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Myeloid-specific GPCR kinase-2 negatively regulates NFκB1p105-ERK pathway and limits endotoxemic shock in mice

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Abstract

G-protein coupled receptor kinase 2 (GRK2) is a member of a kinase family originally discovered for its role in the phosphorylation and desensitization of G-protein coupled receptors. It is expressed in high levels in myeloid cells and its levels are altered in many inflammatory disorders including sepsis. To address the physiological role of myeloid cell-specific GRK2 in inflammation, we generated mice bearing GRK2 deletion in myeloid cells (GRK2^{Δmye}). GRK2^{Δmye} mice exhibited exaggerated inflammatory cytokine/chemokine production, and organ injury in response to lipopolysaccharide (LPS, a TLR4 ligand) when compared to wild type littermates (GRK2^{fl/fl}). Consistent with this, peritoneal macrophages from GRK2^{Δmye} mice showed enhanced inflammatory cytokine levels when stimulated with LPS. Our results further identify TLR4-induced NFκB1p105-ERK pathway to be selectively regulated by GRK2. LPS-induced activation of NFκB1p105-MEK-ERK pathway is significantly enhanced in the GRK2^{Δmye} macrophages compared to GRK2^{fl/fl} cells and importantly, inhibition of the p105 and ERK pathways in the GRK2^{Δmye} macrophages, limits the enhanced production of LPS-induced cytokines/chemokines. Taken together, our studies reveal previously undescribed negative regulatory role for GRK2 in TLR4-induced p105-ERK pathway as well as in the consequent inflammatory cytokine/chemokine production and endotoxemia in mice.

Introduction

G-protein coupled receptor kinases (GRKs) are enzymes that phosphorylate activated G-protein coupled receptors (GPCRs) and cause desensitization of G-protein-dependent signaling. GRK2 is one of seven members of GRKs and is widely expressed (De Blasi et al., 1995; Loudon et al., 1996). GRK2 levels are altered in immune cells from human patients with a variety of inflammatory disorders, as well as, in a number of animal disease models (Giorelli et al., 2004; Lombardi et al., 2001; Lombardi et al., 1999; Vroon et al., 2005;

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Vroon et al., 2003). In particular GRK2 levels are markedly increased in neutrophils from septic patients (Arraes et al., 2006). Treatment of neutrophils and macrophages (M ϕ) with TLR ligands upregulates GRK2 levels significantly (Alves-Filho et al., 2009; Loniewski et al., 2008). This increase in GRK2 levels has been postulated to be important in limiting chemokine receptor (a GPCR)-induced chemotaxis of immune cells. In fact, neutrophils from human septic patients show significantly attenuated chemotaxis (Arraes et al., 2006). Several other studies have also determined the role of GRK2 in immune cell chemotaxis owing to the fact that chemokine receptors belong to the GPCR family and that the observations are remarkably in-tune with the known classic role for GRK2, i.e. GPCR desensitization. In spite of these seminal advances in GRK2 biology, role of GRK2 in M ϕ , particularly in response to non-GPCRs, is not well understood. More importantly, the role of myeloid cell-specific GRK2 in lipopolysaccharide-induced inflammation and endotoxemia *in vivo* is not known.

Lipopolysaccharides (LPS) activate a class of innate immune receptors called the Toll-like receptors (TLRs) which act as the first line of host defense against bacterial infections (Beutler, 2009). Among the TLRs, TLR4 is activated by LPS from gram-negative bacteria that triggers an inflammatory response (Beutler, 2009). Under endotoxemic conditions, however, this system is over-stimulated and the exaggerated cytokine response elicited by the host turns harmful and leads to endotoxic shock and eventual death (Salomao et al., 2008). In addition to endotoxic shock and sepsis, TLR4 is now proposed to be an important player in a number of human and animal inflammatory diseases (Beutler, 2009). Activation of TLR4 by LPS triggers the recruitment of adapter proteins such as TRIF and Myd88 as well as other TIR domain containing proteins that eventually activates the inhibitor of κ B kinase (IKK) complex (O'Neill and Bowie, 2007). The activated IKK complex then phosphorylates I κ B α (an inhibitor of NF- κ B) thereby targeting it for ubiquitination and proteasomal degradation. I κ B α degradation enables the release and nuclear translocation of NF- κ B, which then regulates the expression of genes involved in inflammation and innate and adaptive immune responses. In macrophages, activation of IKK complex also phosphorylates NF κ B1 p105 (another I κ B protein), which normally is stoichiometrically bound to a MAP3K called TPL2. LPS stimulation and phosphorylation of p105 leads to partial degradation and subsequent release of TPL2. P105-free TPL2 activates MEK1/2, and ultimately the ERK1/2 pathway (Beinke et al., 2004; Waterfield et al., 2004). In addition to these pathways, LPS also mediates the activation of p38, JNK, and Akt signaling pathways (Symons et al., 2006). TLR4-induced activation of these signaling pathways and the subsequent activation of transcription factors, such as NF κ B, AP-1 and EGR-1, mediate the pathogenesis of inflammation and endotoxemia and shock (Salomao et al., 2008; Symons et al., 2006; Wong and Tergaonkar, 2009).

