CRITICAL ROLE OF G-PROTEIN COUPLED RECEPTOR KINASE 5 IN SEPSIS

By

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ABSTRACT

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G-protein coupled receptor kinase-5 (GRK5) is a member of the GRK family of kinases, originally discovered in the context of G-protein coupled receptor (GPCR) desensitization. Recent studies from our lab and others have demonstrated critical role of GRK5 in inflammatory pathways particularly involving Toll-like receptors. Because Toll-like receptor signaling pathways are critical in the regulation of sepsis pathogenesis, it was hypothesized that GRK5 is a critical regulator of inflammatory pathways in sepsis pathogenesis. Thus the overall goal of the thesis project was to decipher the role of GRK5 in sepsis pathogenesis using mouse models of sepsis. In initial studies, we demonstrated GRK5 regulates cytokine production *in vivo* by both MyD88 and TRIF dependent pathways, suggesting that GRK5 can regulate a broad range of TLRs. In the next set of studies, we used poly-microbial and mono-microbial sepsis models to further understand the role of GRK5. Indeed, our studies using polymicrobial sepsis delineates key roles for GRK5 in modulating sepsis outcome. We demonstrated that GRK5 is a critical positive regulator of inflammatory mediator production; sepsis induced thymic apoptosis and development of immune suppression. More importantly, we established that GRK5 deficiency protected mice from polymicrobial sepsis-induced mortality suggesting that GRK5 could be amenable for therapeutic targeting in sepsis. Having demonstrated the role of GRK5 in polymicrobial sepsis, we further examined the role of GRK5 in a mono-microbial acute lung infection model. Here we demonstrated that GRK5 has distinct roles in regulating progression of inflammation depending on the dose of bacteria. Our study revealed GRK5 as a crucial regulator

of neutrophil chemotaxis early during infection likely *via* modulation of CXCL1/KC levels. In addition, we further show that GRK5 modulates bacterial burden in lungs depending on the initial dose of *E. coli* infection. And the regulation of bacterial burden was further dependent on the *in vivo* neutrophil activation status. Overall, our studies have uncovered novel roles of GRK5 during acute inflammatory disease processes and further work in discerning the involved molecular mechanisms will likely lead to therapeutic strategies to target GRK5 during sepsis-induced inflammatory conditions.

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KEY TO ABBREVIATIONS

AdGRK5-NT: Adenoviral GRK5-N-terminal construct

Akt: Protein Kinase B (PKB)

ANF: Atrial natriuretic factor

ANOVA: Analysis of Variance

AP-1: Activator protein-1

Apaf1: Apoptosis protease-activating factor 1

ATCC: American type culture collection

β2AR: β2 -adrenergic receptor

BAD: Bcl-2 associated death promoter

Bcl-2: B cell lymphoma protein 2

Bcl-3: B cell lymphoma protein

Bcl-xl: B cell lymphoma-extra large protein

CaM: Calmodulin

cAMP: cyclic AMP

dsRNA: double stranded RNA

E. coli: *Escherichia coli*

ERK: Extracellular signal-regulated kinase

GPCR: G protein-coupled receptor

GRK: G protein-coupled receptor kinase

GRK5-NT: G protein-coupled receptor kinase 5 – amino terminal

GTP: Guanosine triphosphate

HDAC: Histone deacetylase

HEK293: Human embryonic kidney 293

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid

HPRT: Hypoxanthine-guanine phosphoribosyl transferase

IB: Immunoblot

ICAM: Intracellular adhesion molecule

IκB: Inhibitor of κB

IKK: Inhibitor of κB kinase

iNOS: inducible nitric oxide synthase

IRAK1: Interleukin-1 receptor-associated kinase-1

IRAK4: Interleukin-1 receptor-associated kinase-4

IRF3: Interferon regulatory factor 3

Jaks: Janus kinases

LPS: Lipopolysaccharide

MAPK: Mitogen-activated protein kinase

Mdm2: Murine double minute oncogene 2

MEK1: MAPK/ERK kinase

MyD88: Myeloid differentiation primary response gene 88

n: Amino

NADPH-oxidase: Nicotinamide adenine dinucleotide phosphate-oxidase

NES: Nuclear export sequence

NFκB: Nuclear factor kappa enhancer of activated B cells

NLS: Nuclear localization sequence

NT: Amino-terminal region

p: phosphorylated

p38-MAPK: p38 mitogen activated protein kinase 5

p65: Transcription factor p65 (RelA)

p50: NF-KappaB1

PAMPs: pathogen-associated molecular patterns

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PH: Pleckstrin homology

PIP2: Phosphatidylinositol 4,5-bisphosphate

PI3K: phosphoinositide 3-kinase

PKA: cAMP-dependent protein kinase

PKC: Protein kinase C

PKD: Protein kinase D

PLC: Phospholipase C

PLK1: Polo-like kinase 1

PMSF: Phenylmethanesulfonyl fluoride

qRT-PCR: Quantitative reverse transcription – polymerase chain reaction

RGS: Regulator of G protein signaling

RH: RGS homology

ROS: reactive oxygen species

SDS: Sodium dodecyl sulphate

SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

TAK1: Transforming growth factor- β-activated kinase 1

TBK1: TANK binding kinase 1

TEMED: Tetramethylethylenediamine

TIR: Toll-interleukin-1 receptor

TIRAP: TIR domain-containing adaptor protein

TLRs: Toll-like receptors

TNFα: Tumor necrosis factor-α

TNFR: Tumor necrosis factor receptor

TPL2: Tumor progression locus 2 TAB: TAK1-binding protein

TRAF6: TNF receptor-associated factor 6

TRIF: TIR domain-containing adaptor inducing IFN-β

CHAPTER ONE

A LITERATURE REVIEW ON G-PROTEIN COUPLED RECEPTOR KINASE 5 (GRK5) AND SEPSIS

G protein coupled receptor kinases (GRKs) are key modulators of G-protein coupled receptor (GPCR) signaling. GRKs are serine/threonine kinases that phosphorylate agonist bound GPCRs, desensitize them and regulate their downstream GPCR signaling. In addition to their classical role in GPCR desensitization, GRKs are known to carry out GPCR and/or G-protein independent roles including phosphorylation dependent regulation of cellular signaling, modifying transcription of genes by virtue of nuclear localizing signal (NLS), or by phosphorylation independent protein-protein interactions. Recent studies emphasized on this GPCR- and G-protein independent role of GRKs kindled a renewed interest in using GRKs as drug targets in inflammatory diseases. Thus, it was hypothesized that GRK5 (one of the GRKs) by virtue of its role in mediating inflammatory signaling can modulate the outcome of inflammatory disease pathogenesis. This thesis investigates, the role of GRK5, a member of GRK family in the pathophysiology of sepsis using mouse microbial sepsis models.

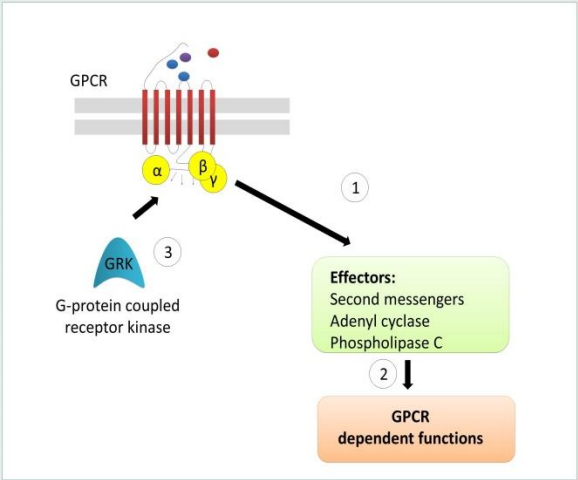
This brief introduction provides an overview of the hypothesis tested in this thesis with a glance on the focus of subsequent chapters. Chapter one provides a comprehensive literature review pertaining to the current knowledge on GRKs and its involvement in inflammatory diseases. Chapters two through four are organized based on the specific aims of this thesis, each consisting of an abstract, introduction, materials and methods, results and discussion. Chapter two focuses on the overlapping and distinct roles of GRK5 in MyD88 dependent and independent pathways using TLR2-, and TLR3- ligand induced inflammatory response *in vivo*. Chapter three focuses on the critical role of GRK5 in mediating polymicrobial sepsis. Chapter four focuses on understanding the critical involvement of GRK5 in modulating severity and

outcome of *E. coli* induced acute pulmonary inflammation. Chapter five highlights the results obtained in this project with a discussion on how these findings contribute to our current understanding of the role of GRK5. Also, discussed are some of the limitations and future directions of this project.

1.1: Introduction: G-protein-coupled receptor kinases (GRKs)

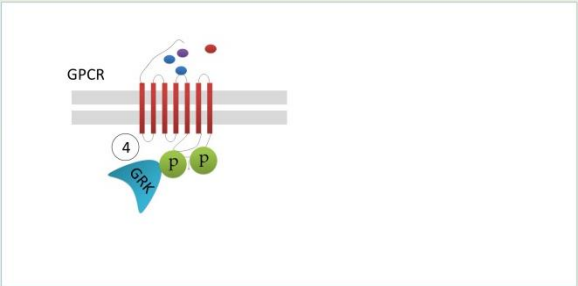
The superfamily of hepta-helical transmembrane proteins (also called G-protein-coupled receptors (GPCRs)) modulate a variety of physiological processes including sense of vision, olfaction, hormonal signal transduction, cellular proliferation, differentiation, survival etc. Upon activation, GPCRs activate heterotrimeric G-proteins to dissociate into Gα and Gβγ subunits. These subunits modulate various effector mechanisms to bring about the physiological responses. Most GPCRs display a rapid loss of responsiveness even in the continuing presence of stimuli; this phenomenon is now widely appreciated and known as desensitization. The mechanism of GPCR desensitization is a specific, controlled two-step mechanism (Figure 1). First, GPCR kinases (GRKs) phosphorylate the activated receptors, marking those targets for binding to arrestins (intracellular scaffolding protein). Secondly, the arrestin bound receptor prevents the GPCR from G-protein-dependent signaling by not only steric interference but also by mediating endocytosis of these receptors[1,2].

A)



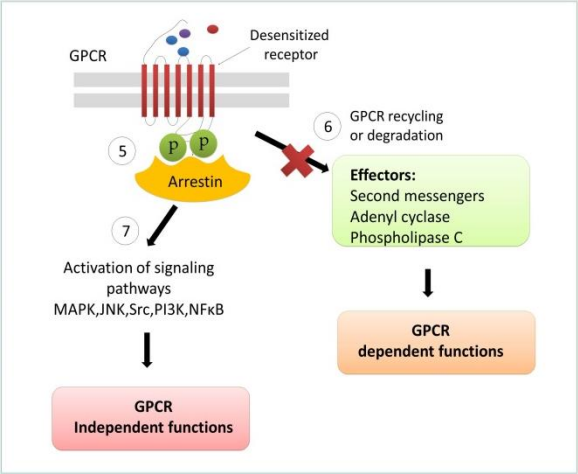
1. GPCR activation leads to recruitment of various second messengers.
2. Second messengers activate downstream signaling, referred as G-protein dependent GPCR functions.
3. GPCR activation recruits GRKs

B)



4. GRKs phosphorylate intracellular domains of GPCRs.

C)



5. Phosphorylated GPCR signals arrestin recruitment and binding.
6. Arrestin binding inhibits G-protein signaling. Arrestin and GPCR form a signalosome complex.
7. Arrestin signalosome activates G-protein-independent signaling.

Figure 1.1: Schematic summary of the role of GRKs/arrestins in carrying out GPCR dependent and independent functions. a) GPCR activation and G-protein dependent function. b) GRK recruitment and phosphorylation of GPCR intracellular domains. c) Binding of arrestin, formation of signalosome complexes leading to G-protein independent functions.

Sustained research in the GPCR field yielded remarkable insights on the GPCR regulation and function leading to development of various pharmacological agents[3,4]. And of all the pharmaceutical drugs out on market more than 50% target GPCRs. In addition to the desensitization functions, recent research indicates that GRKs and arrestins are involved in signaling functions that are desensitization- and G-protein independent. In many cases, it has been shown that arrestins and GRKs mediate these G-protein independent effects by recruiting several signaling mediators or by phosphorylation of signaling molecules by GRKs. In addition to mediating G-protein-independent effects of GPCRs, there is now convincing evidence that GRKs regulate non-GPCR families including tyrosine kinase receptors, TNF receptors and toll like receptors in both phosphorylation dependent and independent manner. In this review, we discuss these emerging functions of GRKs and their potential implications in both physiological and pathological conditions.

1.2: The G-protein coupled receptor kinase family: Identification, organization and structure

During the 1970s and mid-1980s, agonist induced dampening of G protein-mediated signaling was discovered on two receptors, rhodopsin and β2-adrenergic receptor *via* phosphorylation of their cytoplasmic c-terminal domain. The enzyme phosphorylating rhodopsin receptor, which controls vision, was identified in the late 1970s and aptly termed "Rhodopsin kinase" (modern name: GRK1). During 1986, a novel kinase was identified that phosphorylated β2- adrenergic receptor and dampened G protein-mediated signaling[5]. This kinase was initially named β-adrenergic receptor kinase or βARK (modern name: GRK2). In the

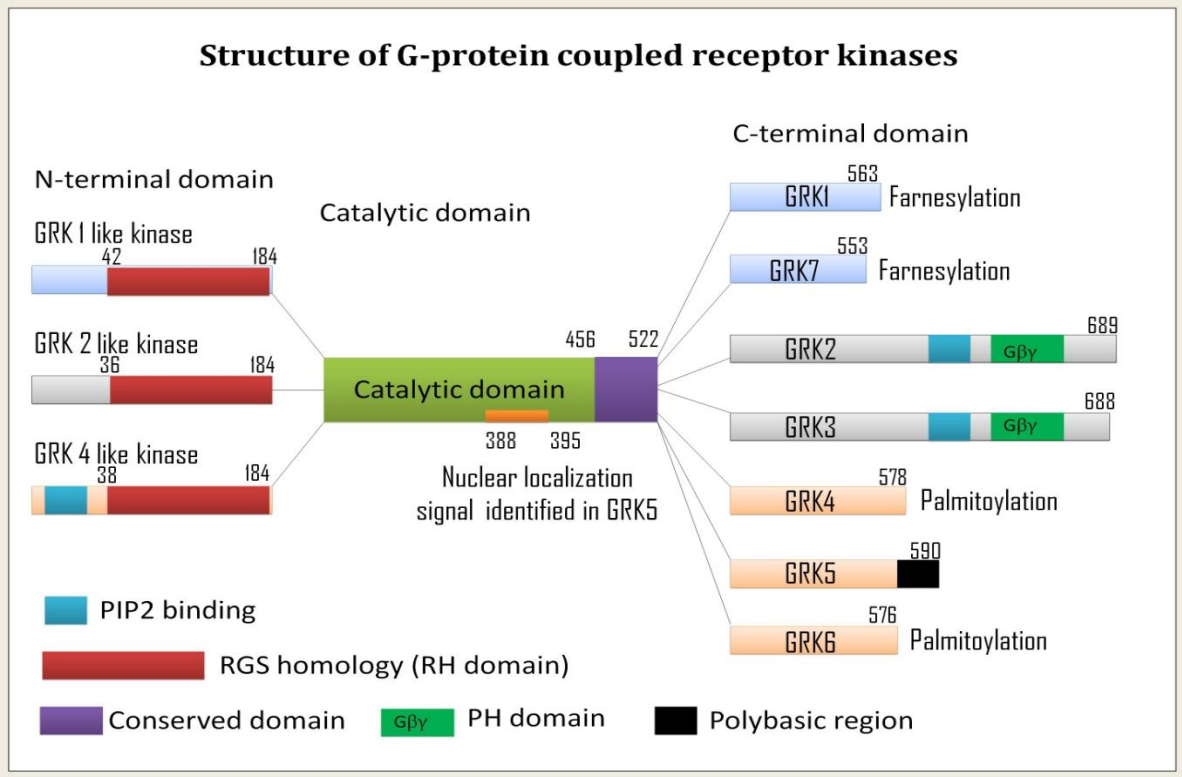


Figure 1.2: Domain structure GRK family of proteins: GRK family of proteins contains a GRK family specific short N-terminal domain and catalytic domain, with variations in C-terminal domains. Regulator of G-protein signaling homology domain (RH) is present in N-terminal region represented in red. Structural differences in the C-terminal domain differentiate GRKs. The Phosphatidylinositol 4,5-bisphosphate (PIP2) sites in C-terminal domain of GRK2/3 family (represented in blue), polybasic and palmitoylation sites in C-terminal domain of GRK4/5/6 family, short farnesylated C-termini of GRK1/7 aids in membrane translocation. GRK5 contains a nuclear localization signal represented in red. Numbers above domains represent amino acid residue based on Lodowski *et al*. (2006)[6].

same year, cloning of the mammalian beta-adrenergic receptor revealed a sequence similarity with the photo receptor, rhodopsin and led to the recognition of a GPCR family, with rhodopsin as its founding member. Another protein (termed S-antigen) was discovered initially for its role in allergic uveitis[7], was found to associate with rhodopsin and dampen G-protein mediated retinal signaling[8,9]. In 1986, with the discovery of this "arresting" function in retinal signaling, the protein was renamed "arrestin". Further functional characterization of GRKs and arrestin firmly established the idea of two-step inactivation of GPCRs. Meanwhile, other members of the GRK family were identified including GRK3 [10], GRK4 [11], GRK5 [12], GRK6 [13] and GRK7[14,15]. GRKs are grouped functionally into two classes: Visual and non-visual GRKs. Expression of the visual GRKs comprising GRK1 and GRK7 is restricted to the eyes and pineal gland. The non-visual GRKs can be grouped into two sub-groups: GRK2-like [GRK2 and GRK3, otherwise known as βARK1 and 2 (β-adrenergic receptor kinases 1 and 2)] and GRK4-like (GRK4, GRK5 and GRK6) based on their structural similarity[16]. Unlike visual GRKs, the non-visual GRKs are widely distributed throughout the body with the exception of GRK4, which is found restricted in the testis and proximal tubule of the kidney. Therefore, most GPCRs in the body are invariably regulated by four GRKs namely GRK2, GRK3, GRK5 and GRK6. And each of the four GRKs may regulate more than one GPCR (there are approximately 800 GPCRs in the mammalian body), increasing the complexity of understanding the role of GRKs.

All GRKs are multi domain proteins, sharing a 25 residue n-terminal region (unique for GRKs) followed by a regulator of G-protein signaling homology domain (RH) and a catalytic ser/thr protein kinase domain responsible for phosphorylating substrates (Figure 1). The n-terminal basic region in the GRK4- like family (GRK4-6) aid in membrane translocation.

However, the c-terminal domain is the most essential domain for targeting the GRKs to the plasma membrane. GRK1 and 7 are membrane associated by virtue of their short farnesylated c-termini. The c-termini of GRK2 like kinases are comparatively longer than the GRK4 like family and contain a 125 amino acid pleckstrin homology (PH) domain, which has binding sites for PIP2 and free Gβγ. Hence, on GPCR activation these primarily cytosolic GRKs (GRK2 and 3) are targeted to the membranes. GRK4 and GRK6 are exclusively membrane associated by their palmitoylated residues within the last 15-20 amino acids of the c-terminus and also by their n-terminal basic region. GRK5 is also predominantly membrane bound, but not through lipidation of c-termini, but through the poly basic regions found in both the c- and n- termini[17].

Early research on GRKs primarily focused on GRK2, GRK3 and it has been widely assumed that GRK4, GRK5 and GRK6 might be just redundant kinases. However, studies focusing on the GRK4- like family have remarkably changed the view of GRKs plainly acting for GPCRs. Recent studies have appreciated the intrinsic kinase activity possessed by these GRKs to regulate non GPCR substrates. In particular, GRK5 and GRK6 of the GRK4 like sub family have been shown to phosphorylate p53, HDAC5, Lrp6, tubulin etc. Interestingly, GRK5 also contains a nuclear localization signal and has been shown to accumulate inside the nucleus. Indeed, GRK5 can phosphorylate class II histone deacetylase and therefore mediate gene transcription[18]. Interestingly, GRK6A, one of the three splice variants of GRK6, was also detected inside the nucleus; however, its physiological role is yet to be ascertained[19]. These unforeseen roles of GRKs and their ability to initiate alternate signaling pathways have invoked a tremendous interest in the field.

1.3: Regulation and Activation of GRKs

Similar to other kinases, GRK activity is regulated by both protein-protein interactions and phosphorylation events. Gβγ subunits were the earliest identified proteins known to interact with and activate GRKs. Other studies have demonstrated that lipids can also bind and activate GRKs. Interestingly, phospholipids (PIP2) binding to the c-terminal PH domain of GRK2 family activates their kinase activity, whereas GRK4 like family members are activated by binding of phospholipids (PIP2) to their n-terminal polybasic regions[20]. Furthermore, calmodulin, caveolin-1, and actin were shown to affect GRK activity by direct binding.

Phosphorylation can lead to either activation or deactivation of GRKs. Visual GRKs (GRK 1 and 7) tend to get deactivated by both auto phosphorylation and phosphorylation mediated by PKA [21]. In contrast, non-visual GRKs tend to get activated or deactivated depending on the stimuli. GRK5 auto phosphorylated by phospholipid binding enhances its ability to phosphorylate receptors, but GRK5 phosphorylated by protein kinase C (PKC) tends to inhibit it [22]. However, the same PKC tends to enhance the activity of GRK2 by inducing phosphorylation[23]. Apart from phosphorylation, GRK2 can also be s-nitrosylated by nitric oxide synthase to inhibit its activity[24]. These findings suggest that multiple pathways are involved in regulating the activities of GRKs.

Recently[25], crystallographic structures of GRK1, GRK2 and GRK6 have been elucidated to give certain vital information on how these GRKs act to cause receptor desensitization. These studies revealed GRKs in a closed conformation in which the n-termini had conserved 18-20 residues that formed an alpha (αN) helix and interacted with the kinase domain. The formation of

this αN helix stabilized the closed (active) conformation of the kinase domain leading to allosteric activation and favored catalysis by the kinase domain. Furthermore, the αN helix is proposed to act as a docking site for the activated receptors and removal of the αN helix abolished GPCR phosphorylation suggesting αN helix formation is indeed the primary step in the activation of GRKs.

1.4: Phenotypes of GRK deficient mouse

Phenotypes of GRK knockout mice enabled researchers to identify the unknown physiological and pathological roles of GRKs. In some cases, GRKs were able to compensate for each other, and were able to produce less obvious phenotypes. The most obvious phenotype was from that of homozygous knockout of GRK2 that led to embryonic lethality due to defective cardiac development[26]. Using targeted knockouts and heterozygous mice, GRK2 has been shown to be important in the development of heart, lymphocyte chemotaxis, experimental auto immune encephalomyelitis, sepsis, atherosclerosis etc. Closer examination of other GRK knockout mice surprisingly revealed distinctive phenotypes- GRK3 in olfaction[27]; GRK6 in behavioral responses, locomotor stimulating effects of cocaine etc.[28]

GRK5 was found to regulate cholinergic responses. GRK5 deficiency caused hypothermia, hypo activity, tremors and enhanced salivation with oxotremorine (a muscarinic receptor agonist)[29]. In addition β2 adrenergic receptor-induced airway smooth muscle relaxation was reduced in GRK5 deficient mouse. Transgenic mice overexpressing GRK5 in cardiac tissues and vascular smooth muscle showed increased β adrenergic receptor desensitization and increase in blood pressure respectively.

Research over the last few years shows, GRKs have multiple functions in different organ systems. However, their role in immune system and its consequences are only beginning to be understood. Here, we will focus on the role of GRKs on the regulation of the immune system and its effects on function.

1.5: GRKs in immune system and inflammation

GPCRs are known to modulate immune functions including inflammatory responses[30-32], chemotaxis[30-32], and apoptosis[33] . GRKs are the turn off signals for G-protein dependent signaling, and along with its function on non- GPCR signaling such as TLR signaling, makes them important proteins to study with respect to the immune system.

1.5.1: Inflammatory signaling and GRKs

Inflammation is a complex biological response of host tissue to any inciting stimuli ranging from pathogens, irritants and even damaged host cells. Inflammatory processes are generally considered to be beneficial in most cases, however, when not controlled (acute inflammation) or persisting for a long duration of time (chronic inflammation) are considered harmful. It is for that reason the inflammation process is tightly regulated by the host. Broadly inflammation can be categorized into three stages: a) onset stage, b) propagation stage and c) resolution stage. Any defect in regulating these stages can have major consequences in the final outcome of the response. During the onset stage, different classes of receptor molecules, recognize the inciting stimuli and activate downstream effector molecules leading to propagation of inflammation *via* production of inflammatory mediators like cytokines and chemokines. These inflammatory mediators amplify the inflammatory process by recruiting immune cells to the site,

which results in prompt removal of inciting stimuli. Once the stimulus is removed, anti-inflammatory mediators are produced which result in resolution of inflammation. If this is not the case, the inflammation will propagate itself causing destruction of host tissues by inflammatory mediators and also by the inciting stimuli eventually leading to death of the host. Sepsis or septic syndromes classically manifest in this manner, wherein the inciting cause is a microbe. Since, my research project, mostly focuses on bacterial inflammation, from here on I will focus on bacteria/ bacterial products induced inflammatory signaling and the role of GRKs in modulating those signaling pathways.

Once the skin or mucosal barrier is breached by bacteria, the phagocytic cells of the innate immune system interact with the pathogens *via* recognizing evolutionarily conserved patterns on the surface of microbes, called PAMP's – pathogen-associated molecular patterns. PAMPs include a wide variety of microbial components ranging from Lipopolysaccharide (LPS) from gram-negative bacteria, flagellin, lipoteichoic acid from Gram positive bacteria, peptidoglycan from the bacterial cell wall, zymosan from fungi, and nucleic acid from viruses. PAMPs bind to pattern recognition receptors (PRR) on or within innate immune cells. Broadly PRR´s are classified into: 1) the toll like receptors (TLR's); 2) the intracellular nucleotide-binding oligomerization domain (NOD) leucine-rich repeat proteins; and 3) the retinoic-acid-inducible gene I (RIG-I)-like helicases. Upon engagement these receptors activate intra cellular signaling proteins like NFκB, AP1, CREB, and c/EBPβ, which will eventually lead to gene transcription and inflammatory mediator production.

1.5.1.1: Toll like receptor (TLR) signaling, NFκB and GRK5

Toll-like receptors are molecular sensors for microorganisms including viruses, bacteria, and fungi. TLR2 and TLR4 recognizes components from Gram positive and negative bacteria respectively, TLR3 senses viral dsRNA, and TLR9 is activated by unmethylated bacterial DNA. Lipopolysaccharide (LPS) binds to the CD14-TLR4/MD2 complex and results in activation of TLR4. Intracellular adaptor molecules containing conserved Toll/interleukin-1 receptor (TIR) domain are necessary to recruit and activate downstream kinases and transcription factors that regulate the host inflammatory response and type I IFN production. LPS/TLR4 signaling has been divided into two pathways: a MyD88-dependent and a MyD88-independent pathway (Also known as TRIF pathway).

