findings that the increased ERK activity is responsible for the enhanced expression of certain pro-inflammatory mediators in GRK2 deficient peritoneal macrophages. The effect of GRK2 on ERK activation has also been shown by other investigators. In HEK 293 cells, increased GRK2 was found to cause reduced ERK activation in response to C-C chemokine ligand 2 (CCL2). On the other hand, splenocytes from GRK2+/- mice, were found to have an enhanced ERK activation in response to chemokines. These studies also showed that GRK2 and MEK are present in the same multi-molecular complex but unlike our studies, GRK2 did not affect MEK activity [28]. In another study, pro-inflammatory cytokine IL-1β was found to induce a 2-3 fold increase in the expression of GRK2 in primary astrocytes and a simultaneous decrease in CCL2-induced ERK1/2 activation whereas astrocytes from GRK2 +/- mice show an increase in ERK1/2 phosphorylation [29]. While these other studies have focused on chemokine receptor (a GPCR)-induced ERK activation, our studies address the role of GRK2 in TLR4 signaling, especially in primary macrophages. Thus although GRK2 has very important roles in GPCR signaling, our studies here demonstrate that GRK2 is equally important in regulating TLR4-induced ERK activation and the consequent cytokine/chemokine production.

In this study we have determined that the mechanism by which GRK2 regulates TLR4-induced ERK activation lies at the level of NFκB1 p105. In macrophages, NFκB1 p105 lies upstream of MEK1/2 kinase and is present in a

complex with Tpl2, a MAP3K. Our studies clearly show that LPS-induced MEK1/2 as well as NFκB1 p105 phosphorylation are significantly enhanced in macrophages from GRK2 deficient mice. We believe that the mechanism lies at the level of NFκB1 p105 for the following reasons: 1. LPS-induced IκBα phosphorylation is not affected by GRK2 deficiency in macrophages. IκBα is also phosphorylated by IKKβ, the same enzyme that phosphorylates NFκB1 p105. Thus if the regulation by GRK2 is at or above the level of IKKβ, then one would expect that IκBα phosphorylation to also be regulated by GRK2. 2. Previous studies have shown that GRK2 and NFκB1 p105 directly interact with each other. GRK2 and NFκB1 p105 have been shown to interact in yeast two-hybrid assays. And the RH domain of GRK2 has been shown to directly interact with the Carboxy terminus of p105 in direct interaction assays. Previous studies, however, have also shown that NFκB1 p105 is a poor substrate for GRK2, suggesting that the regulation might be phosphorylation independent. This is not entirely surprising given the role of RH domain of GRK2 in mediating phosphorylation-independent cell signaling, protentially as a scaffolding protein. In studies using Raw264.7 macrophages cell line, however, we did not find any functional significance of GRK2 in LPS-induced NFκB1 p105-ERK pathway, using RNAi. In the present studies we find that in primary macrophages from genetically modified levels of GRK2, GRK2 does regulate LPS-induced NFκB1 p105-ERK pathway. Thus the differences between the studies could be related to the differences in cell types (cell line v/s primary cells) and/or the method of decreasing GRK2 levels (RNAi v/s genetic).

GRK2 has previously been shown to regulate a number of signaling pathways not necessarily restricted to GPCR signaling. Even with in GPCRs, role of GRK2 has expanded considerably beyond its role in GPCR phosphorylation. Some of these signaling pathways are relevant to TLR4 signaling. For example, Liu et al., have demonstrated that Akt interacts with GRK2 and this interaction inhibits Akt activity, which then limits the activation of eNOS and the production of NO in sinusoidal endothelial cells leading to intrahepatic portal hypertension. Correspondingly, GRK2 deficient mice develop less severe portal hypertension after liver injury [30]. Even though TLR4 activation in primary macrophages induces Akt phosphorylation, we did not observe any difference in Akt phosphorylation between GRK2 deficient and control macrophages. One possibility for this difference is the different cell types examined. In another study, Peregrin et al. showed that GRK2 can interact with and phosphorylate p38 MAPK and this can lead to the inactivation of p38 MAPK. Furthermore, peritoneal macrophages from GRK2+/- mice were found to have an enhanced production of TNFα due to an enhanced activation of p38 [31]. In our studies, however, we did not observe enhanced activation of p38 in the GRK2 deficient macrophages compared to the control cells. The reason for this difference is not clear. In our studies, however, we demonstrate that inhibition of enhanced ERK activation significantly lowers the enhanced cytokine/chemokine levels to that of the wild type levels, suggesting a clear role for this signaling pathway in mediating MIP1α, IL-1α, GCSF and MCP1. Our studies further demonstrate that the NFκB1 P105-mediated p50-NFκB pathway may also be equally important in mediating the enhanced secretion of IL-1β and IL-6 in the GRK2 deficient macrophages because the

IKKβ inhibitor blocked the production of these two cytokines even though they were not affected by the ERK inhibitor.