Even though one consequence of TLR4-mediated regulation of GRK2 levels in M ϕ and neutrophils is modulation of GPCR activity, we postulated that this regulation might have a feed back role in TLR4 signaling. To test this hypothesis, we generated myeloid cell-specific knockout of GRK2 and determined the role and mechanisms by which GRK2 regulates TLR4 signaling *in vivo* and in primary M ϕ . We demonstrate here that myeloid cell-specific GRK2 negatively regulates TLR4-induced inflammatory cytokines/chemokines and limits the pathogenesis of endotoxemic shock in mice. Furthermore, we also provide evidence that the pro-inflammatory phenotype of GRK2-deficient M ϕ is in part due to enhanced NF κ B1p105-ERK pathway.

Materials and methods

Materials

Antibodies were from Santacruz Biotech (ERK, p50 and actin-HRP) or from Cell Signaling Technology (most antibodies). *E. coli* LPS (0111:B4) was from Sigma and ultrapure LPS was from Invivogen.

Animals

Animals were housed four to five mice per cage at 22–24°C in rooms with 50% humidity and a 12-h light–dark cycle. All animals were given mouse chow and water *ad libitum*. All animal procedures were approved by the Michigan State University Institutional Animal Care and Use Committee and conformed to NIH guidelines.

Generation of myeloid cell specific GRK2 deficient mice

GRK2^{fl/fl} mice in which exons 3-6 of GRK2 are flanked by LoxP sites were crossed with LysMCre mice to generate GRK2^{fl/fl} LysMCre mice (Matkovich et al., 2006). A breeding colony was maintained by mating GRK2^{fl/fl} with GRK2^{fl/fl}+LysMCre. The mice were generated on a mixed C57BL6/129sv background. GRK2^{fl/fl}+LysMCre were used in experiments and compared to littermate GRK2^{fl/fl} controls. LysMCre and GRK2^{lox/lox} mice were genotyped as described previously (Clausen et al., 1999; Matkovich et al., 2006). Myeloid cell GRK2 deleted mice will be referred to as GRK2^{Δmye} and littermate controls as GRK2^{fl/fl} mice. Male mice 6-8 weeks of age were used for the experiments.

Peritoneal Macrophage and Neutrophil isolation

To isolate peritoneal cells, mice were injected with 1 ml of 4% thioglycollate (i.p.) for 3 hours (for Neutrophils) or 4 days (for macrophages). Cells were collected as described before (Greten et al., 2007). Cells were plated in 12 well plates and serum starved for 3-4 hours before stimulation.

Cytokine analysis

A mouse 23-plex assay was used to determine the cytokine/chemokine concentrations according to manufacturer's instructions via Luminex 100 technology as described previously (Appledorn et al., 2008). Plasma cytokine levels are expressed as pg/ml of plasma. Cytokine levels in the cell culture supernatants were normalized to cellular protein levels and expressed as pg/μg of total cellular protein.

Bronchioalveolar lavage fluid (BALF)

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

Western blot analysis

Cells were lysed in lysis buffer (20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl) containing 1% Triton X-100 and protease inhibitors (Protease inhibitor cocktail, Roche Diagnostics). Insoluble material was removed by centrifugation (13,000 × g, 10 min., 4°C) and protein concentration of the supernatants determined by Bradford assay. Western blotting was performed as described previously (Loniewski et al., 2007; Patial et al., 2009).

Morphological assessment of liver injury

Livers from GRK2^{fl/fl} and GRK2^{Δmye} mice were fixed in 10% buffered formalin solution for histological examination. Tissue sectioning (5 micron) and staining with Hematoxylin and Eosin were performed at MSU Investigative Histopathology Lab. For glycogen staining, tissue sections were stained to demonstrate the periodic acid-schiff (PAS) reaction in which an intense red dye reacts with aldehyde groups on carbohydrate-rich macromolecules like glycogen, mucus, and basement membrane material. Subsequent treatment with diastase (an amylase) enzymatically dissolves glycogen, leaving a clear or non-staining area where the previously red PAS-positive glycogen had been detected in the cell cytoplasm. Mucus and other non-glycogen continue to stain intensely red after diastase treatment. All sections were blinded with regard to the genotype before microscopic analysis.

Survival study

Six to eight week old mice were injected with LPS (20 μg/gm body weight, i.p.) from Escherichia Coli (serotype 0111:B4; Sigma-Aldrich, St. Louis, MO). The mice were monitored for LPS induced lethality every 6 hours for a period of 48 hours. Differences in survival were analyzed using a Kaplan-Meier test (Prism 5 software, Graph Pad Software, La Jolla, CA).