Activation of the **MyD88-dependent pathway** leads to the early phase of NFκB activation and pro-inflammatory cytokine expression, while MyD88-independent pathway mediates the late phase of NFκB activation and the induction of interferon-inducible genes in TLR4 signaling[34]. Upon binding of ligand to the receptor, MyD88 recruits and activates a death domain-containing kinase IRAK4 which in turn phosphorylates IRAK1. Phosphorylated IRAK1 dissociates from the receptor complex and binds TRAF6 which in turn interact with TAK1. TAK1 then activates downstream IKK (IκB kinase) and MAPK pathways. IKK complex (IKKα, IKKβ, IKKγ/NEMO), induces phosphorylation and subsequent degradation of IκB and translocation of the transcription factor NFκB to the nucleus, which controls the expression of pro-inflammatory cytokines such as IL-1, IL-6, IL-8, TNFα and other inflammatory mediators.

The **MyD88-independent (TRIF) pathway** utilizes the adaptor protein TRAM together with TRIF leading to the activation interferon regulatory factor (IRF) 3 and the subsequent induction of IRF3-dependent gene expression such as interferon-β (IFN-β). Studies using TRIF-deficient macrophages demonstrate that TRIF plays a crucial role in the activation of IRF3, and the late-phase activation of NFκB and MAPK[35].

Nuclear factor κB (NFκB) was first described in 1986 as a nuclear protein binding to the kappa immunoglobulin-light chain enhancer in B cells[36]. NFκB is the central mediator in both MyD88 and TRIF pathway. Most inflammatory signaling pathways converge onto NFκB and so its components are often targeted to treat, inflammatory diseases. NFκB is a transcriptional regulator and their families consist of five members: p65 (RelA), Rel B, c-Rel, p50 and p52[37]. All NFκB family members share a highly conserved Rel homology domain (RHD) that is responsible for DNA binding, dimerization, and interaction with a family of inhibitory proteins known as inhibitors of NFκB (IκBs). Except RelB which only forms heterodimers, the other NFκB proteins can combine as homodimers or heterodimers and activate specific target genes. p50/RelA heterodimer being the common dimer complex is very important in the coordination of inflammatory and innate immune responses[38]. Under resting state, IκBs (IκBα, β, ε, Bcl-3 and the precursor p100 or p105 Rel proteins)[39] by masking the nuclear localization signal (NLS) on the NFκB subunits restricts their entry into nucleus. However, upon phosphorylation of IκBs by IKK or other kinases leads to their ubiquitination and proteasomal degradation releasing NFκB. This process translocates NFκB subunits into nucleus to start gene transcription. The NFκB activation is self-limiting, and is switched off when the inflammatory signal terminates. IκBs, by virtue of their nuclear export sequence (NES) removes the NFκB subunits from nucleus. In

addition, NFκB activation induced IκBα synthesis can in turn prevent further NFκB activation. Also, IκBß and IκBε, are synthesized constitutively and reestablish NFκB inhibition.

NFκB signaling pathway can be activated by a variety of stimuli including bacterial products, growth factors, UV irradiation and oxidative stress. In addition to the aforementioned stimuli, pro-inflammatory cytokines such as IL-1 and TNF induced by NFκB, can amplify NFκB activation *via* IL-1 and TNF receptor mediated pathways[39]. Recent data indicates that NFκB activation and resultant cytokine production contributes to the pathogenesis of organ injury and lethality during sepsis. However, using NFκB inhibitors as a drug therapy in sepsis can have negative effects owing to their involvement in other physiological responses which can be beneficial to the host.

GRKs and NFκB pathway: GRKs, in particular GRK2 and GRK5 have been reported to regulate molecules of NFκB signaling pathway. GRK5, in macrophages was shown to negatively regulate TLR4-induced ERK activation. Knockdown of GRK5 in macrophages significantly enhanced IKK-mediated p105 phosphorylation and degradation[40]. NFκBp105 normally sequesters the MAP3K kinase, tumor progression locus 2 (TPL2) in an inactive state and following phosphorylation and subsequent degradation of p105, TPL2 is free to initiate the MAPK signaling cascade that culminates in ERK1/2 activation. GRK5 was shown to directly interact with phosphorylate p105 and prevent its phosphorylation and degradation by IKKβ. In this way, GRK5 was shown to inhibit TLR4-induced ERK activation.

Later Iaccarino *et al*[41]., reported that GRK5 binds to IκBα and stabilizes it from degrading and this led to nuclear accumulation of IκBα into nucleus, by covering the nuclear export signal (NES) sequence. This effect in turn led to decreased NFκB activation since, IκBs in the nucleus bind to NFκB preventing it from transcription of genes. The IκBα binding site is mapped to the RH domain in the N-terminal of GRK5 and over expression of this domain alone (GRK5-RH) is sufficient to inhibit NFκB. Also, the catalytic function of GRK5 doesn't seem to play a role in inhibiting NFκB as overexpression of a kinase dead GRK5 mutant, GRK5ΔK215R, in bovine aortic endothelial cells, behaved like GRK5 wild type with regards to IκBα nuclear accumulation and the phosphorylation.

However, in contrast to the previous studies GRK5 can also positively regulate NFκB signaling in human cells (HeLa cell line and HEK293 cells), mouse macrophages, zebra fish embryos and drosophila[42]. Also, GRK5 is reported to be required for normal microbial resistance *in vivo* in zebra fish and drosophila[42]. Additionally, *in vitro* studies [43] in macrophages have shown that GRK5 acts as an IκB kinase and phosphorylates the same residues (serine 32/36) on IκBα as that of the canonical IKKβ. An IκBα S32A/S36A mutant exhibited approximately 60% reduced *in vitro* GRK5-mediated phosphorylation, compared to wild type IκBα suggesting that serine32/36 sites are phosphorylated by GRK5. Cytokine and chemokine production was largely attenuated in GRK5 knockout mice and p65 nuclear translocation and IκBα phosphorylation were significantly reduced in LPS treated GRK5-/- macrophages, compared to GRK5+/+ macrophages. Overall these data suggest, GRK5 regulates both positively and negatively, depending on the cell type and disease models involved. GRK5 modulates inflammatory signaling depending on the cell type involved. This is intriguing because, NFκB signaling can be

beneficial and modulating its signal depending on the cell type might prove beneficial. Additionally, results from chapter 2 and chapter 3 suggest that in a whole animal system, GRK5 primarily plays a positive regulatory role in NFκB signaling and its eventual inflammatory responses. In summary, GRK5 modulates NFκB signaling either by phosphorylating (IκBs or NFκB1-p105) or stabilizing (IκBs) components of NFκB signaling and regulates downstream events which can have a multitude of effects on inflammatory processes.

GRK2 has also been shown to interact with IκBα *via* the n-terminal domain of IκBα and phosphorylates IκBα at very low stoichiometric ratios compared to IKKβ and GRK5[44]. However, GRK2 seems to negatively regulate NFκB signaling *via* interaction with NF-κB1p105, and by down regulating the MEK-ERK pathway[45] leading to decreased inflammatory response. Incidentally, *in vitro* studies carried out on macrophages treated with TLR2, TLR4 ligands[46] and lymphocyte samples from septic patients[47] show GRK2 levels are up regulated, whereas GRK5 levels are down regulated. These results suggest that GRK2 and GRK5 might have opposing roles in inflammation *via* their action on NFκB signaling pathways.

1.5.1.2. Mitogen Activated Protein Kinase (MAPK) signaling

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases that mediate fundamental biological processes and cellular responses to external stress signals. Three major groups of distinctly regulated MAP kinase cascades are known that lead to altered gene expression: ERK1/2, JNK, and p38 MAP kinase. ERK is activated by MAP kinase kinase (MKK) and MKK2, JNK by MKK4 and MKK7, and p38 MAP kinase by MKK3, MKK4, and MKK6. Upon activation of the MAP kinases, transcription factors present in the cytoplasm

or nucleus are phosphorylated and activated, leading to expression of target genes resulting in a biological response.

1.5.1.2.1: Extracellular Signal Regulated Kinases (ERK) and GRK5

ERK1/2 is induced under inflammatory conditions by both LPS and TNFα and is known to regulate production of cytokines like TNFα, IL-1, IL-8 and PGE2. ERK inhibitors are found to be useful in abrogating inflammatory responses in various disease models. GRK5 and 2 can negatively regulate NFκB1 p105-ERK pathway in macrophages stimulated with LPS[40,45]. Also overexpression of GRK5 and/or GRK6 has been found to enhance β-arrestin 2-mediated ERK1/2 activation, whereas overexpression of GRK2 and/or GRK3 abolishes β-arrestin 2-mediated ERK1/2 activation[48]. This response is observed with activation of β2 adrenergic receptor, cannabinoid receptor 2[49], Angiotensin 1A receptor[43] and Insulin like growth factor 1 receptor[50].

1.5.1.2.2: p38 MAPK and GRK5

The p38 MAP kinase pathway shares many similarities with the other MAP kinase cascades, being associated with inflammation, cell growth, cell differentiation, and cell death. A number of pathogenic stimuli, including LPS, staphylococcal peptidoglycan and enterotoxin B, echovirus 1 and herpes simplex virus 1, activate p38 through different toll like receptors. The main biological response of p38 activation involves the production and activation of inflammatory mediators to initiate leucocyte recruitment and activation. p38 MAPK positively regulates expression of many genes involved in inflammation, such as those coding for TNF-α, IL-1β, IL-6, IL-8, cyclooxygenase-2, and collagenase-1, -3. Inhibition of p38 MAPK with SB203580 reduced pro-inflammatory cytokine production in monocyte/macrophages,

neutrophils, and T lymphocytes. Expression of pro-inflammatory cytokines can be regulated at both the transcriptional and post-transcriptional levels. Also, p38 directly affects arachidonate signaling, which can stimulate and suppress inflammation. Several studies have implicated p38 MAPK in polymicrobial sepsis. p38 MAPK activity markedly increases in splenic and peritoneal macrophages and inhibiting it improved survival rate during sepsis. GRK5 and GRK2 are shown to regulate a constitutively active virally encoded GPCR (US28)[51]. GRK2 and GRK5 phosphorylate the active receptor and inhibit GPCR activation which is favorable for viral propagation, and increases activation of p38 MAPK which is favorable for the host. This result is predicted to increase cellular detection of viral particle by producing p38 MAPK dependent factors.

1.5.1.2.3: c-Jun N-terminal kinase (JNK) and GRK5

JNKs, while activated by mitogens, are also activated by a variety of environmental stresses (heat shock, ionizing radiation, and oxidants), genotoxins (topoisomerase inhibitors and alkylating agents), ischemic reperfusion injury, mechanical shear stress, vasoactive peptides, pro-inflammatory cytokines and PAMPs/DAMPs. JNK induces transcription of 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 lymphocytes and hence plays a vital role in immune system. The role of GRK5 in JNK signaling is not well characterized. However, studies with transgenic mice overexpressing cardiac specific GRK5 and a constitutively active mutant of the α1B-adenergic receptor showed attenuation of JNK activation compared to controls. GRK5 had variable effects on α1BAR

signaling, and the complexity of GRK5 regulation of *in vivo* α1BAR signaling remains to be fully elucidated[52].

1.5.2: Chemotaxis and GRKs

Chemotaxis is an important function, which enable immune cells to arrive at the site of inflammation. Cells producing chemokines, act on the chemokine receptor, and initiate a chemotactic response. The chemotactic response depends on the amount of chemokines produced and also on the expression levels of chemokines receptor. Chemotaxis would depend on the integrated modulation of different steps of the chemotactic process (receptor sensing, cell polarization, membrane protrusion, adhesion/ de-adhesion cycles) in given cell types and in response to specific stimuli. Chemokine receptors being GPCRs also undergo desensitization upon continuous presence of stimuli. It is likely that GRKs, which regulate GPCRs, can also modulate chemotaxis and thereby modulate immune responses. Intriguingly, GRK2,-3,-5 and -6 are expressed in high levels in immune cells suggesting modulation of these GRKs during disease might change the outcome or progression of the disease.

1.5.2.1: GRKs and cell migration

Of the GRKs, GRK2 is highly studied and more characterized. Studies have shown that GRK2 regulation of cell migration is not straightforward; it depends on the stimuli and also on the cell type. In most cell types[53-55], GRK2 negatively regulates chemotactic responses consistent with its canonical negative role in GPCR signaling. However, in a few cell types[56], GRK2 positively regulates chemotactic responses. These roles of GRK2 might depend on the cell type and its polarization state. In more polarized cells like epithelial cells, GRK2 positively regulates

chemotactic responses, whereas in the less polarized cells such as immune cells, GRK2 does the opposite. Interestingly, the positive regulation of chemotactic responses by GRK2 doesn't require catalytic functions, rather requires recruitment of GRK2 to the plasma membrane denoting a protein –protein interaction. In keeping with this notion, a membrane-targeted kinase mutant strongly enhances cell motility, and GRK2 indeed interacts with GIT1 (GRK2 interacting factor), and is present at the leading edge of polarized/migrating epithelial cells in wound-healing assays. Also, this transient association of GRK2 with GIT1 is critical for proper ERK1/2 activation and efficient cell migration. Also, GRK2 directly phosphorylates histone deacetylase 6 (HDAC6), a cytoplasmic histone deacetylase responsible for deacetylation of tubulin and other substrates involved in cell migration. Furthermore, GRK2 phosphorylates ERM proteins ezrin and radixin, which contribute to the F-actin polymerization dependent motility. These novel roles of GRK2 in cell migration might throw some light on comprehending the non-canonical positive regulation of motility by GRK2

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GRK5 regulates GPCRs that are critical in chemotaxis in both a canonical and non-canonical manner. GRK5 is shown to regulate migration in very few cell types. GRK5 regulates monocyte migration by regulating the CC chemokine receptor-2 (CCR2), a GPCR for the monocyte chemo attractant protein-1[57]. GRK5 in also shown to modulate monocyte chemotaxis in a non-GPCR dependent fashion by regulating the colony-stimulating factor-1 receptor (CSF-1R), a receptor tyrosine kinase. GRK5 is reported to attenuate atherosclerosis by desensitizing CCR2 in monocytes and inhibiting their migration[57]. An *in vitro* study suggests[1] that GRK5 can regulate CXCR4 (CXC chemokine receptor-4) desensitization indirectly, by phosphorylating HIP (HSP70 interacting protein), a co-chaperone protein which phosphorylates CXCR4 and

mediates desensitization. However, *in vivo* evidence of GRK5-mediated desensitization of CXCR4 is currently lacking. Furthermore, in a model of polymicrobial sepsis[2], GRK5 plays a role in modulating inflammation and mortality but doesn't seem to play a role in chemotaxis of immune cells. GRK5 regulates prostate cancer cell migration and invasion[58]. GRK5 forms a complex with moesin (ERM (ezrin–radixin–moesin) proteins and plays important roles in the cytoskeleton remodeling and cell adhesions to neighboring cells and to matrix), phosphorylates moesin principally on threonine 66 residue, and regulates cellular distribution of moesin[58]. In addition, GRK5 is required for the *in vivo* growth, invasion, and metastasis of prostate cancer.

GRK6 is shown to regulate chemokine receptors like CXCR2[59], CXCR4[60] and LTB4[61]. By desensitizing these receptors, GRK6 regulates neutrophil and lymphocyte recruitment *in vivo* in different disease models[62-64]. In epithelial cells, a functional screening identified GRK6 as a critical mediator in integrin-mediated cell adhesion and migration of tumor cells[65], and GRK6 deficiency reportedly promotes CXCR2 receptor-mediated tumor progression and metastasis in a lung carcinoma model[66]. These roles of GRK6 are mostly related to their canonical role of GPCR desensitization, and considering the non-canonical roles played by GRK2, it is likely for GRK6 to have non canonical functions in chemotaxis. However, currently there are no sufficient studies to suggest a non-canonical role for GRK6 in chemotaxis.

Although, the role of GRK5 in chemotaxis seems less well-studied compared to GRK2 and GRK6, its role may be relevant to the cell type and the disease models in question. A recent study demonstrated that GRK5 functions as an actin-bundling scaffold and promotes neuronal filopodial formation, neurite outgrowth, etc[67]. These functions in neuronal tissues closely resemble the initiation events of chemotaxis in immune cells. GRK5 may replicate these

functions in other cells. Also, it is also possible that other GRKs might play a redundant role in chemotaxis of immune cells and further study is needed to assess the role of GRK5 in other cells including epithelial cells.

1.5.3: Apoptosis and GRKs

Apoptosis or programmed cell death regulates development, selection, maturation of immune cells at various stages of their life cycle. Hence, normal rates of apoptosis are considered important in immune system development. Inappropriate apoptosis (either too little or too much) is a mediating factor in many human conditions including sepsis, neurodegenerative diseases, ischemic damage, autoimmune disorders and many types of cancer. In addition to the role in development of immune system, apoptotic bodies/cells can alter the course of the inflammation. Apoptotic cells are usually taken up by macrophages and Dendritic cells (DC). In response to the uptake, macrophages induce production of and release immunosuppressive cytokines such as IL-10, TGF-β, prostaglandin E2 and PAF [68] and suppress production of proinflammatory cytokines IL-1β, IL-12 and TNFα [69] . Similarly, ingestion of apoptotic cells by DCs also results in suppression of IL-12 and IFN-γ expression, up regulation of co-inhibitory molecules and production of anti-inflammatory cytokines[70]. Thus, apoptotic cells have significant impact on the function of these phagocytes and in turn modulate inflammatory disease pathogenesis.

The mechanisms of apoptosis are highly complex and sophisticated, involving an energy-dependent cascade of molecular events. There are two major apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. The extrinsic, intrinsic, and

granzyme B pathways converge on the same terminal, or execution pathway. This pathway is initiated by the cleavage of caspase-3 and results in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, cross-linking of proteins, formation of cytoplasmic blebs and apoptotic bodies and finally phagocytosis of the apoptotic bodies by adjacent parenchymal cells, neoplastic cells or macrophages.

GRKs are reported to play a role in apoptosis of immune cells. GRK5 was shown to phosphorylate p53 and regulate irradiation-induced apoptosis[71]. p53 is crucial in determining the cellular response to stress, not only by inducing apoptosis but by also having a role in growth arrest. Induction of p53-dependent apoptosis occurs *via* extrinsic or intrinsic pathways depending on apoptosis signals and converges on the activation of caspase proteins. P53 triggers the expression of Apaf1 and BH3 proteins (proteins involved in the intrinsic pathway) and up regulates caspase expression. P53 also up regulates the expression of extrinsic pathway receptors, Fas, DR5 and PERP, by increasing mRNA levels, and promoting trafficking of the Fas receptor to the cell surface from the Golgi. GRK5 phosphorylates p53 *in vitro* and *in vivo* at Thr55, which results in reduced p53 levels. Treatment with the proteasome inhibitor, MG132, prevented the reduction in p53, thus confirming its degradation is *via* the proteasome. GRK5 knockout mice displayed tissue-wide aberrant up regulation of p53 suggesting GRK5 negatively regulates p53 *in vivo*. GRK5-mediated p53 degradation directly affects apoptosis, as knockdown of GRK5 in the osteosarcoma cell line, U2OS, increases apoptosis by 40% following cisplatin treatment. Apoptosis of Saos-2 cells (sarcoma osteogenic cell line), which are p53 null, were unaffected following GRK5 knockdown, thus suggesting that GRK5 induces apoptosis *via* p53[71]. In contrast, results from chapter 3 suggest GRK5 positively regulates thymocyte apoptosis *in*

vivo in polymicrobial model of sepsis[2]. Specifically, lack of GRK5 caused decreased apoptosis of CD4CD8++ cells in thymus, which is attributed to decreased sepsis induced corticosterone levels in GRK5 deficient mice. Additionally, in concordance with the results in chapter 3, GRK5 has been shown to negatively regulate Bcl-2 transcription (anti apoptotic protein) in SH-SY5Y (neuroblastoma cell line) cells[72]. This result is predicted to increase cell death thereby shows a positive role for GRK5 in apoptosis, although the impact of this regulation on apoptosis is yet to be elucidated[72]. Even though, the above studies provide an opposing role for GRK5 in apoptosis, the disease context is different and hence the role might differ. However, these results suggest GRK5 modulates apoptosis of cells through multiple mechanisms and also plays an important role in inflammatory disease conditions.

Other GRKs have also been reported to modulate apoptosis of cells. GRK2 overexpression increased caspase 3 levels and induced increased cardiomyocyte apoptosis following, ischemia/reperfusion injury to myocardium. Conversely, GRK2 inhibition reduced apoptosis by increased NOS activity, NO production and AKT levels in cardiomyocytes[73]. Also, GRK2 levels were increased in apoptotic lymphocytes obtained from heart failure patients. These data suggest a positive regulatory role of apoptosis by GRK2 [74]. GRK6 is reported to regulate clearance of apoptotic cells[75]. GRK6 cooperates with GIT1 to activate Rac1, which promotes apoptotic engulfment. GRK6 greatly contributed to removing unnecessary B cells by splenic white pulp macrophages and removing senescent red blood cells by splenic red pulp macrophages. GRK6-deficient mice also have increased iron stores in splenic red pulp in which F4/80+ macrophages are responsible for senescent red blood cell clearance. As a consequence, GRK6-deficient mice develop autoimmune disease.

In summary, the above studies suggest, GRKs by their GPCR-dependent and independent signaling pathways regulate apoptosis of immune and non-immune cells and the functional consequences regulates the overall outcome of inflammatory disease conditions.

1.6: Pathological role of GRKs

Altered activity/expression of GRKs might critically contribute to deregulate chemotaxis of immune cells, inflammatory signaling and the apoptotic pathway. And overall these functions have greater impact in altering the course of many diseases. GRKs expression levels vary in many immune mediated diseases and are known to play a significant role in many pathologies including, sepsis, cardiovascular diseases, neurodegenerative disorders and auto immune diseases. In particular GRK2 and GRK5 are known to play an significant role in the pathogenesis of sepsis[47,76-79], cystic fibrosis of lung[80], neurodegenerative diseases[81-83], hypertension[84], myocardial ischemia/infarct[85], heart failure[86,87] and hypothyroidism[88]. Additionally GRK5 also regulates development of atherosclerosis [57], colitis (unpublished data) in mice, GRK2 in secondary bacterial infections[89] and tuberculosis[90], GRK2 and GRK6 in rheumatoid arthritis[64], GRK6 in colitis[63] and auto immune diseases[75]. These roles are attributed both to their canonical GPCR dependent functions and to their role in non-GPCR dependent functions. As more and more novel interactions of GRKs with non-GPCR substrates are uncovered, GRKs are becoming identified as diverse targets for many disease conditions. It is very important to take into consideration of their interactions with diverse targets, when GRKs are targeted for therapeutic purposes.

Altogether these studies provide a broad overview and rationale for looking at the role of GRK5 in inflammation. In this dissertation, I present evidence that GRK5 plays critical roles in TLR induced inflammatory response, polymicrobial sepsis and *E. coli* induced pneumonia.

1.7: Acute Inflammatory diseases

1.7.1: Sepsis - Incidences and causes

Severe sepsis and septic shock - the serious manifestations of infectious diseases are in the majority of cases caused by bacteria. Gram positive and gram negative infections each constitute about half of verified bacterial infections. Fungi are emerging as important pathogens in subgroups of patients and viruses underlie sporadic cases. The term sepsis, severe sepsis and septic shock have been used for decades, but it was not until 1992[91] that a consensus meeting agreed on universal definitions of these syndromes. Sepsis was defined as systemic inflammatory response syndrome together with a clinical suspicion of infection; severe sepsis as sepsis in addition to signs of acute reduction of organ perfusion (not related to primary septic focus or underlying chronic disease); and septic shock as severe sepsis in addition to hypotension requiring vasopressor support, or lack of response to adequate fluid resuscitation.

Overall in the United States, 2% of patients admitted to hospital end up with severe sepsis representing 10% of all ICU admissions. The number of cases in the United States exceeds 750, 000 per year and was recently reported to be rising[92]. In an analysis of four large sepsis trials, 14-day mortality averaged 26% and 28 day mortality 42%, which is equivalent to 215, 000 deaths per annum in the United States. In this study, mortality in childhood was around 10%, rising to 38.4% in those over age 85.

Severe sepsis occurs as a result of both community- acquired and health care–associated infections. Pneumonia is the most common cause, accounting for about half of all cases, followed by intra-abdominal and urinary tract infections. *Staphylococcus aureus* and *Streptococcus pneumoniae* are the most common gram-positive isolates, whereas *Escherichia coli*, Klebsiella species, and *Pseudomonas aeruginosa* predominate among gram-negative isolates[93-95]. A recent epidemiologic study involving 14,000 ICU patients in 75 countries showed gram-negative bacteria predominate in patients with sepsis[96].

1.7.2: Pneumonia- Incidences and causes

Pneumonia is an inflammation of the lung often caused by infection with bacteria, viruses, and other organisms, although there are also non-infectious causes. It is the number one cause of sepsis-induced mortality in humans. Most septic cases originate as a mild lower respiratory tract infection, which progresses to pneumonia and eventually spread the etiological agent to other organs and causes sepsis. The World Health Organization estimates that lower respiratory tract infection is the most common infectious cause of death in the world (the third most common cause overall), and has estimated that about 450 million cases occur every year with almost 3.5 million deaths yearly[97]. Together, pneumonia and influenza constitute the ninth leading cause of death in the United States, resulting in approximately 50,000 estimated deaths in 2010[98]. This data likely underestimates the incidence, since deaths from other conditions like cancer, Alzheimer's disease, in which pneumonia is the terminal event are coded separately[92]. Similar to sepsis, incidence of pneumonia is the highest among very young and elderly. Identification of the etiology of pneumonia/lower respiratory tract infection is challenging as few

develop bacteraemic illness and high prevalence of nasopharyngeal colonization by potentially pathogenic bacteria, limits the use of respiratory samples for diagnosing bacterial pneumonia.

Sepsis and pneumonia has often been viewed to involve excessive immune inflammation that leads to death, mostly due to multiple organ failure. Sepsis is initiated itself as a systemic inflammatory response syndrome (SIRS) to an infection and proceeds to the development of the compensatory anti-inflammatory response (hypoinflammatory status) syndrome (CARS)[99,100]. Hyper-inflammation mediated multiple organ failure is a characteristic feature of SIRS, whereas immunosuppression-mediated opportunistic/ nosocomial infection and the resultant multiple organ failure is a characteristic feature of CARS (Figure 4). Development of multiple organ failure, refractory hypotension and loss of homeostasis are the later events in sepsis contributing towards the high fatality rates observed during septic syndrome. Current approach to treatment is early goal directed therapy (EGDT). The plan of action upon detection of sepsis is: broad-spectrum antibiotics for targeting infection-causing bacteria, fluid resuscitation to alle*via*te hypotension and external life support to maintain homeostasis. Discussed below are the common major events considered crucial to development and progression of both pneumonia and sepsis.