It is not clear why we see a slightly different response in terms of the cytokines /chemokines which are secreted in plasma verses those in cell culture supernatants. However, one can envision several possible reasons for this discrepancy: 1. A variety of cell types are involved in producing cytokines under *in vivo* conditions and the levels observed in plasma are an overall contribution of these various cell types as compared to only one cell type used *in vitro* in our studies. 2. LPS stimulation of TLR4 onto macrophages is a direct signaling mechanism leading to the expression of genes for cytokines / chemokines as compared to indirect signaling mechanisms due to cross talks involved under *in vivo* conditions. 2. We used only one type of macrophages i.e. thioglycollated elicited peritoneal macrophages under *in vitro* conditions. However, there are a variety of macrophages (tissue specific) which contribute to the effects seen under *in vivo* conditions.

Several studies have shown that sepsis patients have increased circulatory levels of TNFα, MIP1α, MIP1β, IL-6 and IL-10. Furthermore, in one study it was found that neutrophils from septic patients show a reduced chemotaxis due to a failure to induce an increase in tyrosine phosphorylation and actin polymerization in response to chemokines. These neutrophils were found to have high expression levels of GRK2 and GRK5. Moreover, neutrophils from healthy individuals treated with LPS and cytokines were also found to have an increased expression of GRK2 and GRK5. This suggested that the pro-inflammatory mediators produced during sepsis increase the expression of GRKs leading to desensitization of neutrophils to chemokines [8].

However, our studies suggest that GRK2 is protective against sepsis in the initial phase since deletion of GRK2 in myeloid cells increases susceptibility to sepsis. In an already established septic condition however, the expression levels of GRK2 might increase in an effort to protect the system from the lethal consequences of sepsis. Reduced chemotaxis might be detrimental since neutrophils are required to help fight infection. The system, however, needs to maintain a balance and it is tempting to hypothesize that this might be mediated by increasing GRK2 levels under septic conditions. Obviously further studies are necessary to delineate these issues.

In summary, mice with a deficiency of GRK2 in myeloid cells are more susceptible to LPS-mediated endotoxemia and this is associated with enhanced secretion of several cytokines/chemokines. We further demonstrate that the enhanced cytokine/chemokine in the GRK2 deficient mice is related to enhanced activation of the p105-ERK pathway in macrophages. Our results suggest that the increase in GRK2 levels observed in immune cells under septic conditions might limit the progression of sepsis.

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CHAPTER 6

SUMMARY AND CONCLUSIONS

6.1 Specific aims and results of the study

The major aim of this thesis was to investigate the roles of GRKs in inflammatory signaling. It was hypothesized that GRKs are important regulators of inflammatory signaling in macrophages and therefore GRKs might play essential roles in the regulation of inflammatory diseases. Summarized below are the specific aims along with the results obtained:

Specific Aim 1: To determine the biochemical mechanisms by which GRKs regulate TNFα-induced signaling pathway in mouse macrophages

Results:

1. Knockdown of GRK2 and GRK5 inhibits TNFα-induced NFκB signaling in Raw 264.7 mouse macrophage cell line
2. Overexpression of GRK2 and GRK5 increases TNFα-induced NFκB activity
3. GRK2 and GRK5 interact with and phosphorylate IκBα, an inhibitor of NFκB
4. GRK5 phosphorylates serine 32 on IκBα which is the same site that is phosphorylated by IKKβ in classical NFκB signaling

Specific Aim 2: To determine the role and mechanism by which GRKs regulate inflammation in a mouse model of disease (Endotoxic shock)

Subaim 1: To study the role of GRK5 in inflammation in a GRK5 knockout mice using endotoxic shock model

Results:

1. Primary peritoneal macrophages from GRK5$^{-/-}$ mice exhibit an inhibition of LPS-induced NFκB activation as compared to primary peritoneal macrophages from GRK5$^{+/+}$ mice
2. GRK5 gene deletion does not affect LPS-induction of MAPKs viz. ERK, JNK and p38
3. Peritoneal macrophages from GRK5$^{-/-}$ mice show reduced secretion of key inflammatory cyokines in comparison to those from GRK5$^{+/+}$ mice
4. LPS-induced secretion of plasma cytokines is also inhibited in GRK5$^{-/-}$ mice compared to GRK5$^{+/+}$ mice
5. Reduced secretion of cytokines and chemokines in the plasma of GRK5$^{-/-}$ mice was associated with reduced ALT levels (an indicator of liver injury), suggesting diminished liver injury in GRK$^{-/-}$ mice

Subaim 2: To generate and characterize a myeloid cell specific GRK2 knockout mice and to study the role of GRK2 in inflammation using endotoxic shock model

Results:

1. A myeloid cell specific knockout of GRK2 was generated using Cre-LoxP system
2. Myeloid lineage GRK2 deficiency did not affect the development or maintenance of myeloid cells

3. LPS injection caused an enhanced secretion of cytokines and chemokines in the plasma of myeloid cell-specific GRK2 deficient mice in comparison to littermate control mice
4. Primary peritoneal macrophages as well as neutrophils from GRK2 deficient mice showed an increased secretion of cytokines and chemokines in response to LPS
5. GRK2 deficient mice exhibited a greater lung and liver injury and were more susceptible to LPS-induced septic shock
6. LPS induced NFκB1 p105 as well as ERK phosphorylation were found to be enhanced in the peritoneal macrophages from GRK2 deficient mice
7. Pharmacological inhibition of ERK and NFκB1 p105 blocked the enhanced expression of cytokines and chemokines in the peritoneal macrophages from GRK2 deficient mice

6.2 Limitations of the study

1. Knockdown of both GRK2 and GRK5 inhibited TNFα-induced NFκB activation in Raw 264.7 mouse macrophage cell line. Primary peritoneal macrophages from a germline GRK5 deleted mice similarly showed an inhibition of LPS-induced NFκB activity. Primary peritoneal macrophages from myeloid cell specific GRK2 deleted mice however, showed no change in LPS-induced NFκB activity. Results from these three different studies should be compared with some degree of caution due to the following reasons:

a) Three different methods of inhibiting the expression levels of GRKs were used viz. siRNA, germline deletion and Cre-LoxP mediated gene deletion.

b) Three different types of cells were utilized: Cultured mouse macrophages, peritoneal macrophages from mice of different genetic backgrounds

2. LPS injection model of sepsis was utilized in this study. Although this is a widely used model due to its simplicity, it should be noted that it does not mimic human sepsis which is characterized by bacteremia with multiple species of bacteria.

3. Since these studies were done on mouse macrophages, its relevance to humans is not known at present.

4. Myeloid cell specific GRK2 deficient mice were used in this study due to the non-availability of homozygous GRK2 knockout mice. Hence, results obtained in this study cannot be generalized as negative regulation of sepsis by GRK2. This is because GRK2 present in non-myeloid cells might regulate sepsis differently.

6.3 Positive outcomes of the study

This thesis research investigated the roles of GRK2 and GRK5 in inflammatory signaling in macrophages. The three individual studies in this project characterized the biochemical mechanisms by which GRKs regulate inflammatory signaling as well as the physiological consequence of such regulation at *in vivo* levels.

This study found that GRK5 can function as an IκB kinase and phosphorylate serine 32, one of the same sites phosphorylated by IKKβ in the classical NFκB pathway. Although further studies are needed to investigate the phosphorylation of IκBα by GRK2 and 5 at *in vivo* levels as well as identify the sites of phosphorylation

on IκBα, our studies provide preliminary evidence that GRK5 can act as an IκB kinase with a capacity similar to IKKβ in macrophages and can thereby regulate TNFα and TLR4-NFκB signaling. Since NFκB signaling plays an important role in several inflammatory disease conditions, our findings in macrophages could have great impact in terms of regulation of the pathogenesis of several inflammatory diseases. Indeed, our *in vivo* studies using LPS injection mouse model of sepsis validates our *in vitro* findings in macrophages.