Statistical analysis

All values are represented as mean ± SEM. Each “N” represents individual mouse. 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 and Analysis of Variance (ANOVA) with Bonferroni post-test was used to compare more than two groups. P value of less than 0.05 was considered significant.

Results

Generation of myeloid cell specific GRK2 deficient mice was accomplished by breeding mice expressing Cre recombinase under the control of the Lysozyme-M (LysM) promoter [LysM-Cre mice express Cre-recombinase specifically in myeloid cells (Clausen et al., 1999)] with mice homozygous for Lox-P flanked GRK2 alleles (GRK2^{fl/fl}) (Matkovich et al., 2006). Double heterozygous mice obtained from this breeding (GRK2^{fl/-} LysMCre^{+/-}) were further intercrossed to obtain GRK2^{fl/fl} LysMCre^{+/-} mice (myeloid -specific GRK2 deficient: GRK2^{Δmye}) and GRK2^{fl/fl} (control mice). A breeding colony was then maintained to generate both GRK2^{fl/fl} LysMCre^{+/-} as well as GRK2^{fl/fl} mice littermates needed for these studies. Using this strategy, peritoneal macrophages (Mφ), neutrophils and bone marrow derived macrophages (but not lungs and spleen homogenates) showed marked decrease in GRK2 protein (~95-99%) in GRK2^{Δmye} mice compared to GRK2^{fl/fl} mice (Fig 1).

LPS-induced MIP-1α, IL-12p40 and IL-10 are enhanced in GRK2^{Δmye} mice *in vivo*

To determine if GRK2 regulates TLR4 signaling *in vivo*, GRK2^{Δmye} and GRK2^{fl/fl} mice were injected with LPS (30 μg/g body weight, i.p., (Greten et al., 2007)) and levels of various cytokines/chemokines determined in the plasma using 23-plex cytokine assay. Of the various cytokines, plasma levels of MIP1α, IL-12(p40) and IL-10 were significantly enhanced in the GRK2^{Δmye} mice compared to GRK2^{fl/fl}. In particular, levels of MIP1α were markedly enhanced in the GRK2^{Δmye} mice at 1 and 3 hours post-LPS injection, whereas IL-12p40 and IL-10 levels were significantly elevated in the GRK2^{Δmye} mice at 12-hours post-LPS injection (Fig 2). Although levels of MIP1β, IL-6 and IL-17 were also enhanced in

GRK2^{Δmye} mice, it did not reach statistical significance. A similar trend was also observed for IL-1 α , IL-1 β , eotaxin, RANTES, MCP1 and IL-9 (data not shown). In contrast, plasma levels of IL-2, IL-3, IL-4, IL-5, IL-12(p70), IL-13, TNF α , IFN γ , GCSF, GMCSF and KC were not different between the two genotypes (data not shown). Taken together, these results suggest that myeloid cell GRK2 negatively regulates a group of inflammatory cytokines/chemokines *in vivo*.

Enhanced tissue injury and mortality in GRK2^{Δmye} mice

The profound increase in inflammatory factors elicited by LPS in mice is in part responsible for organ injury observed in this model (Rittirsch et al., 2007). To determine if the enhanced cytokine levels observed in the GRK2^{Δmye} mice also leads to exaggerated tissue injury, we focused on liver and lung after LPS injection.

Liver injury—To assess liver injury, we performed histopathological analysis of the liver samples from LPS-injected, GRK2^{fl/fl} and GRK2^{Δmye} mice. As predicted, the inflammatory response to LPS in the GRK2^{Δmye} mice was considerably enhanced as evidenced by a much more intense infiltration of inflammatory cells within the parenchyma and a prominence of portal areas due to focally marked perivascular edema with accumulation of fibrin strands. (Fig 3A-D). Furthermore, we also observed that LPS-induced glycogen depletion in the liver was markedly attenuated in the GRK2^{Δmye} mice compared to the GRK2^{fl/fl} mice (Fig 3E-J), suggesting that myeloid cell-specific GRK2^{Δmye} mice also have dysregulated glycogen metabolism.

Lung Injury—Acute lung injury in sepsis is associated with lung edema with extravasation of plasma proteins due to increased vascular permeability. Thus, detection of proteins in bronchoalveolar lavage (BAL) fluid serves as one useful indicator of lung injury (Duniho et al., 2002). GRK2^{fl/fl} and GRK2^{Δmye} mice were injected with LPS for 12 hours and total protein in bronchoalveolar lavage (BAL) fluid was determined. As shown in Fig. 3K there was a significant increase in protein content in GRK2^{Δmye} compared GRK2^{fl/fl} mice suggesting that the lung injury in response to LPS is worse in the GRK2^{Δmye} mice. Taken together, these results suggest that the inflammatory response and tissue injury to LPS develops much more excessively in myeloid cell deficient GRK2 mice.