1.7.3: Inflammatory response and NFκB signaling during sepsis/pneumonia

During an infection, pathogen recognition receptors (PRRs) mediate recognition of microbial components by immune cells leading to their activation and downstream production of inflammatory mediators and recruitment of additional effector cells. Innate immune cells viz., neutrophils, macrophages and dendritic cells form the first line of defense against microbes and aids in clearing infection by production of inflammatory mediators. Inflammatory mediators aid in defense against infection *via* mediating up regulation of PRRs and potentiation of

phagocytosis[101]. Cytokines and secondary metabolites (NO, ROS) produced by immune cells during sepsis can lead to vasodilatation, increased permeability and hypotension producing collateral damage to the host[102]. On the contrary, defective inflammatory response leads to uncontrolled growth of bacteria ultimately damaging the host[103,104]. Therefore, a regulated inflammatory response is critical for resolution of infection with minimal damage to the host[105].

Microbial recognition by Toll-like receptors (TLRs) activates kinases and transcriptional factors, leading to the expression of immunoregulatory mediators. Among the transcription factors activated during sepsis, NFκB plays a major role in immunoregulation. NFκB is critical for the transcription of many molecules involved in inflammatory responses, including cytokines, chemokines, adhesion molecules, nitric oxide synthase, and complement components. Although the classic transcriptionally active form of NFκB hetero-dimer is p65 (RelA) and p50 subunits, other compositional forms of NFκB exist, and utilize RelB, c-Rel, or p52 as hetero- or homo-dimers. Studies have shown that during sepsis, greater nuclear accumulation of NFκB in peripheral blood mononuclear cells (PBMC) is accompanied by higher mortality and worse clinical course in patients with sepsis[106,107]. Clinical studies have also demonstrated that there is an early and persistent activation of NFκB in non-survivors compared to survivors. Furthermore, in preclinical models of sepsis, it has been shown that inhibition of NFκB improves organ function and survival, indicating that NFκB activation contributes to cellular and organ dysfunction[108,109].

1.7.4: Cytokines and their cellular sources

Cytokines and other inflammatory mediators mediated by microbial sensing pathways play a central role in the inflammatory process. Cytokines are soluble proteins, act as intercellular messengers and regulate proliferation, differentiation and functional activity of individual cells and hence have a profound effect on organ systems. Cytokines have variously been called interleukins, chemokines and lymphokines based on their presumed origin, effects or targets. Cytokine production is well regulated under homeostatic conditions; however, in sepsis its production is often disregulated. Macrophages are often the cell type involved in initial microbial recognition (Alveolar macrophages in lungs and peritoneal resident macrophages in peritoneum). After being triggered by bacteria and bacterial components, macrophages induces release of a number of early pro-inflammatory mediators, such as TNF-α, IL-1β, IL-6, IFN-γ, IL-8, and MCP-1, as well as secondary mediators for tissue injury, e.g., NO and ROS.

TNF-α is mainly secreted by macrophages, but also by a range of other cell types. It is rapidly released in animals and humans after bacterial infection or inoculation and has strong pro-inflammatory properties, and is probably the major driving force in the early cytokine storm of severe infections. IL-1β, secreted by monocytes and macrophages, appears shortly after TNF-α and has similar pro-inflammatory properties. Endothelial activation with vasodilation and increased capillary permeability is one example of IL-1 and TNF effects. IL-6 is secreted by T-cells and macrophages. It is one of the most important mediators of the acute phase response. IL-8 is secreted by innate immune cells in response to TLR-signaling and attracts other immune cells through chemotaxis.

The anti-inflammatory cytokines, e.g., IL-10 and TGF-β, which are released in sepsis from macrophages and PMNs, play immune regulatory functions. However, excessive production of these anti-inflammatory mediators may later cause immune dysfunction against pathogens.

IL-12 and IL-18 are also important cytokines in sepsis. They are released from monocytes/macrophages and stimulate NK cells and T-cells to produce IFN-γ and various cytokines. They also promote the development of cell-mediated immunity. Upon interaction with APCs, the naïve T-helper (Th) cells proliferate and differentiate into Th1 cells that produce large amounts of TNF-α, IL-2, IL-12, IFN-γ, and leukotrienes. IL-17A is a pro-inflammatory cytokine that is mainly produced by Th17 cells. IL-17A is involved in mediating pro-inflammatory responses by triggering the production of other cytokines (IL-1β, IL-6, and TNF-α) and provokes cross-talk between lymphocytes and phagocytes.

On the other hand, IL-4-driven Th2 cells give rise to immunomodulatory cytokines, such as IL-4, IL-5, IL-9, IL-10, and IL-13. A majority of the Th1 cytokines are produced at the early phase of sepsis, followed by excessive production of Th2 cytokines. A shift in the balance from Th1 to Th2 cytokines can cause immune suppression, which is evident at the late stage of sepsis, rendering the host susceptibility to nosocomial infection and tertiary pneumonia.

1.7.5: Immunosuppression & Apoptosis

It is now appreciated that anti-inflammatory immunologic events develop concurrently or subsequently during the time-course of sepsis. Although anti-inflammatory responses likely

are essential to the restoration of immune homeostasis following an inflammatory stimulus such as infection, this anti-inflammatory state can result in immunosuppression and subsequent death because of the inability to fight secondary infections in the post-septic period. Production of anti-inflammatory cytokines and impairment of immune cell function are the two major events leading to the immunosuppressive phase of sepsis. Anti-inflammatory cytokines including IL-4, IL-10, IL-13, IL-1 receptor antagonist (IL-1ra), and transforming growth factor-beta (TGF-β) are detected during the immunosuppressive phase and have the ability to inhibit synthesis of IL-1, TNF-α and other pro-inflammatory cytokines. Also, IL-10 exerts a wide range of anti-inflammatory effects including inhibition of cell surface expression of class II major histocompatibility complex (MHC) expression by monocytes, down regulation of TNF receptors, and inhibition of macrophage bactericidal activity. Septic milieu impairs the ability of the DCs to initiate a TH-1 cytokine response and converts to TH-2 type cytokine response[110] which is observed during immunosuppression. It has been shown that during later stages of sepsis, neutrophilic adherence and migration is impaired[111],[112]. Furthermore, expression of neutrophil activation markers is significantly lower in patients who do not survive sepsis[113].

During sepsis, apoptosis is extensively observed in lymphoid organs (spleen and thymus) and lymphoid tissues of large intestine, both in humans[114] and in animal models[115]. Apoptosis of lymphoid cells results in immunosuppression due to decrease in the number of effector cells and decreased co-stimulatory potential of macrophages that phagocytose apoptotic cells. In addition to lymphoid tissue, apoptosis is observed in gut epithelium, lung endothelium, kidney tubular cells and skeletal muscles[115]. Apoptosis of non- lymphoid cells contributes to multi-organ dysfunction, leading to fatality. Strategies to inhibit apoptosis, including bcl-2 overexpression

and caspase inhibition, have shown encouraging results in animal models[116] and are promising targets for future therapeutics.

1.7.6: Coagulation and Endothelial response

Pathogens, besides activating the innate immune cells can also trigger the coagulation cascade. During sepsis, tissue factor (TF) expression is up regulated in monocytes and tissue macrophages, resulting in the activation of the extrinsic coagulatory pathway. Fibrin formed during this process immobilizes pathogens on the surface of the immune cells, facilitating their phagocytosis and subsequent killing. Coagulation initiated through the extrinsic pathway is amplified through the intrinsic pathway by mechanisms that involve cross-talk and feedback. Nevertheless, in immunocompromised hosts or when the bacterial growth exceeds bacterial killing, the coagulation is dysregulated and induces host tissue damage by disseminated intravascular coagulopathy (DIC). The activated vascular endothelium is also capable of modulating systemic inflammation and coagulation, thereby inducing DIC[117]. The endothelium can undergo apoptosis and acquire a more pro-coagulant phenotype[118]. Apoptosis induced loss of membrane integrity of endothelium is believed to induce organ dysfunction and global tissue hypoxia[119]. Moreover, recent studies suggests that vascular endothelium mainly acts as a target organ rather than a mediator of systemic inflammation and endothelial NFκB activation contributes minimally to the systemic inflammation, but plays a pivotal role in contributing to multiple organ damage[120,121].

1.7.7: Cardiac dysfunction

Cardiac dysfunction significantly contributes to mortality in septic patients. Early sepsis and septic shock are characterized by circulatory abnormalities that usually relate to intravascular volume depletion and vasodilation. Consequent underfilling of the heart leads to a reduced cardiac output. Development of cardiac dysfunction is multifactorial and involves multiple interrelated pathways, including circulating inflammatory mediators[122], NO production[123], impaired mitochondrial function[124,125], complement activation, and apoptosis[126]. Cellular hypoxia mediated by systemic hypotension caused by cardiac dysfunction is one of the major reason for cell death and eventual multiple organ failure (MOF)[127].

1.7.8: CLP as a model for polymicrobial sepsis

Murine model of Cecal Ligation and Puncture (CLP) is a clinically relevant model that emulates human sepsis induced as a result of perforation in the intestine (septic peritonitis, post-operative intestinal wound dehiscence, ruptured appendix etc.,). It closely mimics the clinical parameters associated with sepsis including inflammatory response, cardiovascular dysfunction, bacteremia, immunosuppression, multiple organ dysfunction and mortality[128]. The procedure involves ligating and puncturing the cecum below the ileocecal valve in anaesthetized mice. The patency of the puncture is maintained by extruding a small amount of cecal content through the puncture. The severity of sepsis can be altered depending on the position of ligation, size of needle used for puncture and the number of punctures[11]. The cecum is then returned to the peritoneal cavity and is closed using a silk suture. This procedure introduces a mixture of live and dead enteric bacteria, dead epithelial cells and digested food material into the sterile peritoneal cavity leading to septic peritonitis. Control surgeries (Sham) involve exteriorizing and

manipulation of the cecum, without ligation and puncture. To understand the role of GRK5 in the onset and progression of sepsis, we used CLP to induce poly microbial sepsis.

1.7.9: Per-oral inoculation of *E. coli*- as a model for pneumonia

Per-oral inoculation of bacteria into trachea is one of the many mouse models[129] used to mimic human pneumonia. Per oral tracheal instillations may be the preferred methodology, over inhalation or trans tracheal protocols. The former method primarily targets the upper respiratory tract and possibly exposes the investigator to potentially hazardous substances. On the other hand, trans tracheal involves surgical manipulation and therefore increase surgical technique-induced modulation of inflammatory responses. In the per oral technique, the anaesthetized mouse is placed on an angled wooden platform hanging by its incisors on the wire with the mouth open. With the help of a laryngoscope or a light source, and a forceps, the tongue is pulled gently out to visualize the tracheal opening. With the other hand, the inoculum is injected into the trachea slowly. After injection the mouse is observed for signs of proper aspiration of the inoculum (gasping and alteration in breathing pattern). The mouse is held upright for few minutes and then carefully placed in a heating pad until anesthetic recovery. This method models oropharyngeal aspiration and deliver a bolus of organisms to a localized area of lung parenchyma, resulting in a higher ratio of infecting organisms to phagocytes and other local defenses at the site of infection. Conversely, per-oral intubation is some associated with contamination of the lower respiratory tract with oropharyngeal flora. Hence, this technique must be perfected before using for experiments.

To characterize the role of GRK5 in the onset and progression of pneumonia, we used this method to induce pneumonia. Even though we cannot compare this model of pneumonia directly with CLP, we can uncover certain additional roles of GRK5 in bacterial infections; since the inoculum size and composition is characterized, the miniscule alteration in the immunological events can be identified with ease, which is otherwise masked.

1.8: Significance

Treatment of sepsis involves an early-goal directed therapy that revolves around using antibiotics and supportive care. Additionally, specific therapies have been developed during the years to tackle derangements in coagulation and metabolism and hyper-inflammation observed in sepsis using activated protein C, insulin therapy and low dose corticosteroids respectively. Despite advances in the treatment and supportive care, sepsis still remains a persistent clinical problem with reported mortality as high as 30-50%[130]. Sepsis is the number one cause of death observed among ICU patients. In 2008, an estimated $14.6 billion was spent on hospitalization and treatment of septic patients and the treatment cost is increasing at an average of 11.9% annually[131]. Moreover, hospitalization rate has doubled from 2001 to 2008, implying that incidence of sepsis and associated conditions are still on the rise. Current research on sepsis focuses on understanding the pathophysiology of molecular events happening during sepsis and eventually to target those events to improve the outcome of sepsis. According to recent report of NIH GLUE grant[132], GRK5 expression in immune cells correlates with the clinical attributes of sepsis in human patients. Also, another independent study reported that GRK5 expression is increased in blood neutrophils from septic patients[47]. Moreover, in animal models, GRK5 is an important regulator of inflammation during endotoxic shock[4]. So, all these studies suggest that

GRK5 might play a crucial role in the development and progression of sepsis. Thus, understanding GRK5's role in the pathogenesis of sepsis/pneumonia and deciphering the involved mechanisms would provide clues for development of therapeutics for effective sepsis management.

HYPOTHESIS AND SPECIFIC AIMS

The major aim of my thesis project is to understand the overall patho-physiological role of GRK5 in acute inflammatory diseases mainly, sepsis. Based on the current knowledge on GRK5, I propose the following overall hypothesis:

Hypothesis: GRK5 is an important regulator of NFκB signaling pathway *in vivo*, and therefore play an essential and critical role in the pathogenesis of sepsis.

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

Specific Aim 1: To determine the role of GRK5 deficiency in MyD88 dependent and TRIF dependent cytokine production *in vivo*.

Specific Aim 2: To determine the role of GRK5 in a clinically relevant model of intra-abdominal sepsis induced by cecal ligation puncture technique.

Specific Aim 3: To determine the role of GRK5 in acute lung injury/pneumonia induced by per oral intra tracheal *E. coli* injection.

CHAPTER TWO

OVERLAPPING AND DISTINCT ROLES OF GRK5 IN TLR2-, AND TLR3-INDUCED INFLAMMATORY RESPONSE *IN VIVO*

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2.1: Abstract

We recently demonstrated that G-protein coupled receptor kinase-5 (GRK-5) is an important regulator of Toll-like-receptor-4 (TLR4) signaling *in vivo* and *in vitro*. It is not known if role of GRK5 is selective for TLR4 or whether its role can be extended to other TLRs. In addition, it is unclear if GRK5 mediates its effects *via* MyD88- or TRIF-dependent TLR pathways. To address this, we tested the role of GRK5 in TLR2- (MyD88-dependent) and TLR3- (TRIF-dependent) induced inflammatory response *in vivo* using GRK5 knockout and the corresponding wild type mice. To activate TLR2, we injected Pam3CSK4 and to activate TLR3 we administered Poly(I:C). Both ligands induced a broad range of inflammatory cytokines in the wild type mice. However, in the Pam3CSK4 treatment group plasma levels of IL-5, IL-12p40, MCP-1, and G-CSF were significantly inhibited in the knockout mice. Whereas, in the Poly(I:C) treatment group IL12p40, TNFα, and G-CSF were inhibited in the GRK5 knockout mice compared to the wild types. Together, these studies suggest that plasma IL-12p40 and G-CSF levels are regulated by GRK5 at a level that is common to both MyD88 and TRIF. However, our results also indicate that GRK5 regulation of IL-5, and MCP-1 are MyD88-dependent, while regulation of TNFα is TRIF-dependent. Together these results provide *in vivo* evidence that GRK5 has overlapping as well as selective roles in TLR signaling pathways.

2.2: Introduction

Toll-like receptors (TLRs) are a well-characterized family of receptors involved in the recognition of pathogen-associated molecular patterns (PAMPs) derived from microbes. Eleven TLRs in the human and 13 in the mice have been identified[133]. Activation of TLRs by PAMPs leads to transcriptional regulation of many genes and the pattern of gene regulation and the outcome is crucially dependent on the TLR that is activated and the specific cell type. In general, TLR signaling can be broadly classified into MyD88-dependent and TRIF-dependent pathways[133]. MyD88 signaling has generally been linked to NFκB and MAPK signaling, whereas TRIF-dependent signaling has been linked to activation of interferon response factors[134]. Studies have also shown crosstalk and overlap between these two pathways depending on the cell type involved. All TLRs except TLR3 utilize MyD88-dependent signaling, whereas TLR4 and TLR3 utilize TRIF-dependent signaling. TLR4 is unique in that it utilizes both MyD88- and TRIF-dependent pathways[135]. The different TLRs are activated by different components of various microbes. For example, TLR3 is activated by dsRNA from viruses, whereas, TLR2 is activated by lipoproteins from bacteria.

TLRs are involved in a number of inflammatory diseases including non-infectious diseases. Therefore, understanding the mechanistic basis of TLR signaling is a rapidly expanding field. In this regard, we recently showed that TLR4 signaling is regulated by G-protein coupled receptor kinases (GRKs) *in vitro* in macrophages and *in vivo* in mice[40,41,136,137]. GRKs are a family of serine/threonine kinases discovered for their role in G-protein coupled receptor desensitization. Of the seven GRKs, recent studies have suggested that GRK5 has a unique role in regulating TLR4-stimulated NFκB signaling in different cell types and *in vivo* in mice[136]. In

addition, studies have also shown important roles for GRK5 in signaling from microbes in Drosophila and Zebra Fish models[42]. Even though initial studies suggest critical role for GRK5 in TLR4 signaling, it is not known whether the role of GRK5 is specific for TLR4 or whether it regulates other TLRs. In addition, since TLR4 activates both MyD88- and TRIF-dependent pathways, an important question still remains as to whether GRK5 is specific for one of these pathways. To address these issues we utilized our previously described GRK5 knockout mice as model and administered a TLR1/2 ligand (Pam3CSK4) that activates only the MyD88 pathway, and a TLR3 ligand (Poly(I:C)) that activates only the TRIF pathway. Surprisingly, our results suggest that GRK5 has unique and overlapping roles in inflammation induced by TLR2, and TLR3.

2.3: Materials and Method

2.3.1: Animals and protocol

All animal procedures were approved by the Michigan State University Institutional Animal Care and Use Committee and conformed to NIH guidelines. Generation and characterization of GRK5 null mice has been described before[136]. Male mice, 6-8 weeks of age were used for the experiments with appropriate littermate controls. Animals were housed 4–5 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. Wild type GRK5+/+ and Knockout GRK5-/- were segregated into two different groups for intra-peritoneal injection of Pam3CSK4 (100 µg/mouse) and Poly I:C (200 µg/mouse) ligands. Both ligands were obtained from Invivogen (San Diego, CA). The animals were euthanized and samples collected 9 hours post-injection.

2.3.2: Cytokine analysis

A mouse 23-plex multiplex based assay (from Biorad, Hercules, CA) was used to determine the cytokine/chemokine concentrations according to manufacturer's instructions *via* Luminex 100 technology as described previously[138]. Plasma from mice treated with Pam3CSK4 and Poly(I:C) were used to assess the cytokine/chemokine levels.

2.3.3: Statistical methods

All values are represented as mean ± SEM. Data were analyzed and statistics performed using GRAPHPAD PRISM software (La Jolla, California). The Student's t-test was used to compare mean values between two experimental groups. P value of less than 0.05 was considered significant.

2.4: Results and Discussion

Although originally discovered as a regulator of G-protein coupled receptor desensitization, GRK5 has now been shown to broadly regulate other aspects of cell signaling, independent of GPCRs[40,46,136,137]. In this regard, recently we showed that GRK5 is an important regulator of NFκB signaling in mice and this was further confirmed in Drosophila, and Zebra fish, suggesting an evolutionarily conserved role for GRK5 in NFκB signaling. Using cells from mice that are deficient in GRK5, we showed that lipopolysaccharide (TLR4 ligand)-induced activation of NFκB signaling and the consequent cytokine production are critically regulated by GRK5. In addition, in the endotoxemia model, several cytokines/chemokines were significantly attenuated in the GRK5 knockout mice[136].

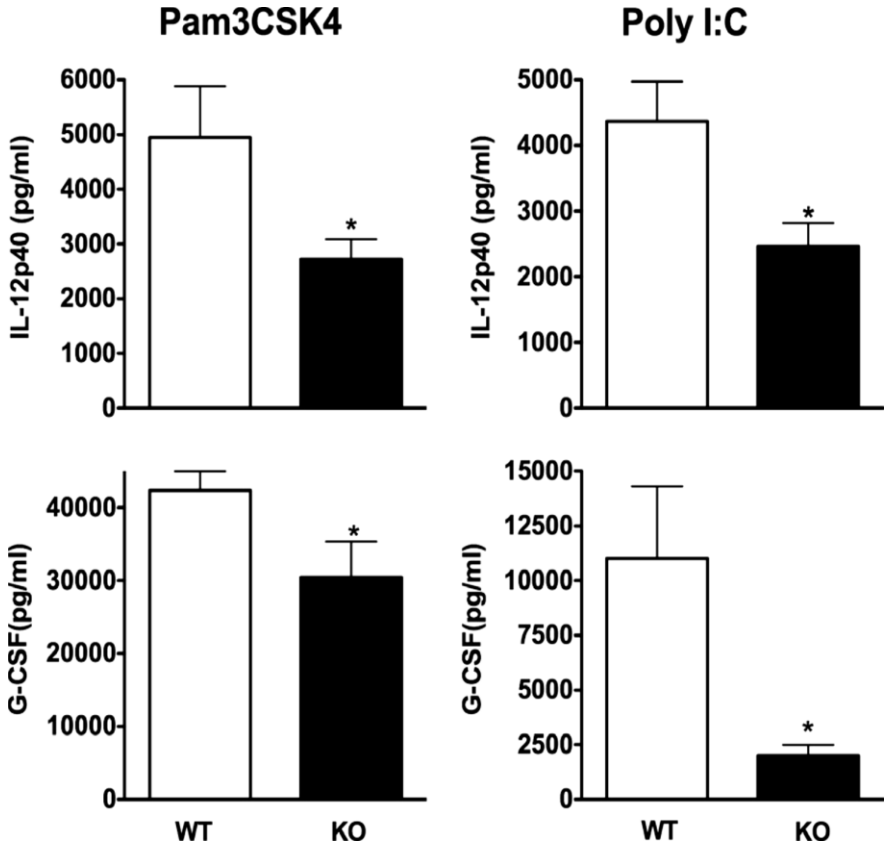

Figure 2.1: Plasma IL-12p40 and G-CSF are regulated by GRK5 in both Pam3CSK4 and Poly (I:C) treatment groups: WT and GRK5 knockout mice were injected with Pam3CSK4 and Poly(I:C) and sacrificed 9 hours after administration. Plasma samples were collected and cytokines measured as described in the methods. n=6 per group. *$p<0.05$

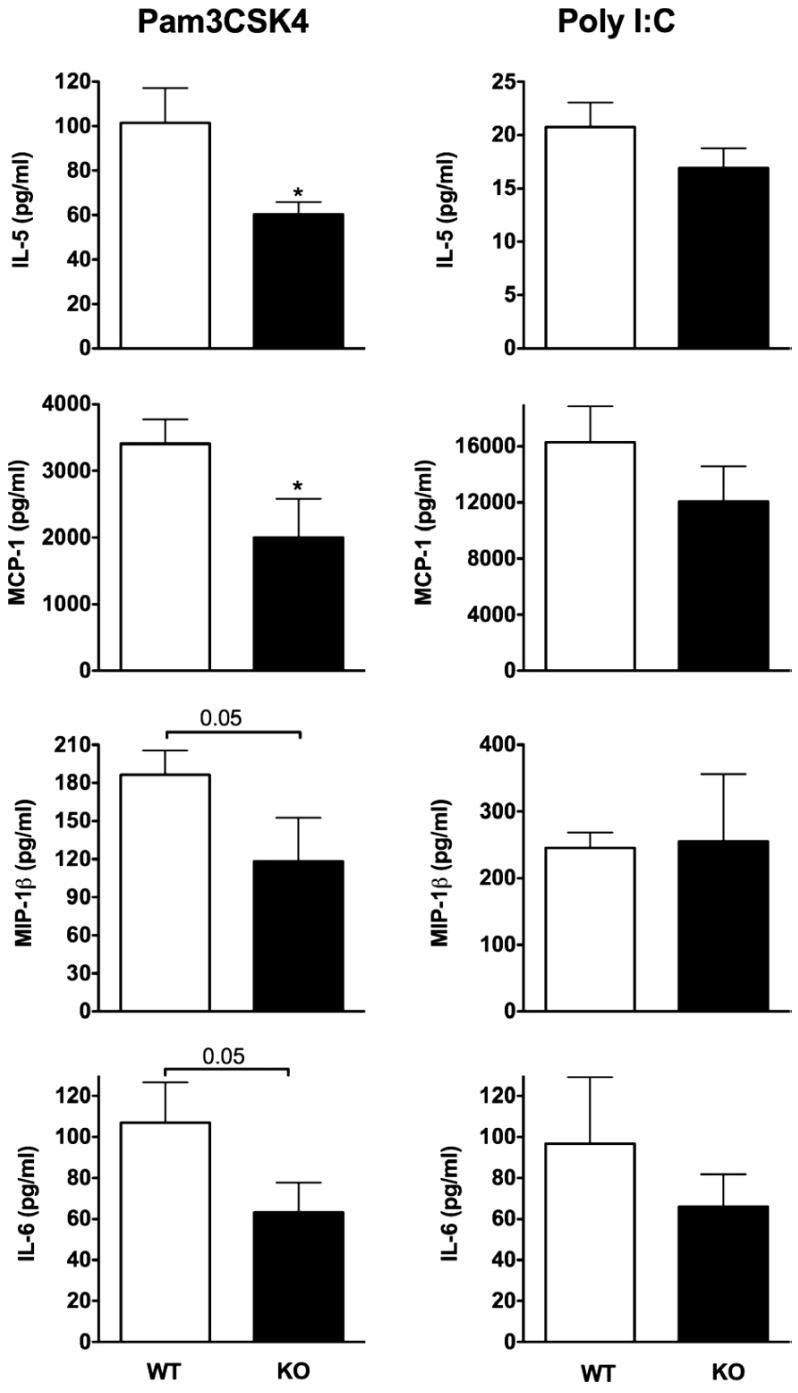

Figure 2.2: Plasma IL-5, MCP-1, MIP-1β and IL-6 production are regulated by GRK5 only in the Pam3CSK4 treatment group: Mice were injected with respective ligands and samples collected and analyzed as described in Fig 2.1. n=6 per group. *p<0.05.