Studies of modulation of LPS-induced sepsis by GRK5 suggest that GRK5 positively regulates LPS induced signaling under *in vivo* conditions. Our studies also show that the regulation of expression of inflammatory mediators by GRK5 occurs at later time points. This is evident from the fact that the levels of most of the cytokines and chemokines in the plasma are similar at earlier time points in both $GRK5^{-/-}$ as well as $GRK5^{+/+}$ mice. This suggests that at earlier time points cytokines are secreted from stored granules in response to LPS and hence GRK5 does not affect their secretion. However, by 12 hours the levels of these inflammatory mediators are reduced in $GRK5^{-/-}$ mice but not in $GRK5^{+/+}$ mice suggesting regulation by GRK5 at the transcriptional levels. This could be very important in terms of disease pathogenesis since cytokines and chemokines are required during early phases of infection. An increased or continued production, however, leads to tissue injury and chronic inflammation. Our studies, thus suggest that GRK5 could be a potential drug target because inhibition of GRK5 might result in lowered but not complete suppression of cytokine levels. This might then subsequently lead to a resolution of

inflammation. The next logical steps here are to establish the role of GRK5 in human sepsis and other human inflammatory diseases.

Unlike GRK5, GRK2 was found to negatively regulate LPS induced signaling under *in vivo* conditions. Previous studies showed that the expression levels of both GRK2 and GRK5 increase in the neutrophils from sepsis patients suggesting that GRKs are involved in sepsis, our studies define a protective role for myeloid cell GRK2 in sepsis. Interestingly, our previous findings in peritoneal macrophages showed that LPS stimulation increases GRK2 but decreases GRK5 levels. Intriguingly, GRK2 appears to be a negative regulator and GRK5 a positive regulator of endotoxemia. Taken together, this suggests that endotoxemia is regulated by GRK2 and GRK5 by a feedback mechanism in an effort to maintain homeostasis. That is, the negative regulator (GRK2) is upregulated whereas the positive regulator (GRK5) is downregulated to prevent further TLR4 signaling and damage.

Because inhibition of NFκB and MAPK pathways have not been successful in limiting all inflammatory diseases, investigation of novel regulators of NFκB and MAPK pathways is an extensive area of research to treat inflammatory diseases. In our studies we have discovered that GRK5 and GRK2 are two novel regulators of NFκB and ERK signaling pathway respectively. Based on our studies, we propose that GRK2 and GRK5 may be targets for therapeutic drug development in sepsis.

6.4 Future experiments

Some of the future experiments to further characterize the roles of GRK2 and 5 in inflammatory signaling should focus onto investigating the regulation of other

inflammatory diseases by GRKs. In particular, double gene deletions of GRKs with inflammatory disease models such as LDL receptor knockout (mouse model of atherosclerosis) or 1L-10 knockout (mouse model of colitis) would be useful models to define the roles of GRKs in these inflammatory diseases. The regulation of inflammatory signaling by GRK2 and 5 in human cells should be studied. If the results are similar in human cells, drug development can be considered whereby inhibitors for GRK5 would be very useful in reducing the tissue injury seen in sepsis. A microarray approach to investigate the genes that are specifically up or downregulated in LPS stimulated GRK2 and 5 deleted macrophages would be useful to determine the downstream effects of GRKs on inflammatory signaling. Investigating whether signaling by other TLRs such as TLR2, TLR3 and TLR9 is similarly regulated by GRK2 and 5 would add to our understanding of the role of GRKs in inflammatory signaling. Peptidoglycans from gram positive bacteria serve as ligands for TLR2 which then leads to gram positive bacterial sepsis. TLR3 and TLR9 on the other hand are activated by viruses. The results from such studies will help us determine treatment strategies as inflammatory diseases such as sepsis is a complex syndrome involving different species and strains of pathogens.

One of the other factors to be considered is the regulation of signaling by GRK2 and 5 in cells other than macrophages. In our studies, although we found a similar response in terms of production of several inflammatory mediators, both in primary macrophage cell culture supernatants as well as in the plasma (up or down regulation), some of the mediators found to be regulated in macrophages were found to be unaffected in plasma and *vice versa*. One possible explanation for these results is

the fact that other cell types such as T cells as well as cells such as fibroblasts and endothelial cells are also involved in the production and release of inflammatory mediators. Hence, it is also essential to determine the cell type specific regulation of the inflammatory signaling by GRK2 and 5. The results from such studies would help us determine treatment strategies as a cell type specific drug approach or a global targeting of GRKs.