To begin to understand the specificity of GRK5 in various TLR signaling pathways, we used ligands for TLR2, and TLR3 to separately activate MyD88 and TRIF respectively. We administered these ligands in wild type and GRK5 knockout mice. Nine hours after administration, we examined the levels of various cytokine/chemokines using a 23-plex ELISA [136,138]. Pam3CSK4 activated all 23 cytokines/chemokines, whereas PolyI:C activated only 21 of the 23 cytokines (data not shown). In the Pam3CSK4 injected group, IL12p40, IL-5, G-CSF and MCP1 were significantly inhibited in the GRK5 knockout mice (Fig 2.1 and 2.2). IL-6 and MIP1β levels showed similar results but were not statistically significant (Fig 5). Interestingly, all the six cytokines/chemokines (IL12p40, IL5, IL6, GCSF, MCP1, MIP1β) overlapped with our previously reported findings using LPS[136]. Interestingly, when Poly(I:C) was administered, only IL-12p40, TNFα, and G-CSF were significantly inhibited in the GRK5 knockout mice compared to the wild types (Fig 2.2 and 2.3). Poly (I:C)-induced RANTES was also inhibited in the GRK5 knockout, but did not reach statistical significance. Unlike Pam3CSK4, IL1-α, and Eotaxin levels were not at detectable levels in the plasma after Poly(I:C) administration. There was no significant difference in the levels of IL1β, IL-13, IFNγ, IL1α, IL-2, IL-3, IL-4, IL-9, IL12p70 and GM-CSF when compared between the wild type and the GRK5 knockout mice in either of the ligand groups (data not shown).

These results clearly indicate several outcomes as follows: 1. GRK5 regulation of IL-12p40 and G-CSF production occurs at a level that is common to MyD88 and TRIF pathways. 2. Regulation of IL-5, MCP-1 and possibly IL-6 and MIP1β production by GRK5 occurs at a level that is specific to MyD88-dependent pathway. It is important to note that these cytokines/

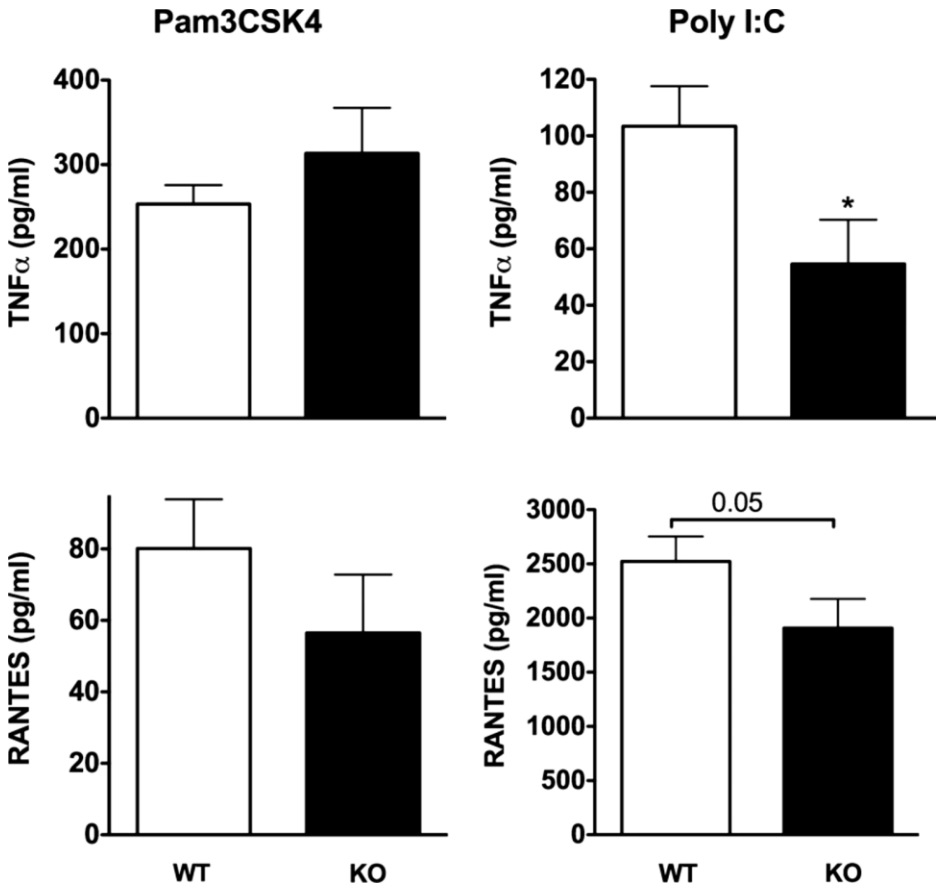

Figure 2.3: Plasma TNFα and RANTES are regulated by GRK5 only in the Poly (I:C) treatment group: Mice were injected with ligands and samples collected and analyzed as described before. n=6 per group. *$p<0.05$.

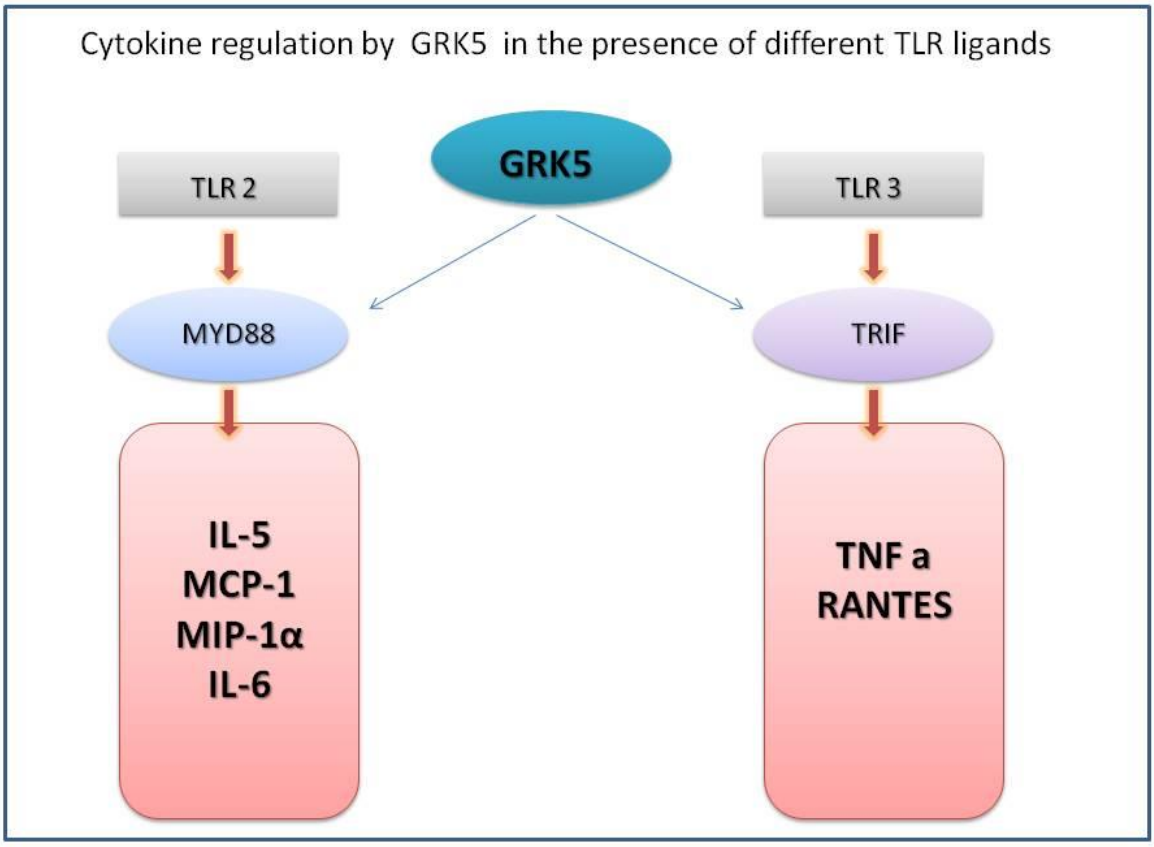

Figure 2.4: Cytokines/chemokines differentially regulated by GRK5 in response to activation of TLR2 and TLR3 signaling pathway.

chemokines were also inhibited in the LPS-induced response, in our previous study. 3. Regulation of TNFα and possibly RANTES production by GRK5 occurs at a level that is likely to be specific for the TRIF-dependent pathway. 4. In previous study, we reported that LPS-induced production of IFNγ, MCP-1, TNFα, IL-1β, IL-12(P40), IL-12(P70), IL-5, IL-17, IL-10, EOTAXIN, IL-2, RANTES, IL-4, IL-9, IL-13 and RANTES are positively regulated by GRK5. Of these IL-17, IL-10, Eotaxin are not inhibited in either TLR2 or TLR3 ligand groups suggesting that regulation of these factors by GRK5 is likely to be unique to TLR4.

Previous studies have shown that GRK5 regulates NFκB signaling and consistent with that many cytokines/chemokines induced by LPS were inhibited in the GRK5 knockout mice. Similarly, in this study many cytokines/chemokines induced by Pam3CSK4- and Poly(I:C)-were inhibited in the knockout mice. While this regulation is likely due to GRK5's role in NFκB pathway, it is possible that other signaling pathways are also being regulated by GRK5, given its roles in many cellular processes[40-42,47,137,139-142]. The observation that GRK5 appears to regulate various cytokines/chemokines at levels that are common to MyD88 and TRIF, as well as unique to MyD88 or TRIF pathways (Fig 2.4) suggests that GRK5 regulates these inflammatory factors *via* multiple pathways. Identifying these novel roles of GRK5 will be the focus of future studies.

CHAPTER THREE

GRK5 MEDIATES INFLAMMATION BUT DOES NOT REGULATE CELLULAR INFILTRATION OR BACTERIAL LOAD IN POLYMICROBIAL SEPSIS MODEL IN MICE

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3.1: Abstract

NFκB-dependent signaling is an important modulator of inflammation in several diseases including sepsis. G-protein coupled receptor kinase-5 (GRK5) is an evolutionarily conserved regulator of NFκB pathway. We hypothesized that GRK5 *via* NFκB regulation plays an important role in the pathogenesis of sepsis. To test this we utilized a clinically relevant polymicrobial sepsis model in mice that were deficient in GRK5. We subjected wild type (WT) and GRK5 knockout (KO) mice to cecal-ligation and puncture (CLP)-induced polymicrobial sepsis and assessed the various events in sepsis pathogenesis. CLP induced a significant inflammatory response in the WT and this was markedly attenuated in the KO mice. To determine the signaling mechanisms and the role of NFκB activation in sepsis-induced inflammation, we assessed the levels of IκBα phosphorylation and expression of NFκB-dependent genes in the liver in the two genotypes. Both IκBα phosphorylation and gene expression were significantly inhibited in the GRK5 KO compared to the WT mice. Interestingly however, GRK5 did not modulate either immune cell infiltration (to the primary site of infection) or local/systemic bacterial load subsequent to sepsis induction. In contrast GRK5 deficiency significantly inhibited sepsis-induced plasma corticosterone levels and the consequent thymocyte apoptosis *in vivo*. Associated with these outcomes, CLP-induced mortality was significantly prevented in the GRK5 KO mice in the presence of antibiotics. Together, our studies demonstrate GRK5 as an important regulator of inflammation and thymic apoptosis in polymicrobial sepsis and implicate GRK5 as a potential molecular target in sepsis.

3.2: Introduction

G-protein coupled receptor kinases (GRKs) are serine/threonine kinases well known for their role in phosphorylation of G-protein coupled receptors (GPCRs)[143]. Functionally GRKs have been linked to a number of cell signaling processes, not only related to their role in GPCR phosphorylation, but also in their ability to phosphorylate or scaffold a number of intracellular signaling proteins[17]. GRKs are functionally grouped into three classes: GRK1-like (GRK1 and GRK7, otherwise known as rhodopsin kinases), GRK2-like [GRK2 and GRK3, otherwise known as βARK1 and 2 (β-adrenergic receptor kinases 1 and 2)] and GRK4-like (GRK4, GRK5 and GRK6). Even though there is some specificity in terms of their tissue distribution, GRKs especially GRK2 and GRK5 are ubiquitously expressed in many cell types including immune cells[17].

Of the seven members of the GRK family, GRK5 was first identified as a kinase that phosphorylates β2-adrenergic receptor (β2AR), m2 muscarinic cholinergic receptor, and rhodopsin[144]. Using GRK5 deficient mice, Gainetdinov *et al*[29] further identified a critical role for GRK5 in muscarinic receptor signaling *in vivo*. Subsequently, however, several studies have demonstrated a broad role for this kinase in cell signaling. For example, GRK5 has been shown to phosphorylate and/or interact with non-receptor substrates including arrestin-2[140], F-actin[67], HDAC5[18], Hip[1], IκBα[43], p105[40], Lrp6[145], nucleophosmin[146], and p53[71]. Based on its role in cell signaling, GRK5 has been proposed to be a critical kinase in the pathogenesis of several diseases including endotoxemia[4], cancer[147], Alzheimer's[148] and atherosclerosis[57]. In addition, GRK5 levels are modulated in a number of diseases including sepsis, heart failure, obesity, cystic fibrosis, cancer and mental disorders[17]. We recently demonstrated that the GRK5 knockout mice

have attenuated ability to produce cytokines *in vivo*, in response to lipopolysaccharide (a Toll-like receptor-4, TLR4 ligand)[4]. We further showed GRK5 to be an important regulator of signaling from multiple Toll-like receptor ligands including TLR2 and TLR3 *in vivo*[77]. Interestingly, a recent study also demonstrated that GRK5 is a critical mediator of inflammation in Drosophila and Zebra fish models[42]. However, role of GRK5 in the pathogenesis of a clinically relevant model of polymicrobial sepsis is not known.

Sepsis is the leading cause of death among intensive care patients[149]. Dysregulated inflammatory response is a prominent modulator of sepsis progression, causing coagulation derangements, apoptosis of lymphoid and non-lymphoid tissues and organ dysfunction[150]. Despite the improvements in resuscitation and antibiotic supportive care, the high incidence and fatality in sepsis underscores the need for better understanding of the pathophysiology of sepsis and to identify new molecular therapeutic targets. Using a clinically relevant polymicrobial sepsis model[151], we demonstrate here that GRK5 is an important modulator of sepsis progression, inflammation, thymocyte apoptosis and mortality. We further demonstrate that GRK5 is an important regulator of sepsis-induced NFκB activation in the liver. Together, our studies implicate GRK5 as an important molecular target in the pathogenesis of polymicrobial sepsis.

3.3: Materials and Methods

3.3.1: Materials

Protease inhibitor cocktail tablets were from Roche Applied Science (Indianapolis, IN); pIκBα, pERK1/2, pP38 and pJNK and tubulin antibodies were from Cell Signaling Technology,

Inc. (Danvers, MA) and Sigma (St. Louis, MO) respectively. Ultra-pure *Escherichia coli* (0111:B4) LPS was from Invivogen (San Diego, CA) and dexamethasone was from Sigma (St. Louis, MO).

3.3.2: Mice

GRK5 KO mice were obtained from Jackson labs and have been previously described[4]. Animals used for experiments were 8-12 week old males. Animals were housed 4-5 mice per cage at 22-24°C with 50% humidity and a 12 hour light-dark cycle. All animal procedures were approved by Michigan State University Animal care and Use committee.

3.3.3: Sepsis model

Polymicrobial intra-abdominal sepsis was induced by cecal ligation puncture technique[152]. Briefly, mice were anaesthetized by administering ketamine (80mg/kg) and xylazine (5mg/kg) intraperitoneally. Cecum was exteriorized, ligated and punctured twice with 20 G needle. Sham surgeries were carried out to serve as control wherein the exteriorized cecum was neither ligated nor punctured. All animals were administered with 1ml of warm saline post-surgery subcutaneously. In one set of survival experiments, antibiotics (ceftriaxone 25mg/kg and metronidazole 15mg/kg) were administered (intraperitoneally) 1-hour post-sepsis and every 24 hours for 5 days.

3.3.4: Peritoneal Lavage fluid and blood collection

Peritoneal exudate fluid was collected at different time points post sepsis as described before[78]. Briefly, peritoneal cavity was lavaged with 7 ml of RPMI media with 10% FBS and

peritoneal fluid collected, centrifuged to separate the cells and the supernatants stored at -80°C until further analysis. Blood was collected by cardiac puncture and plasma was separated (by centrifugation) and stored at -80°C until further analysis.

3.3.5: Cytokine/chemokine measurements

Cytokines and chemokines were measured from peritoneal exudate and plasma using ELISA kits from eBiosciences, Inc. as described before[153].

3.3.6: Bacterial counts

Bacterial load was determined in blood, and peritoneal fluid at different time points as described before[78]. Briefly, blood, and peritoneal fluid were serially diluted and plated on Trypticase™ Soy Agar with 5% Sheep Blood (BD Biosciences) and incubated at 37°C for 48 hours. Colony forming units were counted to determine bacterial load and expressed as colony-forming units per milliliter.

3.3.7: Determination of thymic cell numbers and apoptosis

Thymi were collected from septic and sham operated mice 20- and 36-hours post-sepsis and single cell suspension prepared as described before[154]. For determining cell number changes, cells were counted using hemocytometer. Cells were also labeled with Annexin V and propidium iodide (following manufacturer's instructions (eBiosciences, Inc)) to determine the apoptotic cells by flow cytometry (LSRII, BD biosciences) as described before[155]. In addition, cells were labeled with anti-CD4-PE-Cy7 and anti-CD8-PE for determining CD4+ and CD8+ cells in the

thymus and data were acquired using LSRII (BD Biosciences) and analyzed using Flowjo software (Tree StarInc., Ashland, Oregon).

3.3.8: Caspase activity assays

Thymocytes (obtained as described above) were lysed in buffer (50 mM HEPES, 0.1% CHAPS, 1 mM DTT, 0.1 mM EDTA, 0.4% Triton-X100, pH 7.4) at 4°C for 15 min. The cell lysate was collected and the protein content determined (BIO-RAD). 10 µg of the cell lysate was incubated with the fluorescent substrates (Ac-DEVD-AFC, Z-IETD-AFC and Ac-LEHD-AFC) to determine caspase-3, -8 and -9 activities, at 100 µM in the assay buffer (50 mM HEPES, 1% Sucrose, 0.1% CHAPS, 10 mM DTT, pH 7.4) as described [156]. The fluorescence of the cleaved substrates was determined spectro-fluorometrically (excitation of 400 nm and emission of 505 nm) in Tecan Spectra FluorPlus fluorescence plate reader. Data are presented as picogram of cleaved AFC per mg protein per min calculated from a standard curve plot with free AFC.

3.3.9*: In vitro* stimulation of septic peritoneal cells

Peritoneal cells collected from wild type (WT) and GRK5 knockout (KO) mice 36 hours-post sepsis were washed with PBS and plated in 12-well plate at 1 million cells/well. Cells were then stimulated (or not) with LPS (0.5 µg/ml) for 12 hours and supernatants collected and assayed for the indicated cytokines by ELISA.

3.3.10: Restraint stress

Eight to 10 week old GRK5 WT and KO mice were subjected to stress with physical restraint as previously described[157]. Briefly, mice were placed in a 50 ml centrifuge tube with

multiple openings for ventilation and held horizontally for 30 minutes. After the stipulated time, blood was collected for corticosterone measurement.

3.3.11: Corticosterone measurements

Plasma corticosterone levels were measured using a corticosterone EIA kit from Cayman Chemical (Ann Arbor, MI) as per the manufacturer's instructions.

3.3.12: RNA extraction and Real-time Q-PCR

Liver and lung samples from septic and sham operated mice were collected 12 hours post-CLP, and total RNA was extracted using Qiagen's RNeasy Mini kit. Reverse transcription was carried out with 1 µg of RNA with promega cDNA synthesis kit. Real-time Q-PCR was performed as described before for the expression of IκBα, IL-6, IL-1β, and HPRT[43]. Primers were obtained from IDT DNA Technologies. Following primers were used: IκBα-Forward: TGG CCA GTG TAG CAG TCT TG; Reverse: GAC ACG TGT GGC CAT TGT AG; IL-6-Forward: ACA AGT CGG AGG CTT AAT TAC ACA T; Reverse: TTG CCA TTG CAC AAC TCT TTT C; IL-1β-Forward: TCG CTC AGG GTC ACA AGA AA; Reverse: CAT CAG AGG CAA GGA GGA AAA C; HPRT-Forward: AAG CCT AAG ATG AGC GCA AG; Reverse: TTA CTA GGC AGA TGG CCA CA. Real-time Q–PCR was performed using ABI fast 7500 (Applied Biosystems) and all the genes were normalized to HPRT.

3.3.13: Western blot analysis

Cytoplasmic extracts from frozen liver tissue samples were prepared by homogenizing the tissue in lysis buffer (1M HEPES, 2M KCl, 0.5M EDTA, 0.1M EGTA along with protease

and phosphatase inhibitors). The protein concentrations in the extracts were determined and equivalent amounts of protein were loaded onto the gels for western blot analysis. Immunoblotting was carried out for p-IκBα, p-ERK, p-JNK, p-p38 and tubulin as described before[4]. The bands were quantified using image-J (for chemiluminescence) or Licor's Odyssey program (for fluorescence).

3.3.14: Statistical analysis

All data are presented as the mean±SEM. Two group comparisons were performed using Student's t-test and comparisons of more than two groups were done by ANOVA with post-Bonferroni test. Survival studies were analyzed by log rank test (Mantel-Cox) [158-160] as well as by factorial analysis. All statistical analyses (except factorial analysis) were performed using GraphPad Prism Software (San Diego, CA) and $p<0.05$ were considered statistically significant. Factorial analysis for survival [Two genotypes (wild type and knockout) and two treatments (without and with antibiotics)] was performed in consultation with MSU's Center for Statistical Training and Consulting and using the SPSS software program.

3.4: Results

In previous studies, we demonstrated that the GRK5 deficient mice have an attenuated inflammatory response after *in vivo* stimulation with TLR ligands[4,77]. Given that the progression of polymicrobial sepsis is in part dependent on stimulation of multiple TLRs, we examined the role of GRK5 in a clinically relevant model of polymicrobial sepsis. For this, we subjected wild type (WT) and GRK5 knockout (KO) mice to cecal ligation and puncture and determined the

various pathogenic events including inflammatory response, immune cell infiltration, thymic apoptosis, bacterial load and mortality.

3.4.1: GRK5 mediates sepsis-induced cytokine production

Cytokine and chemokine levels were examined in the peritoneal fluid and plasma from the different groups of mice (sham and CLP in the WT and KO groups) at 12 hours after surgery. As shown in Fig 3.1a, IL-6, IL-10, TNFα and MCP-1 levels in the peritoneal fluid were significantly decreased in KO septic compared to WT septic mice. Peritoneal IL-12/23 (total p40) was also inhibited in KO mice but did not reach statistical significance. Similar to the peritoneal fluid, plasma levels of IL-6 and IL-10 were significantly decreased in KO compared to the WT septic mice (Fig 3.1b). Plasma levels of TNFα, IL-12/23 and MCP-1 did not significantly differ between septic groups. Note that neither of the sham groups showed any detectable levels or showed very low levels of cytokines/chemokines at the time points tested (data not shown). Together these results suggest that GRK5 mediates sepsis-induced local and systemic inflammation.

3.4.2: GRK5 mediates NFκB signaling in polymicrobial sepsis

Previous studies have shown that GRK5 is an important regulator of NFκB signaling and inflammatory gene expression in mouse, zebra fish and Drosophila models[4,42]. Our results in the CLP model of polymicrobial sepsis suggest that GRK5 deficiency attenuates inflammatory cytokine production both in the peritoneal cavity and in the plasma. To examine if GRK5 deficiency leads to attenuated NFκB signaling in this sepsis model, we determined phospho-IκBα levels and mRNA expression of NFκB-dependent genes in the liver from the two

genotypes of mice subjected to sham or CLP surgery. As shown in Fig 3.2a, CLP-induced significant phosphorylation of IκBα in the liver of WT mice and this was significantly inhibited in the GRK5 knockout. To determine the expression of NFκB-dependent genes, we examined the mRNA levels of IκBα, IL-6 and IL-1β in the liver 12 hours after CLP. Consistent with the role of GRK5 in IκBα phosphorylation, mRNA expression of IκBα, IL-1β and IL-6 was significantly inhibited in the GRK5 KO mice (Fig 3.2b). Interestingly, this phenomenon was also observed in the lungs (Fig 3.2b lower panel). To rule out other signaling pathways, we also examined pERK, pJNK and pP38 and found no effect of GRK5 deficiency on these pathways (Fig 3.2c). Together, these results demonstrate a crucial role for GRK5 in NFκB activation *in vivo* and the consequent inflammatory response in this polymicrobial sepsis model.

3.4.3: GRK5 does not regulate chemotaxis to the local site of injury/infection

Studies have shown that following CLP, peritoneal cell infiltration plays a critical role in the progression of sepsis[161] and that modulation of immune cell infiltration can have therapeutic consequences[162]. GRKs have been shown to be important regulators of chemokine receptor signaling and chemotaxis[47]. Therefore, to determine if GRK5 regulates immune cell infiltration into the site of injury/infection (peritoneum), we examined the number of cells in the peritoneal cavity following CLP at different time points in the two genotypes of mice. As shown in Fig 3.3, GRK5 deficiency did not affect immune cell infiltration into the peritoneal cavity at any of the time points tested. In addition there was no difference in infiltration of specific immune cell

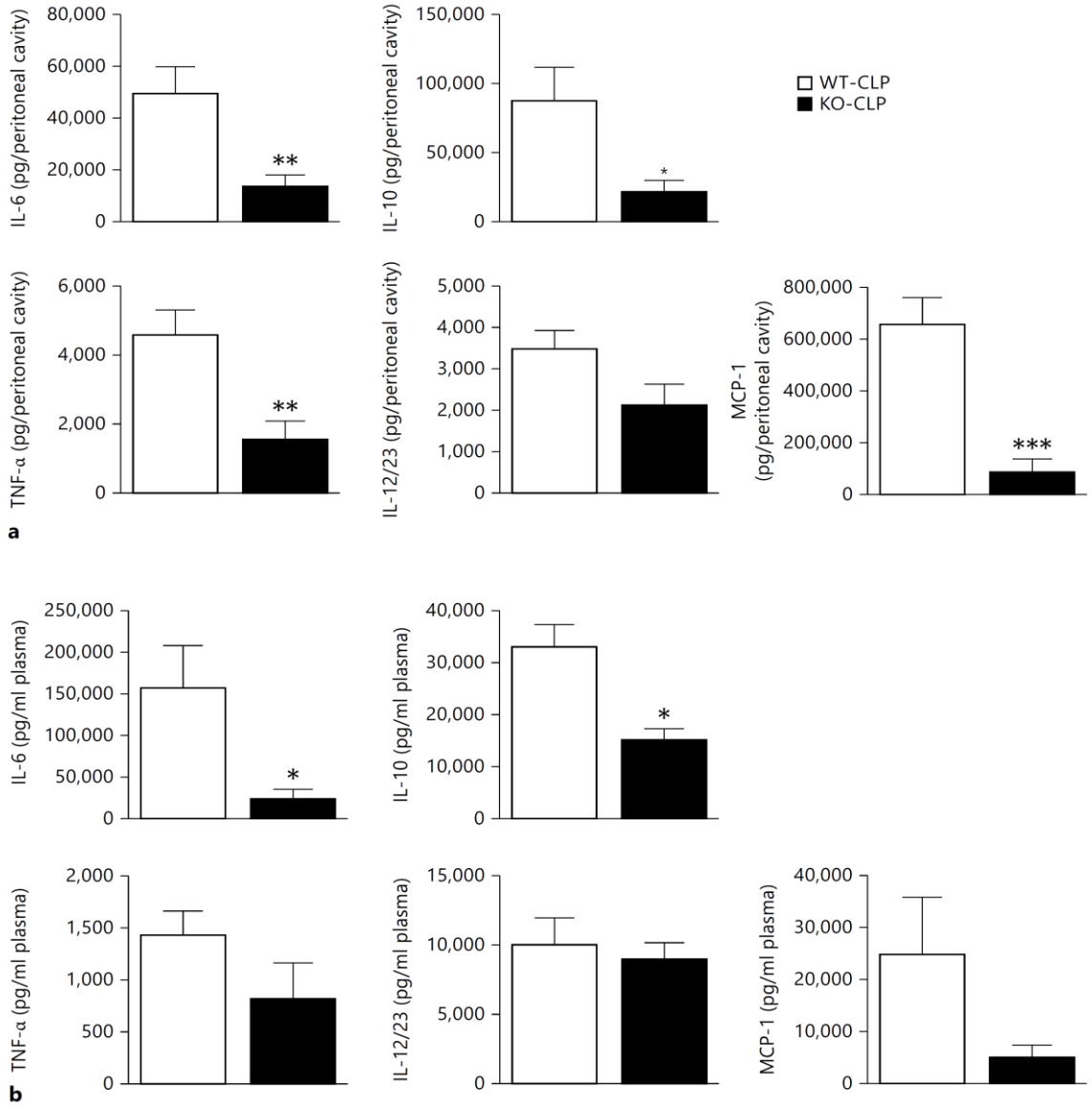


Figure 3.1: GRK5 mediates inflammatory response in cecal ligation and puncture model of polymicrobial sepsis: GRK5 wild type and knockout mice were subjected to CLP surgery and peritoneal (A) and plasma (B) fluids collected 12 hours after surgery as described in the methods. IL-6, IL-10, TNFα, IL12/23 (total p40), and MCP-1 were measured in these two fluid samplesusing ELISA kits from eBiosciences. (n=12 for WT and n=9 for KO for the 12 hour timepoint).*P<0.05, **P<0.01 and ***P<0.001 compared to the corresponding septic wild type groups.

populations (Fig 3.3). Together these results suggest that GRK5 is an unlikely regulator of chemotaxis in this model of sepsis.

3.4.4: Deficiency of GRK5 does not affect bacterial load following sepsis

In order to examine if GRK5 is able to modulate bacterial load after CLP, we plated peritoneal lavage fluid and blood samples from sham and septic mice onto 5% sheep blood agar plates and determined the colony forming units (CFU). Interestingly, bacterial load was not any different between the wild type and the KO mice at any of the time points tested (Fig 3.4a and 3.4b). Taken together, our data so far suggests that even though GRK5 mediates NFκB signaling and inflammation in sepsis, neither chemotaxis nor bacterial load is significantly affected.

3.4.5: Regulation of thymocyte numbers by GRK5 in sepsis

It is now well established that sepsis-induced thymocyte apoptosis contributes to the pathogenic events and the consequent mortality in septic animals[163] and human patients[164]. Importantly, inhibiting thymic apoptosis has been shown to be beneficial in preventing sepsis-induced mortality in experimental models[165]. Previous studies have suggested a role for GRK5 in irradiation-induced thymocyte apoptosis *via* a p53-dependent pathway[71]. Furthermore, NFκB signaling in thymocytes has been shown to be an important regulator of thymocyte apoptosis[166,167]. Together, based on these rationale, we hypothesized that GRK5 could be an important regulator of sepsis-induced thymocyte apoptosis. To test this, we first assessed the number of thymocytes from WT and KO mice subjected to sham or CLP surgery. As predicted from previous studies, in the WT septic mice thymocyte numbers were significantly decreased compared to sham operated mice (Fig 3.5a). Interestingly however, septic KO mice had

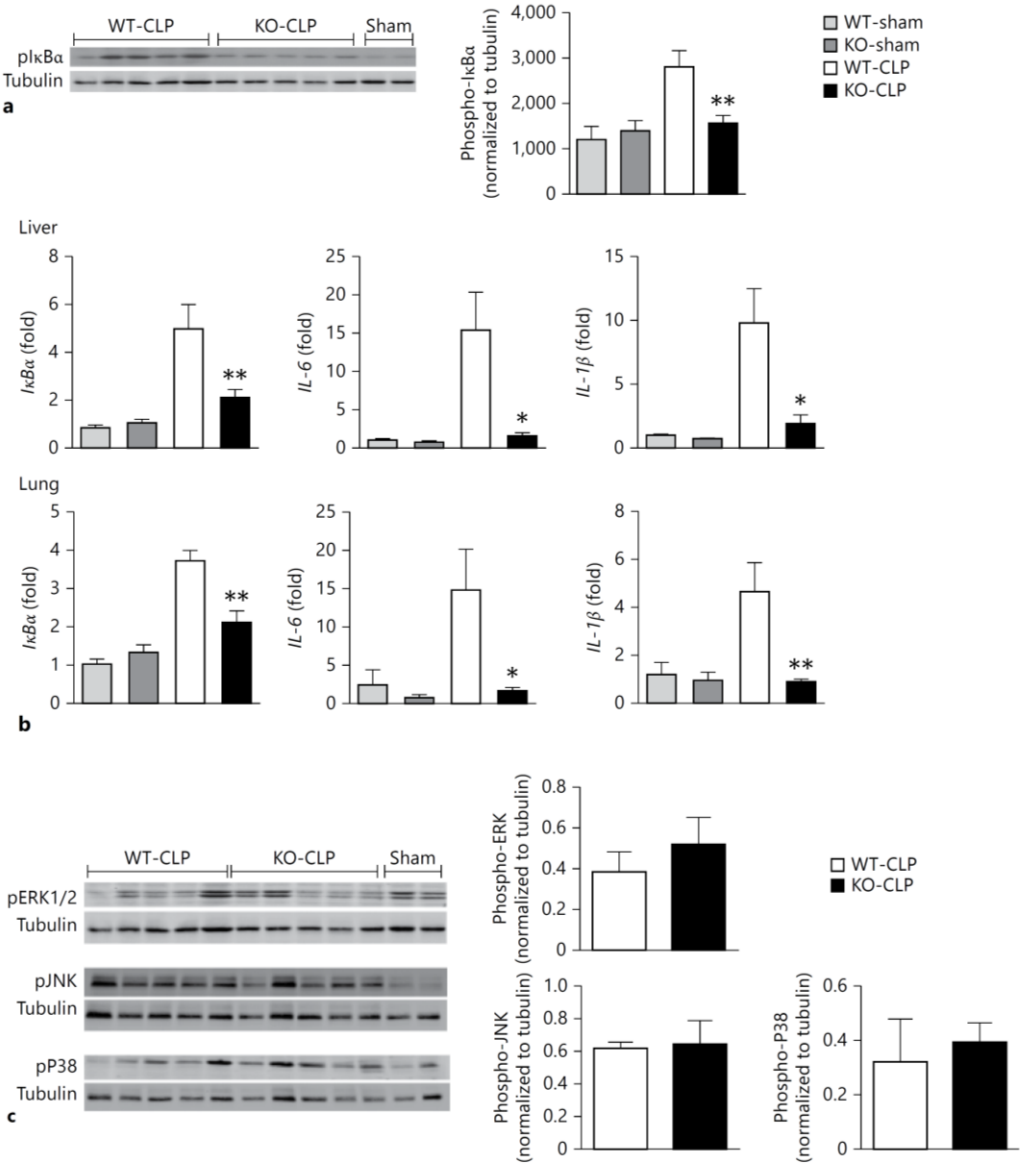


Figure 3.2: GRK5 mediates IκBα phosphorylation and expression of NF-κB dependent genes in liver and lung: (a) Cytosolic extracts of the liver tissue from GRK5 wild type and knockout mice 12 hours post-sepsis were subjected to Western blotting for phospho-IκBα and tubulin (for normalization) as described before[4]. Representative blot is shown in the left and quantitation on the right. n=11-12 each for wild type and KO mice CLP and n=3 for shams; *P<0.05. (b) Liver and lung tissue samples from GRK5 wild type and knockout mice subjected

to sham or CLP surgery were collected 12 hours post-sepsis and analyzed for the expression of NF-κB-dependent genes as described in methods. (n= 8 for CLP and 4 for sham per genotype *P<0.05, **P<0.01 compared to the corresponding septic wild type group. (c) Liver tissue extracts described in (a) above were subjected to immunoblotting for pERK, pJNK and pP38 as described in the methods. Western blot is shown on the left and quantitation on the right. n=5 each for wild type and KO mice CLP.

significantly higher numbers of thymocytes compared to the corresponding WT mice at both time points. In addition to the total cells, the frequency of double positive CD4+CD8+ lymphocytes (loss of these lymphocytes has been linked to poor survival[168]) was also markedly decreased in the wild type septic mice compared to the sham (Fig 3.5b). Importantly, decrease in these double positive CD4+CD8+ cells was significantly attenuated in the GRK5 deficient mice (Fig 3.5b).

3.4.6: Regulation of thymocyte apoptosis by GRK5 *in vivo*

To further confirm the difference in thymocyte numbers is as a result of altered apoptosis between wild type and GRK5 KO mice, we performed two separate assays: Flow cytometry analysis of thymocytes stained with Annexin V and PI; and Caspase activity assays. Consistent with the thymocyte numbers, increased frequency of Annexin V+PI- cells (early apoptotic cells) was observed in wild type septic mice and this was significantly inhibited in the GRK5 KO mice (Fig 3.5c). Furthermore, CLP significantly induced caspase-3 activity in the wild type thymocytes and this was again markedly reduced in the GRK5 KO mice (Fig 3.5c). Unlike caspase-3, activities of caspase-8 and -9 were not significantly induced in sepsis and did not differ between the groups (Fig 3.5c). Together, these results implicate GRK5 in the regulation of caspase-3-mediated thymocyte apoptosis following sepsis.

3.4.7: Mechanism of GRK5-mediated thymic apoptosis

Previous studies have shown that sepsis-induced corticosteroids induce thymic apoptosis in the CLP model[169]. To determine whether GRK5 directly modulates corticosteroid-induced thymocyte apoptosis, we stimulated thymocytes from wild type and GRK5 KO mice *ex vivo*

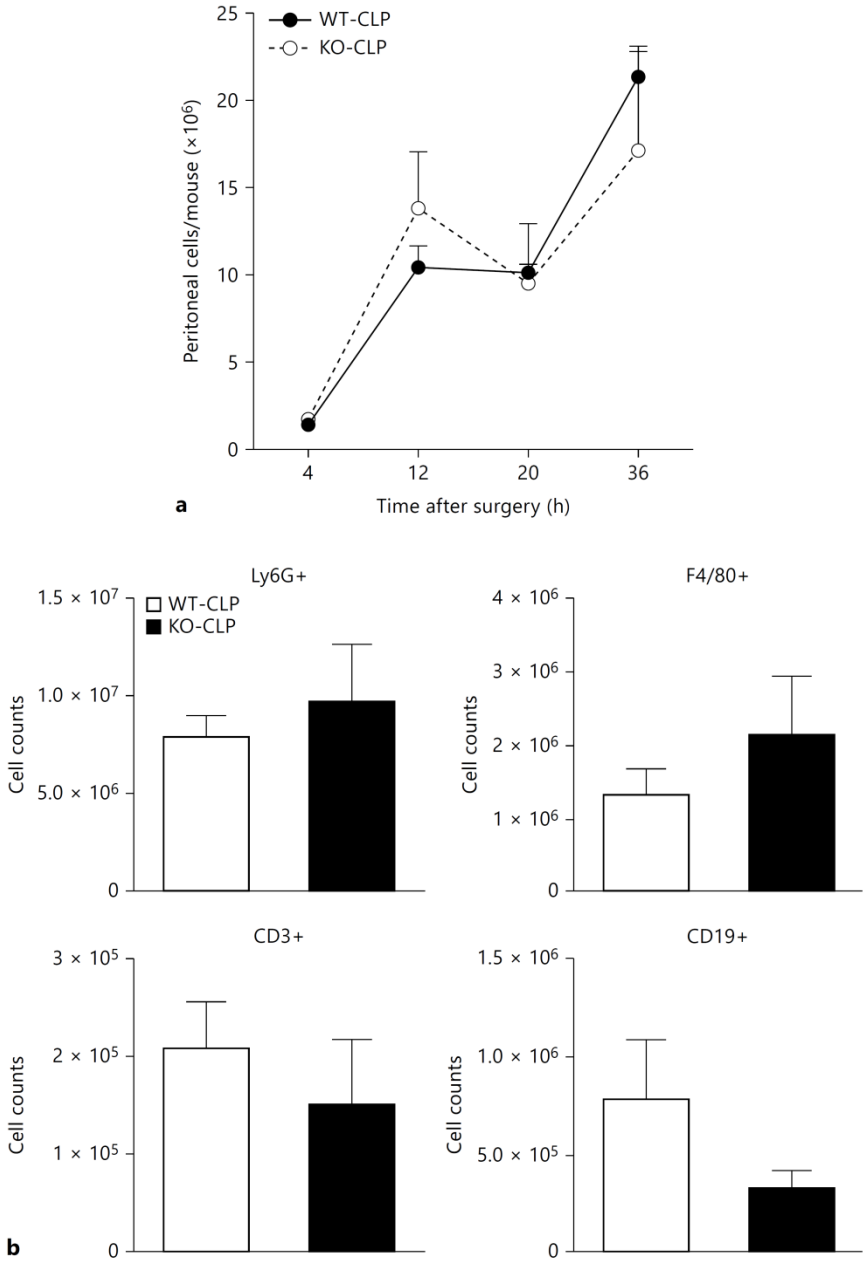


Figure 3.3: Role of GRK5 in peritoneal cell infiltration following cecal ligation and puncture: GRK5 wild type and knockout mice were subjected to CLP surgery and peritoneal cells collected by lavaging peritoneal cavity and cells counted (a) as described in the methods. The different immune populations (b) were assessed by flow cytometry as described in the methods (n=9 for 4 and 12 hour time points and n=5-6 for 20 and 36 hour time points, per genotype).

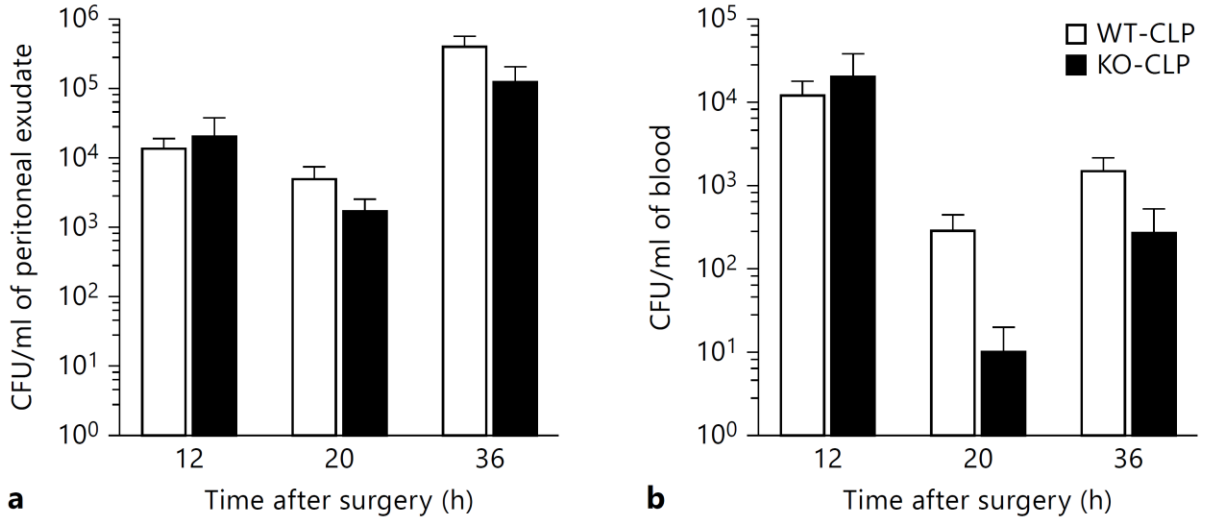


Figure 3.4: Role of GRK5 on bacterial load following cecal ligation and puncture: Blood and peritoneal samples from GRK5 wild type and knockout mice subjected to sham or CLP surgery were assessed for bacterial load as described in the methods. CFU counts from peritoneal (a) and blood (b) for different time points are shown from mice subjected to CLP. (n= 11-14 per genotype at 12 hour time point; n= 5 per genotype at 20 hour time point; n= 9-12 for 36 hour time point). Note that sham mice did not show any bacterial colonies (data not shown).

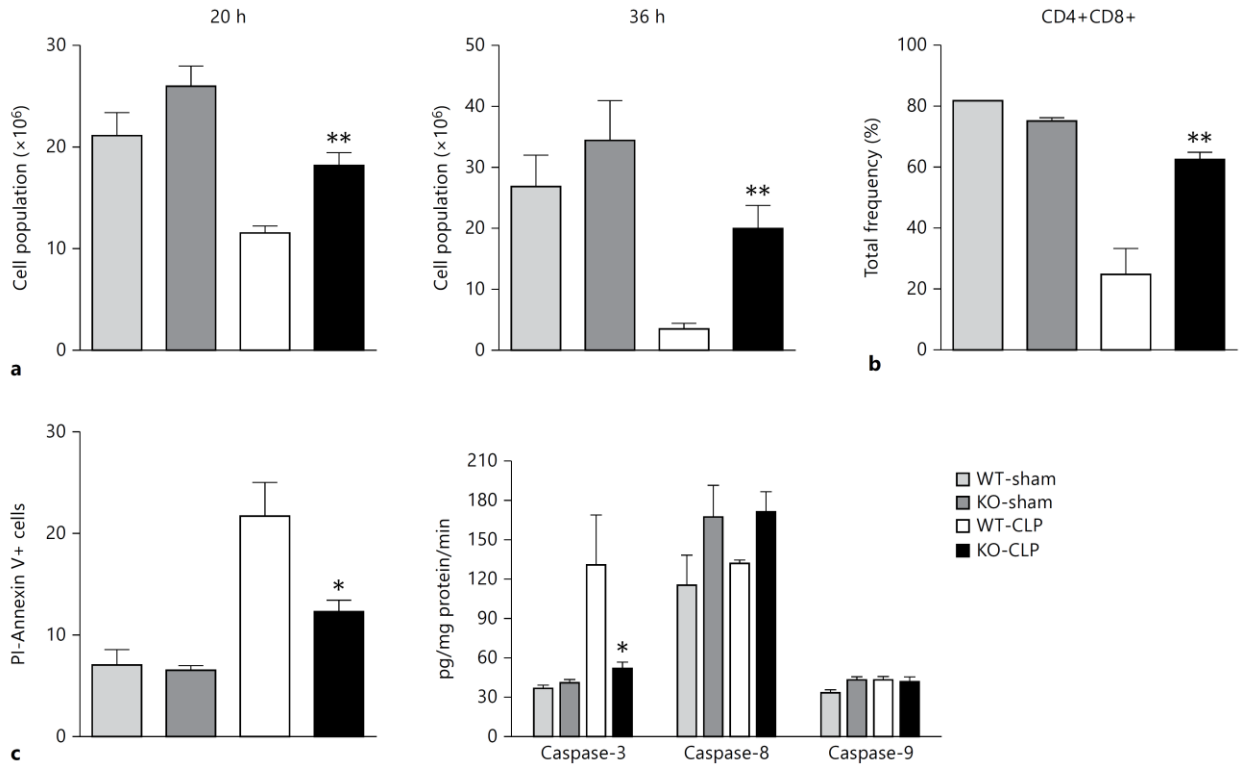


Figure 3.5: GRK5 mediates thymocyte apoptosis in polymicrobial sepsis: Thymi from GRK5 wild type and knockout mice subjected to sham or CLP surgery were collected 20 and 36 hours after surgery as described in the methods. Thymocytes were analyzed for total number (a), CD4+, CD8+ (b), Annexin V+ and PI- staining and for caspase activity (c) as described in the methods. (n=5-6 per genotype for assessing total thymus cell number and Annexin V+ and PI- staining; n=3-4 per genotype for the flow cytometry experiments). *P<0.05, **P<0.01 and ***P<0.001 compared to the corresponding septic wild type groups.

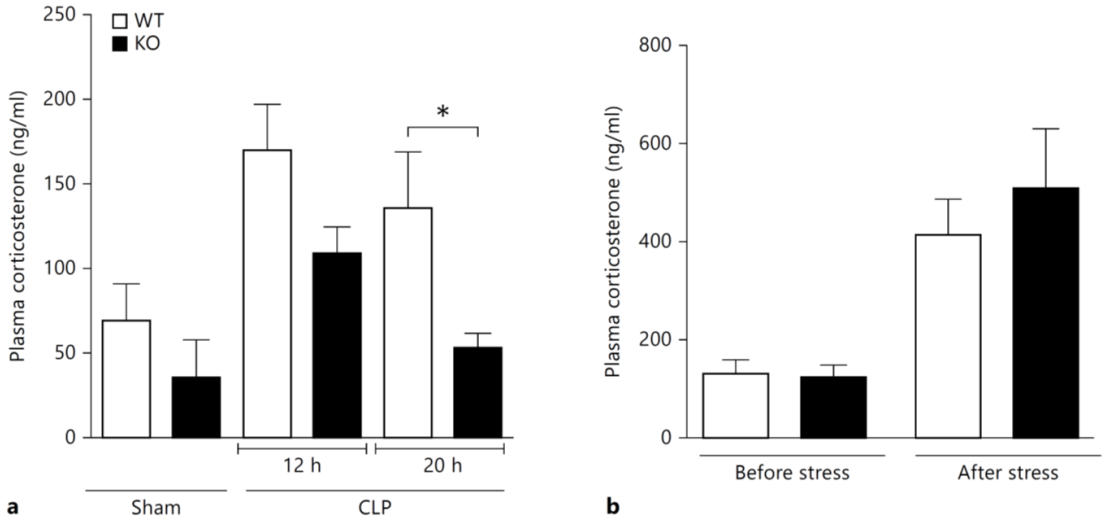


Figure 3.6: Sepsis-induced plasma corticosterone levels are diminished in GRK5 knockout mice: (a) Plasma samples collected 12 and 20 hours post-surgery (sham and CLP) from GRK5 wild type and knockout mice, were assessed for corticosterone levels using Cayman Chemical EIA kit. (n=8 per genotype for both 12 and 20 hour time point for CLP; n=2-3 for sham at 12 hour time point). (b) Corticosterone levels in plasma samples obtained from GRK5 wild type and knockout mice before and after restraint stress (n=7 per genotype). *P<0.05 compared to the corresponding wild type groups.

with dexamethasone (100 nM) and assessed apoptosis using flow cytometry (AnnexinV/PI). Dexamethasone treatment induced significant apoptosis in both the genotypes and surprisingly apoptosis was equivalent between the genotypes (data not shown). Because these results ruled out any direct effect of GRK5 in thymic apoptosis (at least as induced by corticosteroids), we next examined whether the levels of corticosterone are different between the two genotypes during sepsis progression. Interestingly, plasma corticosterone levels were significantly higher in the wild type septic mice (compared to sham) and the levels were markedly attenuated in the GRK5 KO septic mice (Fig 3.6). This was more evident at the later time point (20 h). To determine whether the difference in the corticosterone levels between the two genotypes is specific to sepsis, we induced stress with physical restraint in both genotypes and measured plasma corticosterone levels. We found that 30 minutes of physical restraint induced significant increase in plasma corticosterone, but the levels were similar between the two genotypes (Fig 3.6). Together these results suggest that sepsis-induced corticosterone levels are attenuated in GRK5 deficient mice and this might lead to enhanced thymocyte survival during later stages of sepsis.

3.4.7: GRK5 inhibits immunoresponsiveness of peritoneal cells in sepsis

Decrease in lymphocytes due to apoptosis is thought to be an important pathogenic event in the development of immunosuppression observed during sepsis progression. Because, thymic apoptosis is reduced in the GRK5 deficient mice; we hypothesized that the consequent development of immune suppression may be attenuated in GRK5 deficient mice. To test this *in*

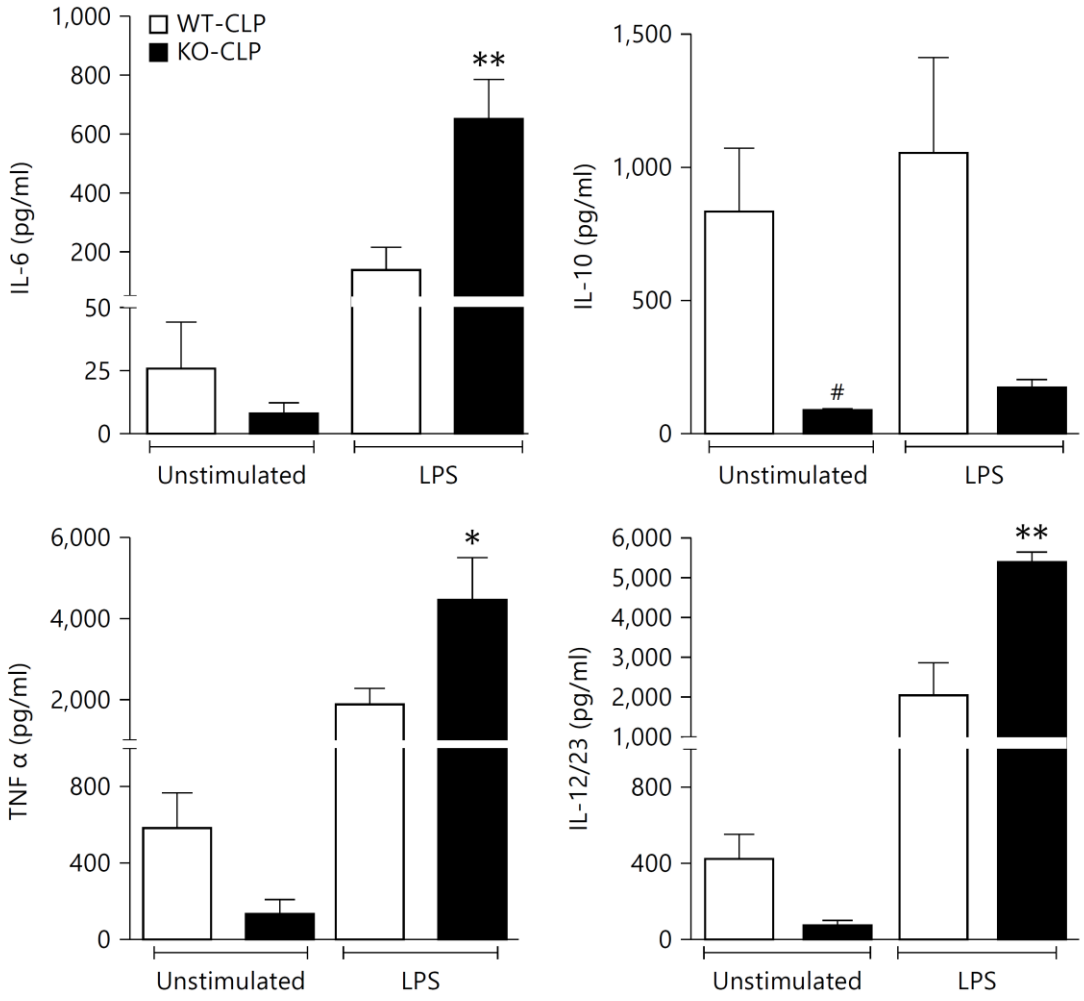


Figure 3.7: GRK5 suppresses immuno-responsiveness of peritoneal cells in sepsis: Peritoneal cells from GRK5 wild type and knockout mice subjected to sham or CLP surgery were collected 36 hours post-sepsis, stimulated with LPS and assessed for cytokine production as described in methods. (n= 7-8 per genotype). *P<0.05, **P<0.01 compared to the corresponding septic wild type peritoneal cells stimulated with LPS. #P<0.05 compared to the corresponding septic wild type peritoneal cells without LPS stimulation.

vitro, we obtained peritoneal cells from the two genotypes of mice subjected to sepsis (36 hours post-CLP) and assessed their immunocompetency in response to *in vitro* lipopolysaccharide (LPS) stimulation. Supernatants from these experiments were assayed for IL-6, IL-10, TNFα, and IL-12/23 using ELISA. As expected, unstimulated GRK5 KO cells (from septic mice) produced lower cytokine levels compared to the wild type septic mice (Fig 3.7). However, upon stimulation with LPS, GRK5 KO cells produced significantly enhanced pro-inflammatory cytokines (IL-6, TNFα and IL-12/23) compared to the wild type cells. This effect was restricted only to the typical pro-inflammatory group and not to IL-10. Basal IL10 level was much higher in the wild type cells from septic mice and LPS stimulation did not further enhance IL10. Together, these results show that even though the initial inflammatory response in the KO cells is attenuated, cells from these septic mice respond better than the wild type cells to *in vitro* LPS stimulation. This suggests that deficiency of GRK5 possibly renders the mice more immune-responsive at later stages of sepsis.

3.4.8: Role of GRK5 in sepsis-induced mortality

Dysregulated inflammatory response, poor bacterial clearance and excessive loss of lymphocytes have all been linked to poor survival in sepsis[150]. Our results using GRK5 deficient mice indicate that GRK5 mediates inflammatory response and thymocyte apoptosis in sepsis. Therefore, we predicted that septic GRK5 deficient mice might exhibit altered survival profile compared to the corresponding wild type mice. Contrary to our expectations, mortality following sepsis was similar between the two genotypes (Fig 3.8A). We then reasoned that because bacterial load was similar between the two genotypes, GRK5 deficient mice might exhibit better survival in the presence of antibiotics. To test this, we subjected wild type and GRK5 deficient

mice to CLP and administered antibiotics (Ceftriaxone: 25µg/g and Metronidazole: 15µg/g body weight) intra-peritoneally 1 hour post- CLP and every 24 hours thereafter for 5 days[170]. Compared to wild type mice that did not receive any antibiotics (~70% mortality), mortality in wild type mice that received antibiotics decreased to ~40%. Interestingly however, GRK5 deficient mice receiving antibiotics had only ~10% mortality (compared to ~90% mortality in GRK5 deficient mice not receiving antibiotics) (Fig 3.8B). Together, these results demonstrate that in the presence of antibiotics, GRK5 deficiency protects mice from sepsis-induced mortality.

3.5: Discussion

Given the high rate of mortality in sepsis, understanding the pathophysiologic events and molecular mechanisms that mediate mortality in sepsis can help in developing new therapies and better treatment strategies. In the CLP model of polymicrobial sepsis GRK5 deficiency inhibited several but not all aspects of sepsis progression. Importantly, GRK5 deficiency significantly enhanced survival only in the presence of antibiotics. Even though GRK5 deficiency attenuated inflammatory response both at the systemic and organ levels, as well as significantly inhibited thymocyte apoptosis, these changes were not sufficient to enhance survival. It is possible that both the severity of sepsis and bacterial dissemination were too high, in spite of the favorable effects on inflammation and thymocyte apoptosis in GRK5 deficiency. This is in part supported by the observation that bacterial load per se, was not different between the two genotypes of mice at early or later time points of sepsis. Thus it is possible that at this level of severity, both antibiotics (to clear bacterial infection) and GRK5 deficiency yield better outcome. A similar phenotype was reported in MyD88 knockout mice[103] wherein deficiency of MyD88 led to a diminished inflammatory response and attenuated lymphocyte apoptosis without any effect

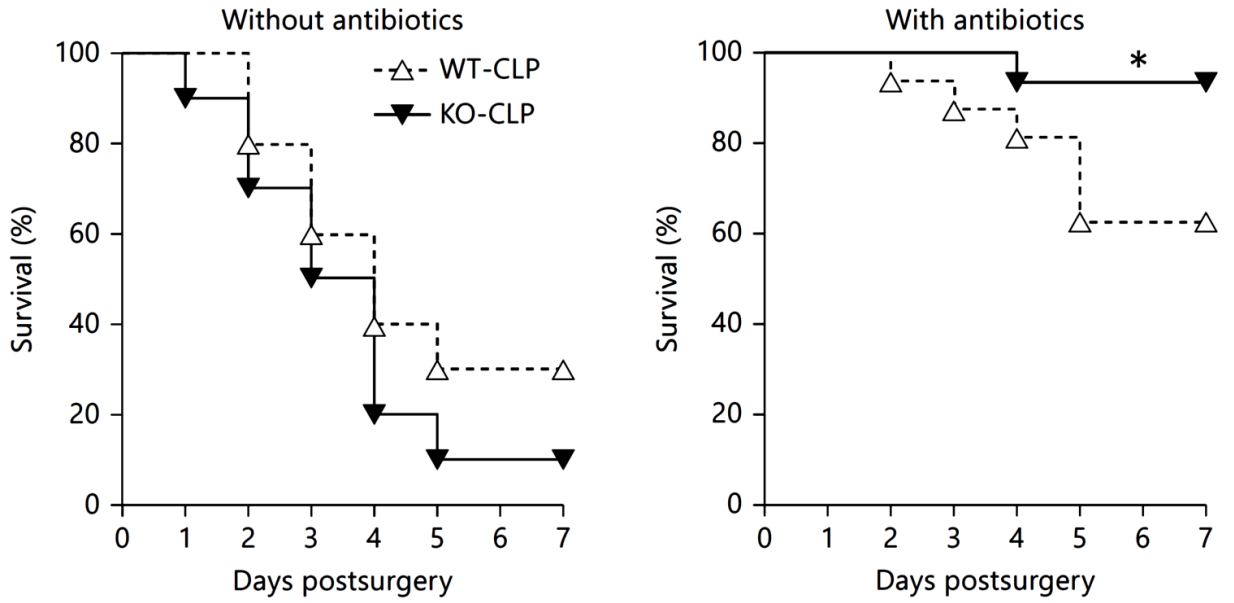


Figure 3.8: Role of GRK5 on survival following cecal ligation and puncture: GRK5 wild type and knockout mice were subjected to sham or CLP surgery and survival was assessed for 7 days in the absence (a) or presence (b) of antibiotics as described in methods. (n=10 per genotype in a; n=15-16 per genotype in b). *P<0.05 compared to the corresponding septic wild type in the antibiotic treated group by log-rank test. Factorial design analysis of the data showed the following: Between genotypes: p=0.204; between antibiotic and non-antibiotic groups: p=0.024; Interaction between genotypes and with antibiotics: p=0.038.

on bacterial load following polymicrobial sepsis. Importantly, similar to our studies, these effects were not sufficient to prevent mortality following CLP. MyD88 is a critical adaptor molecule for many TLRs including TLR4. Consistent with the phenotype of MyD88 knockout mice, blocking TLR4 alone in a CLP model of sepsis did not prevent mortality because of persistent bacterial load[160]. Similar to our model, TLR4 antagonist significantly improved survival only in the presence of antibiotics[160]. Based on our previous studies[4,77] showing that GRK5 is an important regulator of TLR4 signaling, our results are consistent with these other studies in terms of the outcome of sepsis.

Studies have consistently related cytokine response as playing a major role in resolution of sepsis by activation of immune cells and subsequent clearance of microbes. However, excessive production of cytokines with aberrant activation of immune cells can have ill effects on the host. As shown in the endotoxemia model[4], we demonstrate here that GRK5 deficiency attenuates inflammatory cytokines following intra-abdominal polymicrobial sepsis. Consistent with the systemic and peritoneal cytokines, mRNA expression of NFκB-dependent genes in the liver and lungs were also attenuated at 12 hours post-sepsis. In line with our observations in LPS model as well as the demonstrated effects on bacterial infection in Drosophila and Zebra fish models, polymicrobial sepsis-induced IκBα phosphorylation in the liver was significantly inhibited in the GRK5 knockout mice. Together, these studies are consistent with previous studies showing that GRK5 regulates NFκB signaling and therefore is able to modulate NFκB-dependent gene expression.

Contrary to our results in mice and that of Valanne *et al*'s[42] results in Drosophila, Zebra fish and human cells, other studies have also shown that GRK5 is a negative regulator of NFκB signaling in endothelial cells[57] and vascular smooth muscle cells[57]. This effect of GRK5 has been linked to its role in stabilizing nuclear IκBα levels whereas we demonstrated previously that GRK5 is able to phosphorylate IκBα at the same sites as that of IKKβ and mediate degradation[43]. Thus it is possible that GRK5 may have multiple roles in terms of NFκB regulation but the dominance of regulation might depend on the cell type and disease being examined.

Lymphocyte apoptosis has increasingly been recognized as an important step in the pathogenesis of sepsis, by inducing a state of 'immune paralysis' that renders the host vulnerable to invading pathogens[168]. NFκB signaling plays a vital role in lymphocyte development, function and apoptosis. In addition, NFκB signaling can either promote survival[166] or apoptosis[167] of lymphocytes depending on the cell type involved. Even though we found GRK5 to be an important regulator of NFκB signaling in the liver, this phenomenon appeared to be tissue specific since we did not observe any difference in NFκB activity in the thymus from the two genotypes subjected to CLP (data not shown). Since previous studies have shown that sepsis-induced thymocyte apoptosis is mediated by corticosterone[169], we hypothesized that either corticosteroid signaling or its plasma levels may be differentially regulated in the two genotypes. Even though our *in vitro* results rule out any direct effect of GRK5 on corticosteroid-induced thymocyte apoptosis, our *in vivo* results demonstrate that GRK5 is an important regulator of sepsis-mediated increase in corticosterone levels. Since sepsis-induced thymocyte apoptosis has been shown to be mediated by corticosterone, lower levels of plasma corticosterone may in part

explain why GRK5 KO thymocytes are protected from apoptosis *in vivo*. In future studies, we will determine whether the effect of GRK5 on thymocyte apoptosis *in vivo* is due to decreased corticosterone levels or due to differential regulation of other signals that may be involved in thymocyte apoptosis in sepsis.

Consistent with the better survival of thymocytes in the GRK5 deficient mice, septic peritoneal cells from the GRK5 knockout were more immunocompetent than the cells from wild type mice. While our studies don't demonstrate a cause-effect relationship between thymocyte apoptosis and peritoneal cell responsiveness in the septic mice, previous studies have suggested that enhanced lymphocyte apoptosis leads to enhanced immunosuppression in animal models[171]. In addition it is possible that enhanced plasma corticosterone levels in the wild type mice might suppress LPS-induced cytokine production in the wild type cells compared to less suppression in the knockout cells[172]. These mechanisms will be dissected out in future studies. Regardless of the mechanism, our results suggest that under conditions where the innate cells are already exposed to polymicrobial injury, GRK5 deficiency is able to modulate the cells to be more immune-responsive compared to the wild type. The better immuno-responsiveness of innate cells during the stage of sepsis when cells are likely "paralyzed" may lead to a beneficial outcome in sepsis[173]. This has been shown before where innate immune paralysis observed later in sepsis has been attributed to poor outcome[174]. Together, these results suggest that GRK5 deficiency could modulate sepsis at several levels or stages.

In conclusion, we demonstrate here that GRK5 is an important regulator of sepsis pathogenesis and that blocking GRK5 could result in a protective mechanism *via* modulating

several aspects of sepsis. Even though bacterial load is not affected by GRK5, administration of antibiotics in the presence of GRK5 inhibition might lead to a better outcome in sepsis-induced mortality.

CHAPTER FOUR

CRITICAL INVOLVEMENT OF G- PROTEIN COUPLED RECEPTOR KINASE 5 (GRK5) IN *ESCHERICHIA COLI* INDUCED PNEUMONIA

4.1: Abstract

G-protein coupled receptor kinases are serine/threonine kinases involved in a range of pathophysiological processes including inflammation. We recently showed that deficiency of G-protein coupled receptor kinase-5 (GRK-5) improves sepsis outcome in a poly-microbial sepsis model in mice. In this study, we examined the role of GRK5 in mono-microbial pulmonary infection by injecting *E. coli* intra-tracheally at two different doses to induce a low- and high-grade inflammation. We observed distinct phenotypes in GRK5 deficient mice (KO) depending on the dose of *E. coli*. With low-grade inflammation, GRK5 KO mice induced higher CXCL1/KC production and recruited more neutrophils, which were associated with better *E. coli* clearance compared to WT mice. Consistent with the increased clearance of bacteria, the inflammatory response was diminished and resolution of inflammation (as determined by increased number of efferocytosing macrophages) advanced in the KO mice. However, at higher dose of *E. coli*, even though there was increased CXCL1 and enhanced neutrophil recruitment in the KO mice, the bacterial burden remained high in the KO compared to wild type (WT) mice. In addition, the neutrophils from KO mice from the high dose *E. coli* treatment group showed decreased neutrophil activation as determined by CD11b expression. Decreased neutrophil activation and increased bacterial burden in the KO at this dose suggest increased neutrophil exhaustion in the KO mice. Consistent with the increased bacterial burden, the KO mice showed decreased survival. Overall our data suggest GRK5 negatively regulates CXCL1/KC production and modulates the outcome of pneumonia depending on the bacterial dose.

4.2: Introduction

Pneumonia is one of the leading causes of mortality in the United States and is a major cause of severe sepsis[175,176]. Acute lung injury (ALI) is often caused by infection with bacteria, viruses, and other organisms including fungi and chlamydia of which gram-negative bacterial species are the dominating cause of pneumonia-induced ALI[177]. As in any bacterial infection, neutrophil chemotaxis plays a vital role in clearing the infection. Neutrophil recruitment is critical to clear bacteria, and neutrophil migration in the lung requires the production of ELR+ (glutamic acid-leucine-arginine) CXC chemokines like CXCL1/KC, CXCL2/MIP2 and CXCL5/LIX[178-180]. However, excess neutrophilic infiltration or defect in neutrophil removal after clearing infection can propagate inflammatory process, which can be detrimental[181]. A key strategy in reducing mortality is to modulate the innate immune system to enhance the host's ability to combat microbial infection while limiting inflammation-induced tissue injury.

G-protein coupled receptor kinase 5 (GRK5) is a serine-threonine kinase, shown to modulate inflammatory responses *in vivo* and *in vitro* by regulating inflammatory signaling pathways[40,42,43,77]. Of the seven GRKs, GRK5 is highly expressed in normal[12,182] and diseased airways[80]. In addition, GRK5 expression level has been shown to be modulated in conditions such as sepsis, cardiac failure, obesity, cystic fibrosis, cancer etc. [17]. Furthermore, our previous studies revealed critical role of GRK5 in polymicrobial sepsis[2]. Even though GRK5 is expressed in the airways, its role in modulating bacterial pneumonia is not known. Given its role in inflammatory pathways and in polymicrobial sepsis and because GRK5 is highly expressed in the airways, we hypothesized that GRK5 will play a critical role in modulating bacterial pneumonia. We demonstrate here that GRK5 is a critical signaling molecule in the pathogenesis

of bacterial pneumonia by modulating CXCL1/KC levels and neutrophil recruitment; our results however, highlight the complexities by demonstrating that the role of GRK5 is distinct depending on the dose of *E. coli*.

4.3: Materials and methods

4.3.1: Materials

Protease inhibitor cocktail tablets were from Roche Applied Science (Indianapolis, Ind., USA); pIκBα, and tubulin antibodies were from Cell Signaling Technology, Inc. (Danvers, Mass., USA) and Sigma (St. Louis, Mo., USA), respectively. *Escherichia coli* (O6:B1) was obtained from ATCC (Manassas, VA, USA).

4.3.2: Experimental animals

GRK5 deficient mice previously described[4] was used in the study. Healthy, male mice belonging to the age of 8- to 12 weeks were used in the experiments. All mice were housed with a 12-h alternating light-dark cycle at 25°C, with 50% humidity and free access to food and water. All experiments performed were approved by the Institutional Animal Care and Use Committee at the Michigan State University.

4.3.3: *E. coli* infection

A clinical isolate of *E. coli* (O6:B1, ATCC 25922) was used for all experiments. Bacteria were grown in trypticase soy broth for 8 h at 37°C and bacterial colony forming units (CFU) of each culture was determined by comparing the specific OD-600 value to a standard curve. Bacteria were washed twice with sterile phosphate buffered saline (PBS) and adjusted to the

appropriate final concentration. For bacterial injection, mice were anaesthetized using xylazine (5mg/Kg) and ketamine (80 mg/Kg), placed in an intubation stand and gently secured by the incisors. Using a clean forceps tongue was pulled gently out to expose epiglottis and trachea. With the tongue held in position 50 µl of bacterial inoculum was instilled. To avoid bias, WT and KO mice were injected in a blinded fashion. Sham intra-tracheal inoculation were also carried out with the use PBS and served as control. Mice were observed and euthanized at various time points up to 48 hours for cell and tissue analysis.

4.3.4: Bacterial load determination

Mice infected with *E. coli* were euthanized 4, 12, or 24 h post infection. Broncho alveolar lavage fluid (BAL), lung and blood were harvested aseptically. Post-caval lung lobe was collected, weighed and homogenized in 1 ml of sterile PBS. All the samples were plated in Trypticase Soy Agar (BD Biosciences) and incubated at 37 ° C for 48 h. Colony-forming units (CFU) were counted to determine bacterial load and expressed as CFU per milliliter of blood or 50 µl of BAL or gram (g) of lung. In addition, bacterial presence in lungs was also measured indirectly by measuring gene expression of bacterial 16S rRNA using Real-time Q-PCR (Primer sequence for 16s rRNA forward: 5'-GGT GAA TAC GTT CCC GG-3'and reverse: 5'-TAC GGC TAC CTT GTT ACG ACTT-3' HPRT used as housekeeping gene-Forward: 5’-AAG CCT AAG ATG AGC GCA AG-3’; Reverse: 5’-TTA CTA GGC AGA TGG CCA CA-3’).

4.3.5: *In vitro* Bacterial killing assay

Neutrophils were collected from WT and KO mice by processing peritoneal lavage 4h post injection of thioglycollate. A small aliquot was separated to assess the purity of neutrophils

by flow cytometry. Neutrophil numbers were enumerated and incubated with opsonized *E. coli* for 30 minutes to enable bacterial uptake by neutrophils. Post incubation, the cells were treated with gentamicin (200 µg) for 30 and 60 minutes to kill extracellular *E. coli*. The cells were then washed twice with PBS and the pellet was lysed with 0.1% triton-X and plated onto Tryptic soy agar. Plates were incubated at 37° C and Colony forming units were counted 24 hours post plating.

4.3.6: Immune cell infiltration

Lung tissue, Broncho-alveolar lavage fluid cells and blood samples were collected at specified time points post infection to measure the infiltration of immune cells to the lung. Left lung lobe was teased and incubated in collagenase D (1 mg/ml) containing media at 37°C for an hour with gentle shaking and then the tissue was disrupted to release cells. For determining cell number changes, cells were counted using a hem cytometer. Cells were also labeled with cell surface markers for various immune cells (Gr-1, CD11b, F4/80, CD11c, B220, CD3 and Ly6C) and data acquired using LSRII (BD Biosciences) and analyzed using Flowjo software (Tree Star, Inc., Ashland, Oreg., USA) as described before[2].

4.3.7: Chemokine receptor expression

Bone marrow neutrophils were isolated from WT and KO mice using percoll gradient centrifugation. Briefly, RBC lysed bone marrow cell suspension was gently layered on top 62% percoll solution and centrifuged at 2200 rpm for 30 minutes. At the end of the gradient centrifugation, the cloudy layer below the interface were collected and gently washed twice. Neutrophil purity was assessed using flow cytometry. For the expression studies, neutrophils

were treated with controls or chemokine ligands CXCL1 (100ng) and CXCl2 (50ng) (obtained from Peprotech, USA) for the specified time points. The samples were then fixed immediately, processed and analyzed by flow cytometry.

4.3.8: Cytokine/Chemokine measurements

Plasma isolated from blood samples, tissue lysates from lung samples and broncho-alveolar lavage fluid samples were subjected to cytokines and chemokines analysis using ELISA kits from eBiosciences, Inc. as described before[2]. CXCL1 and CXCL2 levels were determined using ELISA kits from R&D system, Minneapolis, MN.

4.3.9: Immuno Blot Analysis

Lung tissue samples for immune blot analysis were prepared by homogenizing the tissue in lysis buffer (1 M HEPES, 2 M KCl, 0.5 M EDTA and 0.1 M EDTA along with protease and phosphatase inhibitors). The protein concentrations were determined and equivalent amounts of protein were loaded onto the gels for Immuno blot analysis. Immunoblotting was carried out for pIκBα and tubulin as described before[2]. The bands were quantified using the image-J (for chemiluminescence) or Licor's Odyssey program (for fluorescence).

4.3.10: Statistical Analysis

All data are presented as the mean ± SEM. Two group comparisons were performed using Student's t test and comparisons of more than two groups were done by ANOVA with the post-Bonferroni Test. All statistical analyses were performed using GraphPad Prism Software (San Diego, Calif., USA) and $p < 0.05$ were considered statistically significant.

4.4: Results

4.4.1: GRK5 modulates bacterial clearance but doesn't mediate intracellular bacterial killing

To determine the function of GRK5 in the host response and subsequent clearance of live Gram-negative infection, we infected WT and KO mice intra-tracheally with log-phase growth live *E. coli* at $1x10^6$ CFU/mouse. After infection, lung homogenates, broncho-alveolar lavage fluid and blood samples were prepared at specified time-points and plated onto tryptic soy agar plates and CFUs determined 24 h later.

Infection of mice with *E. coli* at $1x10^6$ CFU/mouse induced low grade acute inflammation; bacteria were cleared from lungs by 24 h post infection and were not detected in blood at any of the time-points tested. Mice of both genotypes had comparable bacterial load at an earlier time-point (4 h) both in lungs and BAL fluid (Fig 4.1a and 4.1b). However, by 12 hours, bacterial counts were strikingly decreased in the GRK5 KO lung and BAL (Fig 4.1a, b, c). These results suggest that GRK5 deficient mice are able to clear bacteria much better than the WT mice, in this *E. coli* model.

Bacterial burden could be regulated at multiple levels including phagocytosis, bacterial killing or by regulating infiltration of phagocytes. To determine if GRK5 directly regulates phagocytosis or bacterial killing, we performed *in vitro* intracellular bacterial killing assay using thioglycollate-induced peritoneal neutrophils. As shown in Fig 4.2, phagocytosis (as seen in 0 min time point) or intra cellular bacterial killing (as seen in 30 and 60 min time points) were similar between the WT and GRK5 KO neutrophils. These data clearly suggest GRK5-regulated

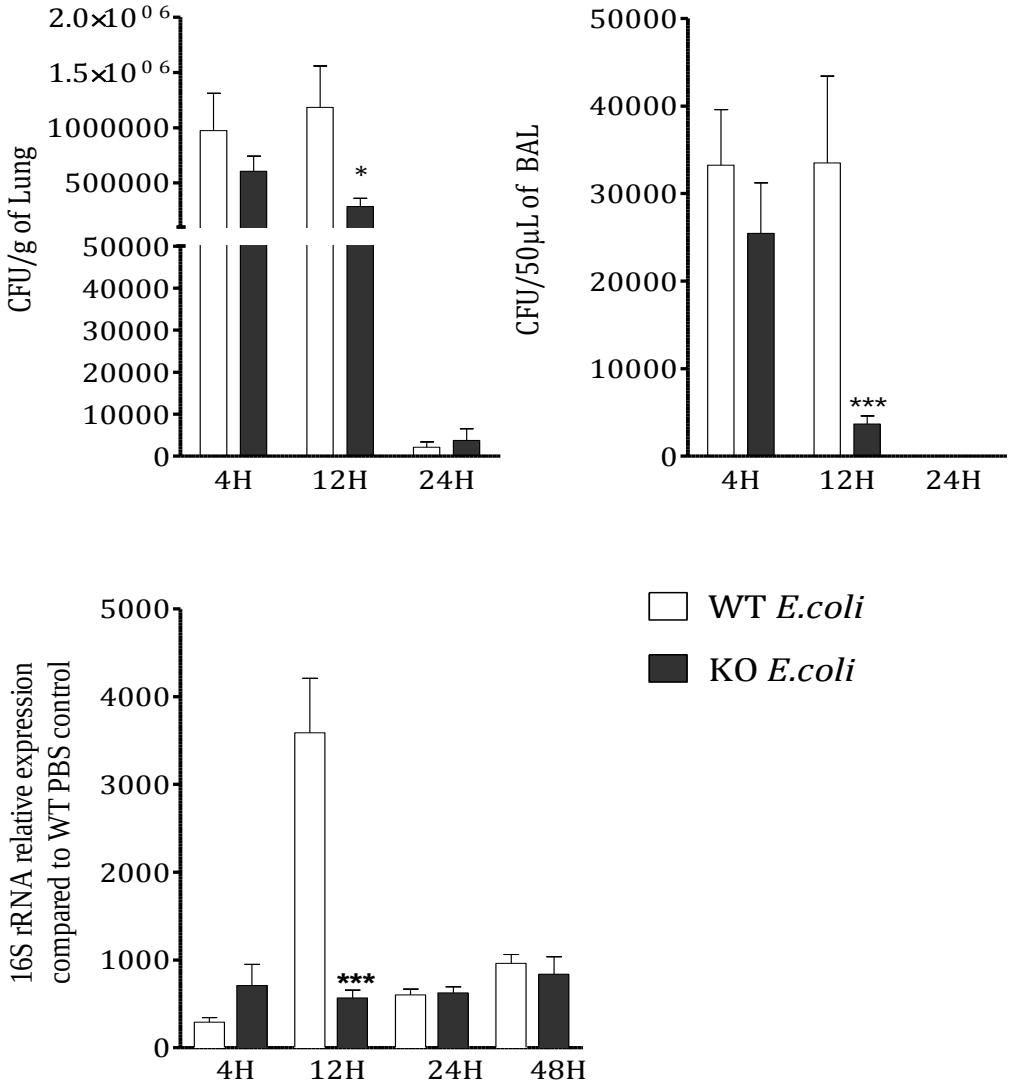


Figure 4.1: Decreased bacterial burden in GRK5 deficient mice at low dose *E. coli*: GRK5 WT and KO mice were intra tracheally injected with 1x 10^6 CFUs of *E. coli* and after the time points mentioned (a) lung and (b) Broncho alveolar lavage fluid were collected and plated for bacterial growth as described in materials and methods. (c) Quantitative relative expression of bacterial 16S rRNA in lung tissue of wild type and KO animals infected with *E. coli*. (n= 9 for 4hr; 12 for 12hr; 7 for 24h; 4 for 48h). * $p < 0.05$, * * * $p < 0.001$ compared to the corresponding infected WT groups.

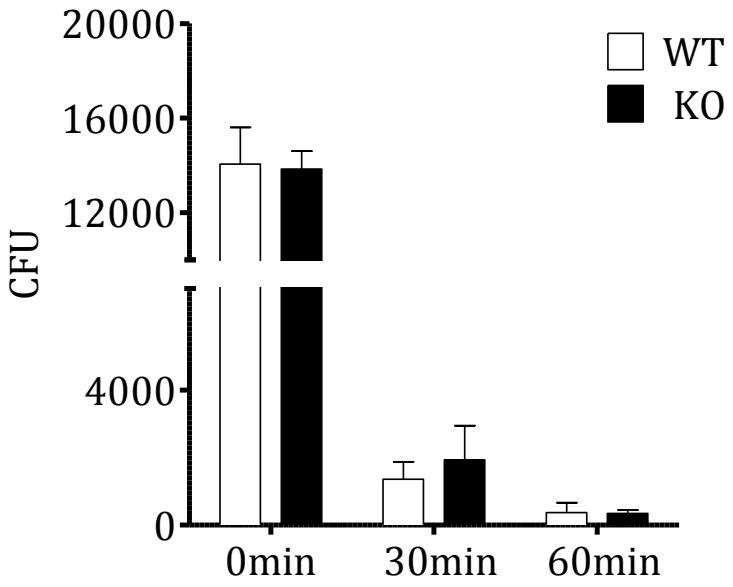


Figure 4.2: GRK5 does not play a role in intra cellular killing of bacteria: Thioglycollate induced neutrophils were collected from GRK5-/- and GRK5 +/+ as described in materials and methods. 1x 10^6 neutrophils were incubated with 4x 10^6 CFUs of opsonized bacteria for 30 minutes to mediate phagocytosis of bacteria. The cells were then incubated with gentamicin for specified time points to kill extra cellular bacteria and later washed twice with HBSS. The washed pellet is then lysed with 0.1% tritonX to liberate intracellular bacteria and then plated onto Tryptic soy agar to enumerate CFUs (n=6).

bacterial burden *in vivo* is unlikely through direct modulation of phagocytosis or intra cellular killing by GRK5.

4.4.2: GRK5 mediates early neutrophil recruitment into lungs

GRK5 is known to regulate many GPCRs, including chemokine receptors. GRK5 was shown to regulate CXCR4 *in vitro* by phosphorylating Hsp70 interacting protein (Hip) which in turn aids in internalization of CXCR4[1]. Since, our data ruled out a role for GRK5 in bacterial killing, we shifted our focus to understand the dynamics of immune cell infiltration, which can alter bacterial persistence during bacterial pneumonia *in vivo*. To study the dynamics of immune cell infiltration, we induced lung infection with *E. coli* in both genotypes and examined the lung tissue for immune cell infiltration using flow cytometry. When infected with *E. coli*, GRK5 deficient mice had a significantly increased recruitment of neutrophils into the lung at 4 h time point (Fig 4.3a). However, at the later time points (12 to 48 h) the number of neutrophils in the lung was similar between the genotypes, suggesting that GRK5 deficiency enhances early neutrophil recruitment. Further to characterize the activation status of these neutrophils, we examined CD11b expression. CD11b expression in neutrophils has been linked to increased reactive oxygen species (ROS) production and increased respiratory burst, which have direct consequence in bacterial killing[183]. Interestingly, 12 hours post-infection, CD11b expression in GRK5 KO neutrophils were significantly higher compared to the WT cells (Fig 4.3d). This coupled with early neutrophil infiltration likely cleared bacteria faster in the GRK5 KO mice.

In addition to these events, we also examined the percentage of CD11b+Gr1+F4/80+ (possibly efferocytosing macrophages)[184] cells in the lungs. Recently, these cells have also been described to phenotype myeloid-derived suppressor cells (MDSCs), which function to restrict

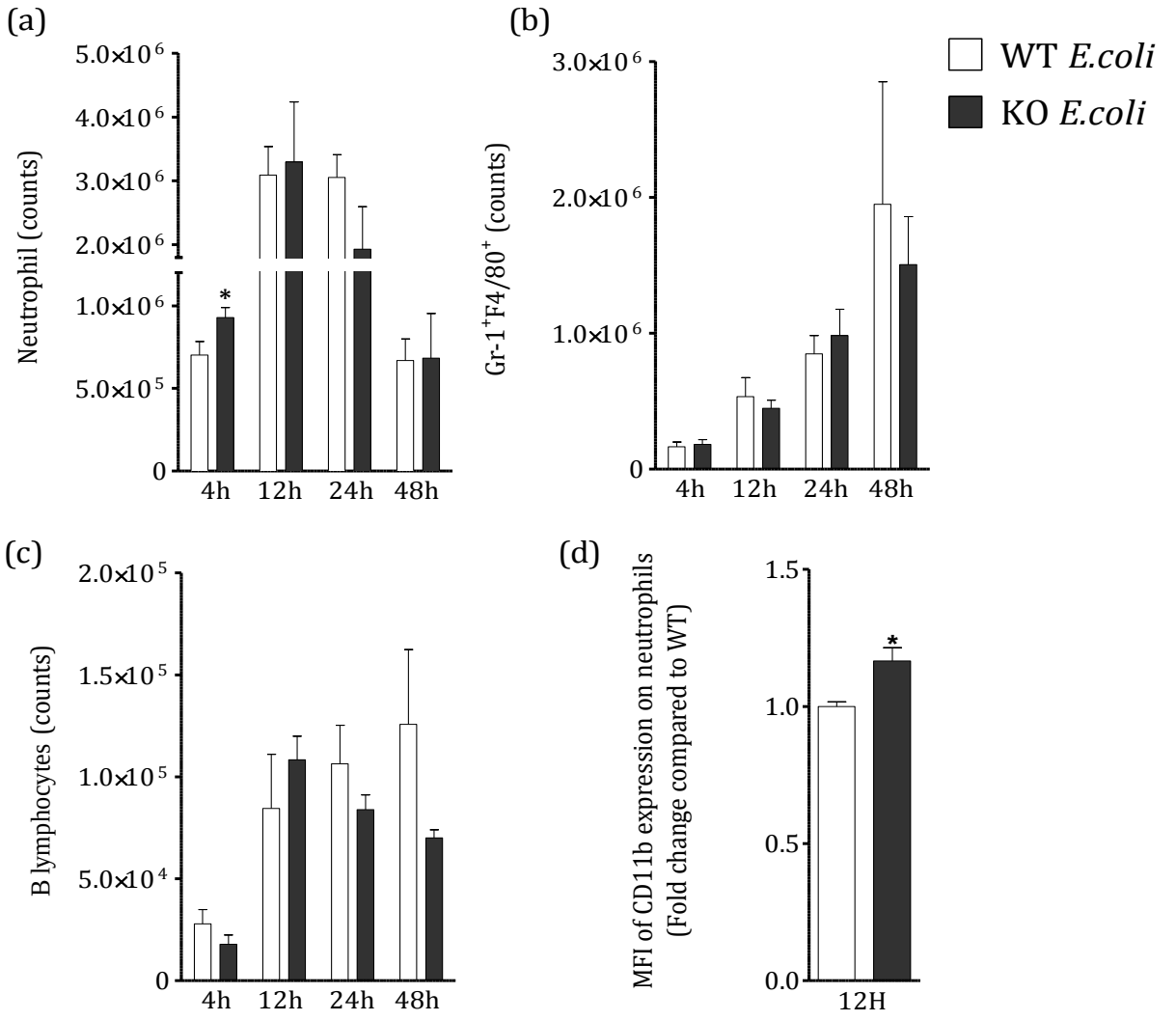


Figure 4.3: GRK5 regulates early recruitment and activation of neutrophil upon *E. coli* infection: Lung samples collected at different time points from *E. coli* infected mice were analysed for cellular composition (a) Neutrophils, (b) Efferocytosing macrophages expressing both Gr-1 and F4/80 and (c) B cells. GRK5 deficiency induced a small but significant increase in neutrophil recruitment only at early time point (4H) and not at later time points.(d) Lung samples collected 12 hours post infections were analyzed by flow cytometry for surface expression (mean fluorescence intensity) of CD11b. (n= 8-10 for 4hr; 6-9 for 12hr; 5 for 24h; 5 for 48h)=* $p < 0.05$, compared to the corresponding WT groups.

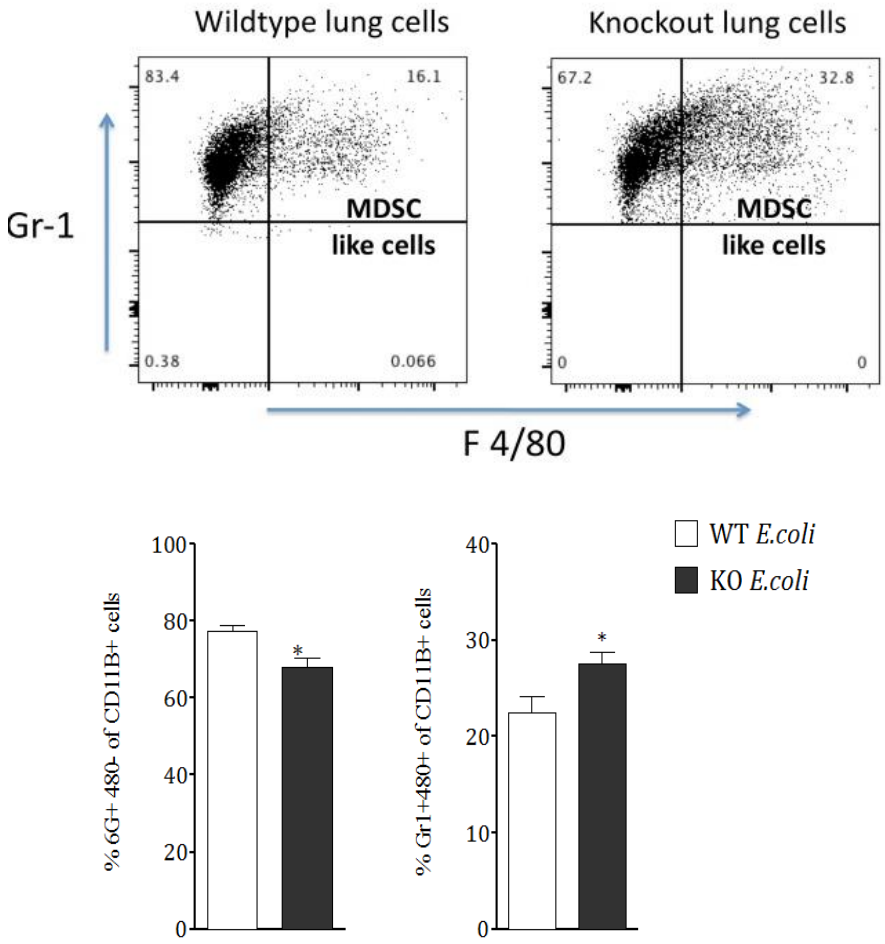


Figure 4.4: Increased proportions of efferocytosing macrophages in GRK5 KO lungs at low dose *E. coli*: (a) Lung samples analyzed by flow cytometry revealed increased percentage of efferocytosing macrophages in GRK5 KO lungs. (b) Percentage of Gr-1+ cells and Gr-1+ F4/80+ cells from lung 12h post infection. (n=8-10). * $p < 0.05$, compared to the corresponding WT groups.

ongoing inflammation and favor resolution[184],[185]. Surprisingly, at 12 hours post-infection, this resolution favoring cell type was significantly higher in the GRK5 KO mice compared to WT (Fig.4.4). These data suggest that the enhanced neutrophil infiltration and activation together with increase in efferocytosing macrophages/myeloid-derived suppressor cells could favor faster resolution of inflammation observed in the GRK5 knockout mice.

4.4.3: GRK5 regulates CXCL1 but does not regulate CXCR2, CXCR4 and CCR1 cell surface expression

Enhanced neutrophil infiltration in the GRK5 knockout could be due to regulation of expression of chemokines or their respective receptors. Therefore, we examined the levels of various chemokines (CXCL1, CXCL2 and MCP1) at different time points after *E. coli* infection. Interestingly, CXCL1 levels were significantly increased in KO mice at 4 h time point coinciding with the increased neutrophil recruitment (Fig. 4.5a). Expression of other chemokines, CXCL2 and MCP1 weren't any different between WT and KO mice at 4 h time point. However, at later time points, the levels of CXCL1, CXCL2 and MCP1 were decreased in KO mice compared to WT mice. To examine if GRK5 also regulated chemokine receptor expression, we assessed cell surface expression of CXCRs in neutrophils under basal and stimulated conditions in the two genotypes. As shown in Fig 4.6, chemokine receptor cell surface expression decreased when the neutrophils were treated with chemokines (likely due to receptor desensitization and internalization). This however was similar between the genotypes under both basal and stimulated conditions. Together these data suggest that in response to *E. coli* infection, GRK5 deficient mice were able to produce higher levels of chemokines early in the process that likely led to enhanced neutrophil infiltration and therefore better clearance.

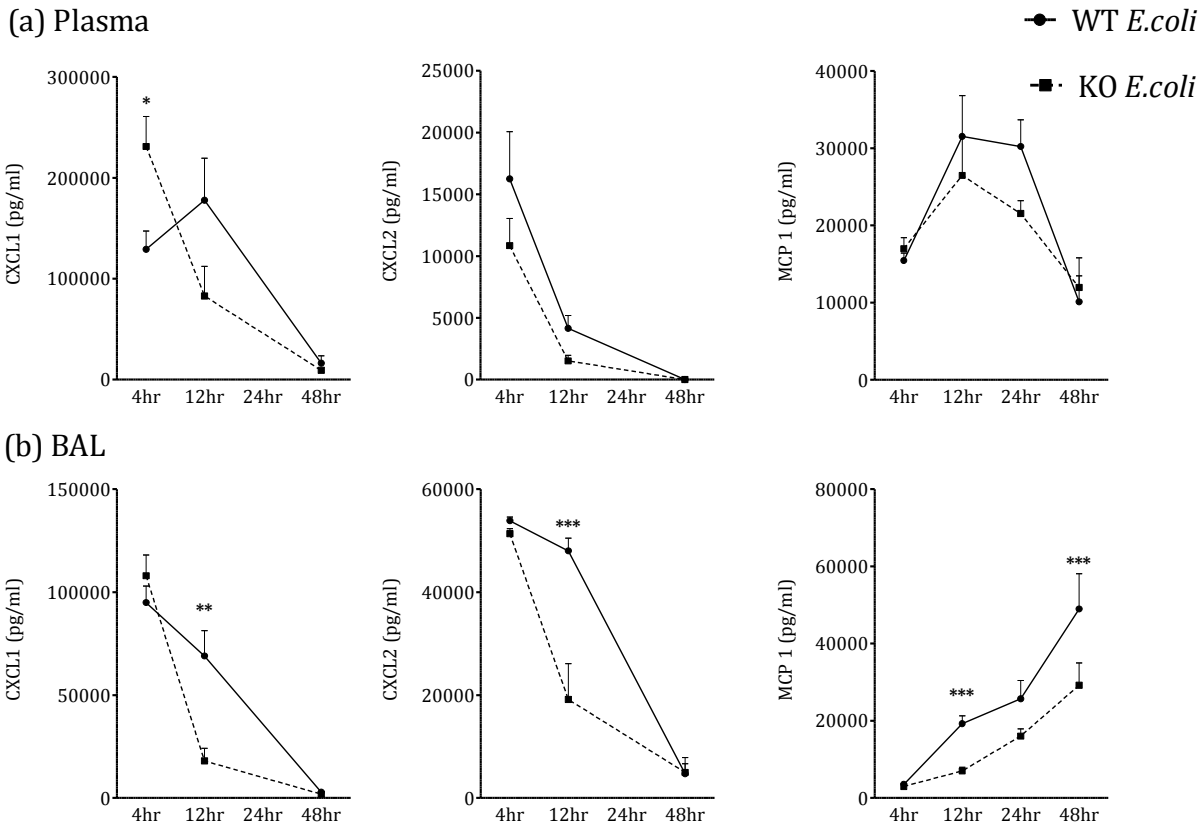


Figure 4.5: GRK5 deficiency induced CXCL1 production early in low dose *E. coli* induced pneumonia: (a) Plasma and (b) BAL samples were analyzed for expression of chemokines CXCL1, CXCL2 and MCP1. (n= 7 for 4hr; 6 for 12hr; 5 for 24h; 4 for 48h).*$p < 0.05$, **$p < 0.01$ and ***$p<0.001$ compared to the corresponding WT groups.

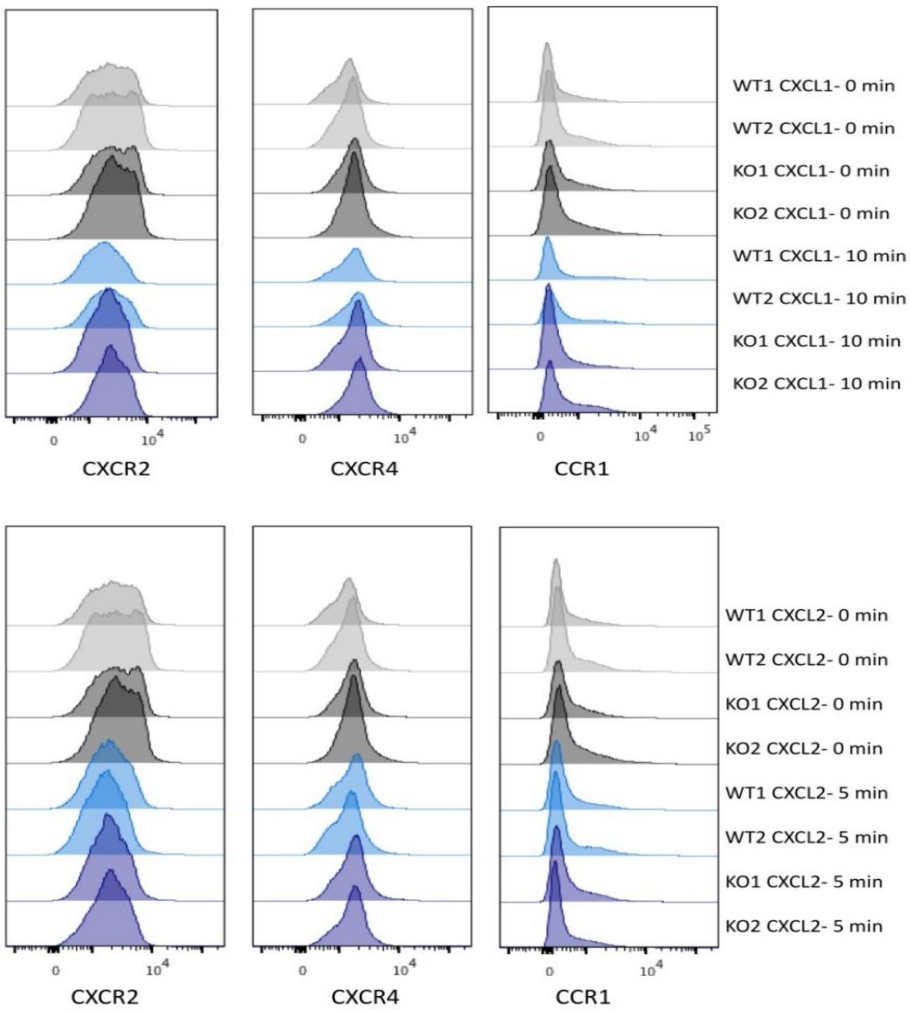


Figure 4.6: GRK5 does not regulate expression of neutrophil CXCR2, CXCR4 and CCR1 : Bone marrow neutrophils were isolated using percoll density centrifugation from wild type and knock out mice and were analyzed for expression of chemokine receptors with and without stimulation using (a) CXCL1 and (b) CXCL2.(n= 5).

4.4.4: GRK5 modulates inflammatory status

The overall phenotype in this lower dose *E. coli* model suggests GRK5 deficiency regulates neutrophil infiltration likely *via* CXCL1 expression and restricts bacterial presence and as sequelae we expected inflammatory cytokine production during active pulmonary infection to be decreased in KO mice. To assess the inflammatory status, we subjected lung, BAL fluid and plasma from *E. coli* infected mice to cytokine analysis by ELISA. Consistent with our prediction, levels of pro-inflammatory cytokines including IL-6 and TNFα levels were significantly decreased in the KO mice, in both plasma and lung (Fig.4.7a and 4.7b). These effects however, were time-dependent. Also, consistent with protein levels, mRNA levels of these cytokines were decreased in KO lung tissue (data not shown). Previous studies[2,4,43] have shown that GRK5 is a critical regulator of NFκB pathway during acute inflammation. To assess the role of GRK5 in NFκB pathway in this model, we measured the levels of phospho-IκBα in the lung tissue. Consistent with the decreased cytokine production, p-IκBα was significantly decreased in the KO lung tissue (Fig.4.7c). These observations on inflammatory status in KO mice are consistent with our previous studies on endotoxemia and CLP induced polymicrobial sepsis[2,4].

4.4.5: GRK5 confers survival advantage during *E. coli* induced pneumonia at higher infection dose:

Our results so far suggest that the sub lethal dose of *E. coli* induces a sub-acute lung inflammation and that the GRK5 knockout mice are protected from bacterial burden and inflammation likely *via* multiple mechanisms. This however did not result in overt histo-

(a)

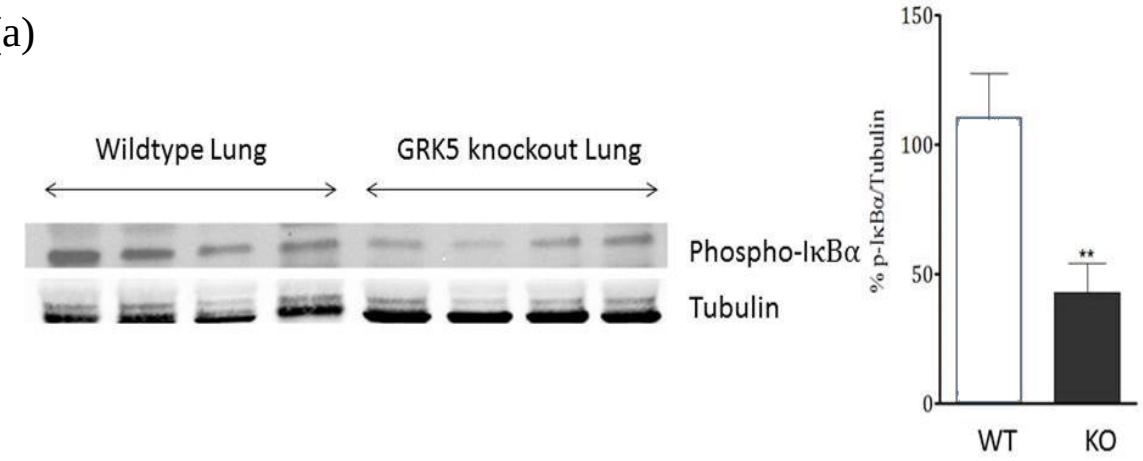
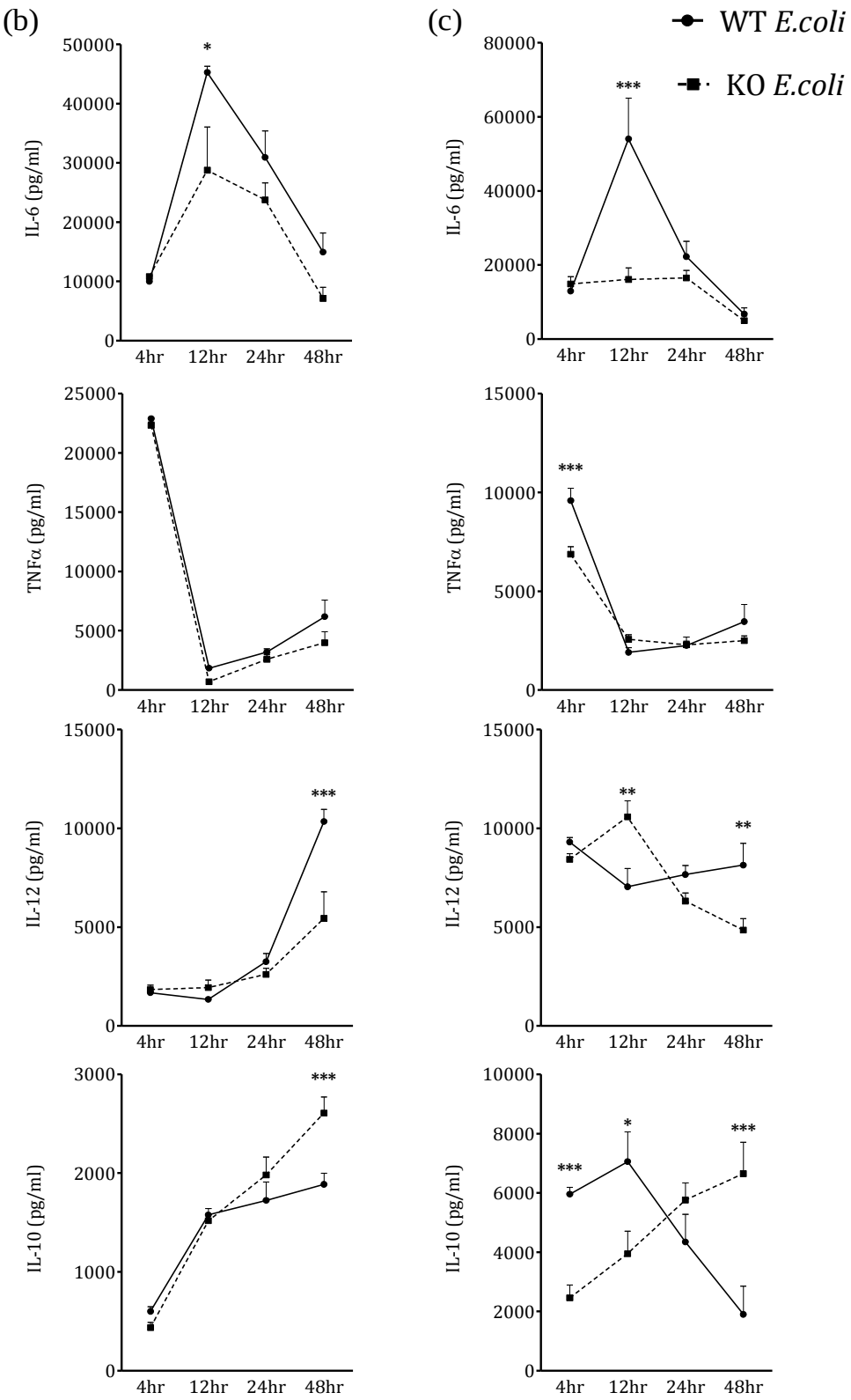


Figure 4.7: GRK5 deficiency induced decreased inflammatory response in low dose *E. coli* induced pneumonia: (a) Immunoblot showing decreased pIκBα levels in GRK5 deficient mice after low dose *E. coli* injection. (n=4). (b) BAL and (c) Plasma samples were analyzed for expression of cytokines IL-6, TNFα, IL-12p40 and IL-10. (n= 10-15 for 4hr; 9-15 for 12hr; 5 for 24h; 5 for 48h) *$p < 0.05$,**$p < 0.01$ and ***$p<0.001$ compared to the corresponding WT groups.

Figure 4.3: (cont'd).



pathological differences in resolution at 48 hours after infection (data not shown). Even though it is possible that a difference in resolution may be evident at later time points, we wanted to further examine if *E. coli*-induced mortality in this pulmonary model is different between the genotypes. We hypothesized that since bacterial burden and inflammation are decreased in the GRK5 knockout mice, this genotype would be protected from *E. coli*-induced mortality compared to the wild type mice. This hypothesis was also based in part on our previous studies in polymicrobial sepsis model, in which we showed that GRK5 deficiency enhances survival especially in the presence of antibiotic therapy. Therefore, we tested the role of GRK5 with a higher dose of *E. coli* that we predicted would cause ~50% mortality based on pilot studies and literature. For this, we used *E. coli* dose of $5x10^6$ CFUs/mouse and performed survival studies. This dose resulted in approximately 50% mortality in wild type in 2 days. In contrast to our prediction, GRK5 deficient mice succumbed to pneumonia much more readily than wild type. At the end of 48 hours post infection GRK5 deficient mice had 90% mortality, whereas wild type mice had only 58% mortality (Fig.4.8). Also, the median survival in WT mice was 33 hours, whereas GRK5 deficient mice had a median survival of 18 hours. To begin to understand the paradox, we first examined bacterial burden using the higher dose of *E. coli*. Surprisingly, and in contrast to the low dose *E. coli*, with the higher dose of *E. coli*, GRK5 deficient mice showed significantly increased bacterial load in the lung and blood (Fig. 4.9). This suggested that with higher dose of *E. coli*, the GRK5 deficient mice are unable to clear bacterial infection. Some key events that were observed with the low dose of *E. coli* were the enhanced infiltration of neutrophils coupled with greater activation and increased CXCL1 production in the GRK5 knockout mice. To confirm if these were altered in the lethal dose model, we examined neutrophil infiltration, CD11b expression and CXCL1 production in the two genotypes following

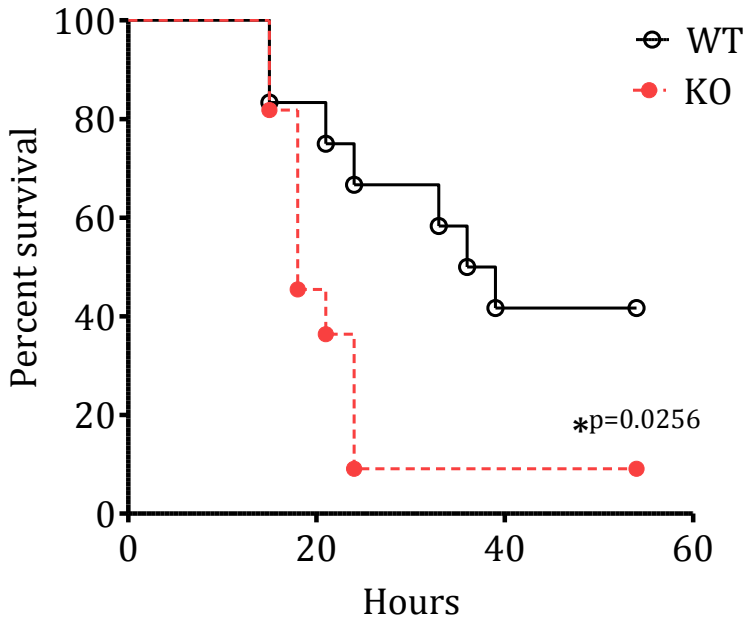


Figure 4.8: Increased mortality observed in GRK5 deficient mouse infected with *E. coli* at $5x10^6$ CFU/mouse: GRK5 deficient mice had approximately 90% mortality as compared WT mice having 58% mortality. (n= 11-12).).*$p < 0.05$, compared to the corresponding WT groups.

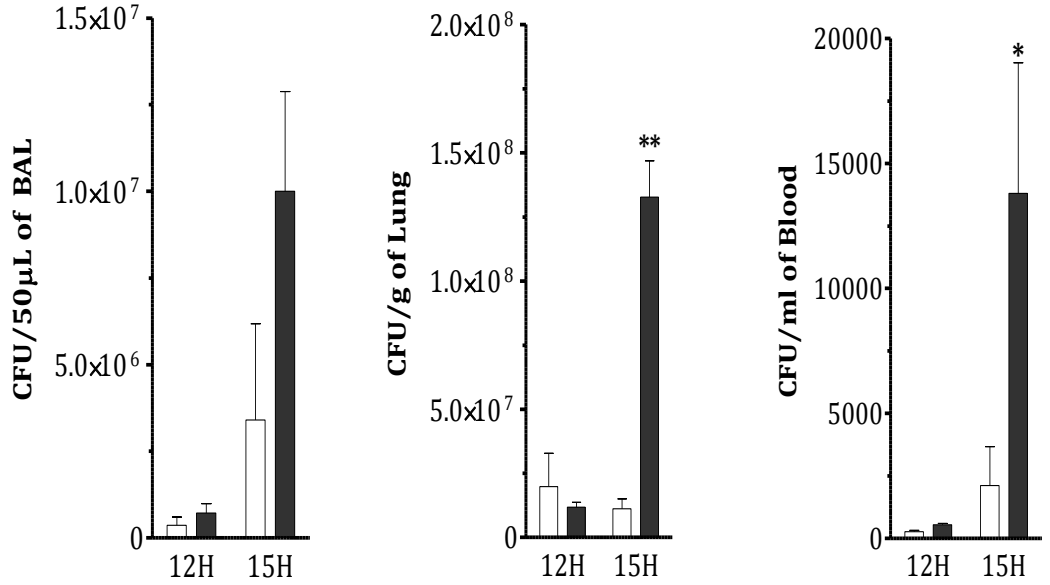


Figure 4.9: Increased Bacterial burden in GRK5 deficient mice at high dose *E. coli*: GRK5 WT and KO mice were intra tracheally injected with 5x 10^6 CFUs of *E. coli* and after the time points mentioned (a) lung, (b) Broncho alveolar lavage fluid and (c) Blood were collected and plated for bacterial growth as described in materials and methods. (n= 3-4 for 12hr; 3 for 15hr).*$p < 0.05$, ** $p < 0.01$ compared to the corresponding infected WT groups.

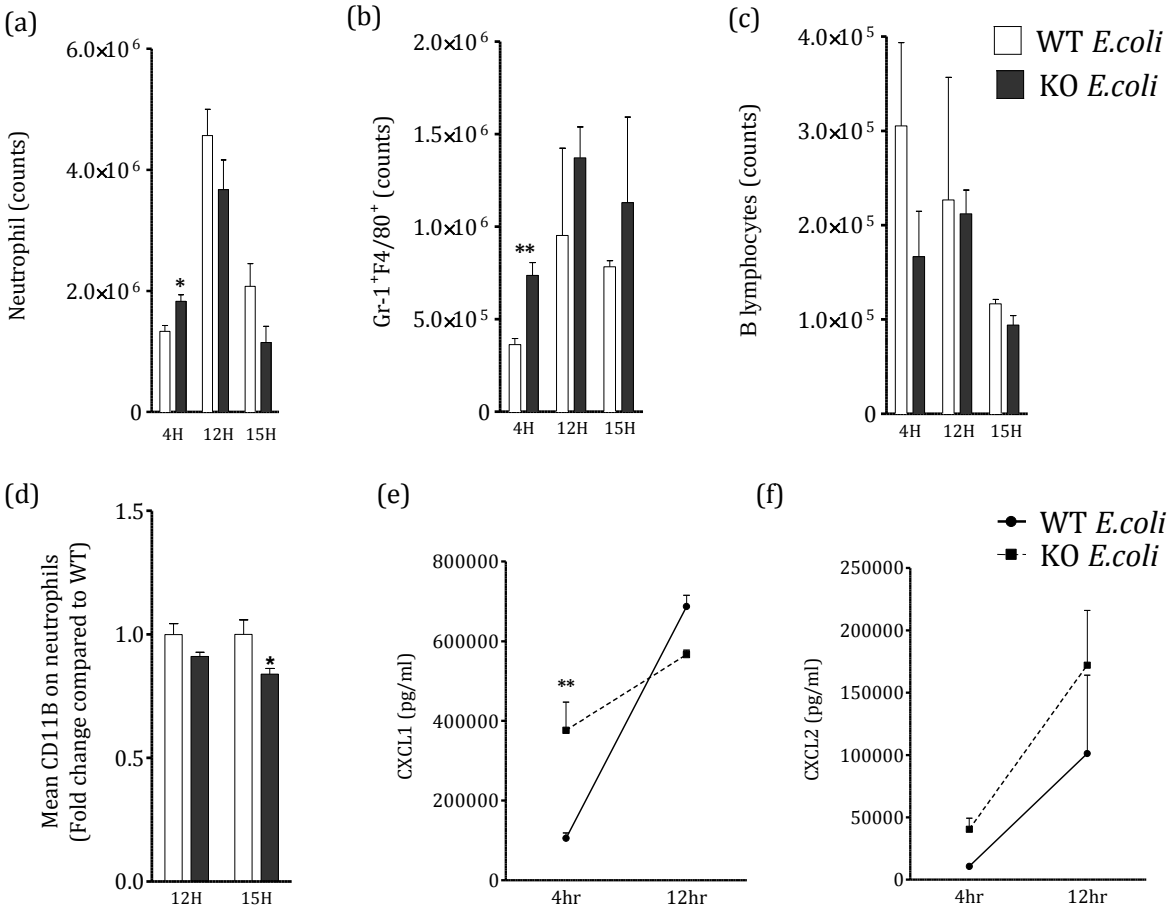


Figure 4.10: GRK5 deficiency mediates early neutrophil recruitment into lungs in high dose *E. coli* infected mice: Lung samples collected at different time points from *E. coli* infected mice were analyzed for cellular composition (a) Neutrophils, (b) Efferocytosing macrophages expressing both Gr-1 and F4/80 and (c) B cells. (d) Lung samples collected 12 and 15 hours post infections were analyzed by flow cytometry for neutrophil surface expression (mean fluorescence intensity) of CD11b, (e and f) Plasma samples from high dose *E. coli* were analyzed for expression of chemokines CXCL1 and CXCL2. (n= 3-4 for 12hr; 3 for 15hr).*$p < 0.05$, ** $p < 0.01$ compared to the corresponding infected WT groups.

infection with $5x10^6$ CFUs/mouse. Interestingly, although neutrophil infiltration and CXCL1 production were enhanced in the GRK5 KO similar to the sub lethal dose, CD11b expression was decreased in the GRK5 KO compared to the wild type mice (Fig.4.10). These data suggest that neutrophils from GRK5 KO mice are likely "less activated" and therefore unable to clear infection efficiently and therefore result in higher mortality. These data however, does not completely rule out other aspects of regulation by GRK5 KO in terms of bacterial clearance, especially under this high dose condition.

4.5: Discussion

Exaggerated inflammation is a characteristic feature of pneumonia and sepsis. Alteration in the initial events of infection like microbe detection, inflammatory signaling, chemotaxis of immune cells can alter the course of disease process. Studies have shown neutrophil recruitment is essential in clearing bacteria from lungs and at the same time exaggerated neutrophil influx is detrimental to the host.

In this study, we show GRK5 plays a paradoxical role in regulating *E. coli* induced pneumonia depending on the dose of *E. coli*. At a sub lethal dose of *E. coli*, GRK5 deficiency leads to efficient clearing of bacteria from lungs by increasing neutrophil recruitment. Being a kinase regulating GPCRs, it is likely that GRK5 can regulate chemokine receptors and hence modulate neutrophil chemotaxis. However, our data showed (Fig 4.8) GRK5 doesn't regulate basal expression of CXCR2 and CXCR4, which are the major chemokine receptor responsible for neutrophil migration. Instead, our data suggest, that the increased bacterial clearance was probably a result of increased CXCL1 production (Fig.4.9a), which likely led to enhanced

neutrophil chemotaxis to the site of infection observed in the KO mice. In addition to the increased bacterial clearance, GRK5 also mediated increased resolution of inflammation as evidenced by increased proportion of efferocytosing macrophages (Fig.4.5), which are known to clear dying and dead cells[185]. We also noticed decreased activation of NFκB (Fig. 4.10) in the lungs of KO mice, which probably could be a result of faster bacterial clearance and therefore less inflammatory signaling pathways being activated or due to previously demonstrated role of GRK5 in NFκB signaling.

Similar to the low dose model, *E. coli* at a lethal dose induced higher CXCL1 and neutrophil recruitment in GRK5 KO mice compared to wild type. However in contrast to low dose model, the increased neutrophil recruitment did not seem to clear bacteria effectively in the KO mice (Fig. 4.2). In fact, high dose *E. coli* resulted in increased bacterial load in both lungs, and blood and this data is consistent with increased mortality observed in KO mice. This data suggests that neutrophils are probably altered in terms of their bacterial killing capacity or their activation status with the high dose of *E. coli*. Our data clearly show marked differences in CD11b expression (linked to neutrophil activation) between the high and low dose *E. coli* infection. Even though at low dose infection, CD11b expression is significantly enhanced in the KO mice neutrophils, this is not observed in the high dose model (which in fact is decreased in the KO, in the high dose model), suggesting less activation GRK5 KO neutrophils in the high dose model. CD11b is an integrin receptor and β2-Integrins (e.g., CD11a/CD18, CD11b/CD18) are generally expressed at low levels basally in the neutrophils, but can be activated to a high affinity state, which enables them to transmigrate from blood to tissues. Also, engagement of CD11b/CD18 in neutrophils triggers generation of reactive oxygen species through NADPH

oxidase in close proximity to the target mediating bacterial killing[186]. Furthermore, CD11b being a component of Complement receptor 3 can influence complement receptor pathways and therefore regulate bacterial burden.

One of the key observations in this study is that deficiency of GRK5 significantly enhances CXCL1 production. CXCL1/KC belongs to a class of ELR+ CXC chemokines, which also includes other major chemokines like CXCL2/MIP2 and CXCL5/LIX. CXCL1 is produced from hematopoietic and non-hematopoietic compartments and was shown to be essential for neutrophil influx and clearing of *Klebsiella pneumoniae* from lungs[187]. Even though the molecular mechanism by which GRK5 regulates CXCL1 production is not known, CXCL1 has been shown to be regulated by TLR-mediated MAPK signaling. p38 MAPK and ERK1/2 were known to regulate CXCL1 production in response to IL-1β[188], FasL[189] activation in a MyD88 dependent manner. Consistent with this, previous studies have shown that GRK5 negatively regulates TLR4 induced ERK1/2 signaling *via* regulation of IKK-NFκBp105 pathway[40]. Thus, deficiency of GRK5 likely leads to over activation of ERK pathway thus leading to increased CXCL1 production. This hypothesis will be tested in future studies.

GRK5 has been shown to positively[43] (macrophages) or negatively[41] (endothelial cells) regulate NFκB signaling and modulate inflammatory response depending on the cell type involved. In endotoxaemia[4] and polymicrobial sepsis[2], we showed GRK5 positively regulates cytokine production and that GRK5 deficient mice exhibited attenuated inflammatory response mostly in the later stages of sepsis. It is likely that, GRK5 can negatively regulate NFκB in endothelial and epithelial cells, both of which can produce CXCL1 and therefore lead to

increased production of CXCL1 in GRK5 KO mice. Nevertheless, in this study we find that IκBα phosphorylation was decreased in the KO lungs suggesting that NFκB is unlikely to be the regulator of CXCL1 production in the GRK5 KO mice

In conclusion, our current study demonstrates GRK5 negatively regulates CXCL1 production (an important chemokine responsible for neutrophil chemotaxis) and thereby regulates neutrophil chemotaxis. In low dose *E. coli* model, this response seems to play a beneficial role in the KO mice, as evidenced by decreased bacterial load and increased resolution of inflammation. However, at the higher dose of *E. coli*, KO mice succumb to lethality due to higher bacterial load in spite of enhanced neutrophil infiltration, likely as a result of decreased neutrophil activation.

CHAPTER FIVE

SUMMARY AND CONCLUSION

More than two decades ago, GRKs were discovered in the context of GPCR desensitization and mostly considered only to play a role in G- protein mediated functions. However, recent studies show GRKs can interact with and phosphorylate non-GPCR targets. However, the knowledge on their physiological and pathophysiological consequences of these newly identified interactions of GRKs especially during inflammatory diseases is scarce. The major focus of this dissertation research project was to understand the overall pathological role of GRK5 in acute inflammatory diseases like sepsis and pneumonia. In this context, our lab showed GRK5 to mediate NFκB signaling by interacting with and phosphorylating IκBα *in vitro*. Furthermore, GRK5 role in TLR4 signaling had been previously identified, where in GRK5 acted as positive mediator of cytokine production. We further wanted to ascertain the role of GRK5 in more clinically relevant models for acute inflammatory diseases like sepsis and pneumonia. The individual aims and main findings from each project are summarized below.

5.1: Role of GRK5 in MyD88-dependent and -independent cytokine production *in vivo*

Toll like receptor signaling can be broadly classified into MyD88-dependent and TRIF-dependent pathways. The activation of the MyD88-dependent pathway leads to the early phase of NFκB activation and proinflammatory cytokine expression, while TRIF dependent pathway mediates the late phase of NFκB activation and the induction of IFN-inducible genes in TLR4 signaling.

GRK5 regulates cytokine production in LPS induced TLR4 signaling pathway *via* positively modulating NFκB signaling. However, it is not known whether the role of GRK5 is

specific for TLR4 or whether it regulates other TLRs. Also, since both MyD88-dependent and TRIF-dependent pathways converge onto NFκB, it is important to know whether GRK5 regulates either one of them or both. To identify the role of GRK5 in these pathways, we injected WT and KO mice with TLR2 ligand and TLR3 ligand separately and measured cytokine production. TLR2 specifically activates MyD88 pathway whereas TLR3 specifically activates TRIF pathway and by comparing the responses we can identify whether GRK5 has any role in either of the pathways.

5.1.1: Results

- GRK5 mediated cytokine production through both MyD88 and TRIF pathways.
- GRK5 positively mediated IL-5, IL-6 and MCP-1 production *via* MyD88 pathway.
- GRK5 positively mediated TNFα and RANTES production *via* TRIF pathway.
- IL-12p40 and G-CSF levels are regulated by GRK5 at a level that is common to both MyD88 and TRIF pathway

5.1.2: Conclusion

Our results show GRK5 has overlapping as well as selective roles in TLR signaling pathways and can regulate multiple TLRs *via* both MyD88 and TRIF pathway. These findings also suggest GRK5 can play a substantial role in acute inflammatory disease conditions like poly microbial sepsis where in microbial TLR is the key players in mediating the disease.

5.1.3: Limitations and future directions

While we showed an evidence of GRK5 playing role in MyD88 and TRIF signaling, we did not take into account the cross talk happening between the two pathways. It is likely that MyD88 activation by itself can interact with partners of TRIF pathway and mediate TRIF dependent responses and vice versa. Our future direction includes identifying role of GRK5 in other pathways activated by the cross talk between MyD88 and TRIF signaling.

5.2: Role of GRK5 in Polymicrobial sepsis

The goal of this project was to understand the role of GRK5 in the pathophysiology of sepsis. Sepsis is characterized by inflammatory cascade, in response to microbial infection or extensive tissue damage. We induced polymicrobial sepsis by cecal ligation and puncture technique in both WT and KO mice to study the role of GRK5 during sepsis.

5.2.1: Results

- GRK5 KO mice had decreased NFκB activation and diminished inflammatory responses during sepsis.
- GRK5 KO and WT mice showed comparable bacterial load.
- GRK5 deficiency decreased sepsis-induced thymocyte apoptosis that correlated with decrease plasma corticosterone production.
- Peritoneal lavage cells from GRK5 KO mice during sepsis showed more immune-responsive phenotype compared to the wild type cells.

- GRK5 deficient mice were able to survive better than WT mice with administration of antibiotics. However, there is no significant survival advantage for GRK5 KO mice in the absence of antibiotics.

5.2.2: Conclusion

Our results demonstrate GRK5 as an important regulator of inflammation in polymicrobial sepsis and implicate GRK5 as a potential molecular target in sepsis. Regulation of inflammatory response, thymocyte apoptosis and immune suppression by GRK5 suggests that it can play multiple roles during sepsis.

5.2.3: Limitations and future directions

Mortality in sepsis is multifaceted and results eventually from cardiovascular, renal and pulmonary collapse. GRK5, being a GPCR regulating kinase could have potentially other beneficial GPCR dependent roles to play during sepsis like regulation of β-adrenergic system and preventing cardio vascular system collapse, regulation of coagulation and preventing disseminated intravascular coagulation (DIC), etc. Role of GRK5 in these processes is unknown and will be the subject of future studies. Non-hematopoietic cells also play an important role in the pathogenesis of polymicrobial sepsis by modulating neutrophil migration and phagocytosis. GRK5 is a ubiquitously expressed protein with a role in cell signaling in both the hematopoietic and non-hematopoietic compartments. Hence, our future direction is to assess the role of GRK5 in non-hematopoietic compartments using bone marrow chimeric mice or cell-type targeted knockout mice.

5.3: Role of GRK5 in *E. coli* induced pneumonia

GRK5 is highly expressed in normal and diseased airways. GRK5 regulates Toll like receptor signaling and airways are often exposed to multiple pathogens. So, the goal of this project is to discern the role of GRK5 in Gram negative bacteria induced pneumonia. We induced pneumonia by per oral tracheal injection of live *E. coli* into WT and KO mice at two different doses to characterize the role played by GRK5 in low grade and high grade airway inflammation.

5.3.1: Results

- At low dose *E. coli*, GRK5 KO mice had low bacterial burden, whereas at five-fold higher dose GRK5 KO mice had increased bacterial load and suffered from increased mortality compared to WT mice.
- GRK5 KO mice recruited more neutrophils to the site of infection in both high and low dose models.
- Increased neutrophil recruitment into lungs at low dose proved beneficial in the GRK5 KO mice, as the KO mice cleared bacteria sooner and mediated early resolution of inflammation.
- Increased neutrophil recruitment into lungs at high dose in the GRK5 KO mice was not effective at clearing bacteria and the mice harbored more bacteria causing early death.
- Increased neutrophil recruitment in both models in KO mice was associated with increased CXCL1/KC and/or CXCL2/MIP2 production.

- Neutrophils activation assessed by CD11b expression showed increased activation of neutrophils in KO mice at low dose *E. coli* but decreased activation of neutrophils in KO mice at high dose *E. coli*.

5.3.2: Conclusion

Our results show GRK5 plays a critical role in bacterial clearance and neutrophil chemotaxis irrespective of low or high doses of *E. coli*. At low doses, GRK5 deficiency induces appropriate neutrophil infiltration to enhance clearance of bacteria, whereas at high dose of *E. coli*, GRK5 deficiency induces exaggerated neutrophilic infiltration leading to neutrophil exhaustion causing bacterial overgrowth and quicker death. Our data exemplify the complexity in the mechanism of progression of pneumonia and suggests modulating GRK5 levels can be beneficial depending on the patient inflammatory status to achieve patient recovery.

5.3.3: Limitations and future directions

Although increased CXCL1 production from GRK5 deficient mice is contrary to the GRK5's positive regulatory role on cytokine production, the overall phenotype in high dose is comparable to essential role of NFκB in pneumonia and suggests GRK5 likely mediates NFκB pathway. Furthermore GRK5 has been shown to regulate NFκB signaling either positively or negatively depending on the cell type. It is likely that GRK5 might negatively regulate NFκB and CXCL1 production from epithelial or endothelial cells of lung, thus altering neutrophil chemotaxis. To answer these questions, GRK5 role in CXCL1 production from hematopoietic and non-hematopoietic compartments must be ascertained.

Together, this dissertation research uncovers novel roles of GRK5 during acute inflammatory diseases. Through this project, GRK5 was shown to regulate both MyD88 and TRIF pathway, modulate immune cell apoptosis and development of immune suppression during poly microbial sepsis, and regulate CXCL1 production thereby enabling neutrophil recruitment and bacterial clearance. These roles are very critical in the progression of acute inflammatory diseases and further work on discerning the involved molecular mechanisms will likely lead to therapeutic strategies to target GRK5 in inflammation.

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