Endotoxic mortality—Organ failure in response to endotoxemia eventually leads to mortality in mice. Because lung and liver injury were exaggerated in the GRK2^{Δmye} mice, we hypothesized that the mortality of GRK2^{Δmye} mice would be higher than the GRK2^{fl/fl} mice. As predicted, 50% of the GRK2^{Δmye} mice died within the first 24 hours of LPS injection compared to only 20% in the GRK2^{fl/fl} group. By 42 hours after LPS injection, 100% of GRK2^{Δmye} mice died whereas 30% of GRK2^{fl/fl} mice were alive by 48 hours (Fig. 3L). Thus, although not statistically significant, there was a trend for higher mortality of GRK2^{Δmye} mice compared to GRK2^{fl/fl} mice after LPS injection.

Enhanced LPS-induced cytokine/chemokine response in GRK2^{Δmye} macrophages and neutrophils

Results so far indicate that myeloid cell GRK2 limits LPS-induced cytokine levels as well as organ injury in mice. To further understand the cellular mechanisms of our *in vivo* findings, we isolated thioglycollate-elicited peritoneal M ϕ from GRK2^{fl/fl} and GRK2^{Δmye} mice and stimulated with LPS (1 μ g/ml) for various time points. As observed *in vivo*, LPS-induced cytokine/chemokine responses were significantly enhanced in primary M ϕ *in vitro* in the GRK2^{Δmye} mice. There was a markedly enhanced secretion of many inflammatory cytokines including IL-1 β , IL-1 α , IL-6, IL-9, IL-5, MIP1 α , KC, RANTES, GCSF and MCP-1 in the GRK2^{Δmye} mice M ϕ compared to GRK2^{fl/fl} cells (Fig 4A). LPS-induced

TNF α showed a biphasic response and was enhanced in GRK2 Δ mye only at the 6-hour time point (data not shown). In contrast to these cytokines, IL-2, IL-12p40, IFN γ and IL-17 did not differ between GRK2^{fl/fl} and GRK2 Δ mye M ϕ (data not shown). Interestingly, neutrophils from GRK2 Δ mye mice showed a significantly enhanced secretion of only G-CSF and KC (Fig. 4B). Other cytokines, such as IL-1 α , IL-1 β , IL-6, and MIP1 β showed a similar trend although not statistically significant. Taken together, these results suggest that GRK2 regulates TLR4-induced inflammatory cytokine production both *in vivo* and *in vitro* in a similar manner.

GRK2 negatively regulates NF- κ B1-p105-TPL2-MEK-ERK pathway in primary macrophages

To understand the mechanisms of the results described above, we tested the effect of LPS in GRK2 Δ mye and GRK2^{fl/fl} M ϕ on various signaling pathways including pERK1/2, pJNK1/2, pP38, pIkB α , pAkt and pGSK3 [pathways/proteins known to be regulated by GRK2, see Ref (Patial et al., 2009; Peregrin et al., 2006; Ribas et al., 2007)]. Interestingly, LPS-induced ERK1/2 phosphorylation (but not JNK, p38, IkB α , Akt or GSK3) was significantly enhanced in the GRK2 Δ mye M ϕ (Fig 5A-C).

To further elucidate the biochemical mechanisms by which GRK2 regulates the ERK pathway, we examined the upstream regulators of ERK phosphorylation in primary M ϕ . Previous studies have demonstrated that ERK activation in M ϕ is regulated via LPS-stimulated IKK β -NF κ B1 p105 pathway (Beinke et al., 2004; Cho et al., 2005; Loniewski et al., 2007; Waterfield et al., 2004). LPS stimulation of p105 phosphorylation (by IKK β) results in the ubiquitination and partial degradation of p105 that then releases TPL2 (a MAP3K, which is stoichiometrically bound to p105 under unstimulated conditions). Free TPL2 phosphorylates MEK1/2, which then activates ERK1/2. Note that since p105 is also bound to NF κ Bp50 under unstimulated conditions, partial degradation of p105 also releases p50 that then evokes changes in NF κ B-dependent gene transcription. We first confirmed the existence of IKK β -mediated ERK signaling pathway in peritoneal M ϕ . As shown in Fig 6, we observed that the pharmacological inhibition of IKK β [with BMS345541 @ 5 μ M, Ref (Burke et al., 2003)] inhibited ERK phosphorylation confirming the existence of IKK β -NF κ B1p105-ERK pathway. To investigate at what level GRK2 regulates this pathway, we examined the phosphorylation of MEK1/2 and p105 after LPS stimulation in GRK2^{fl/fl} and GRK2 Δ mye M ϕ . As shown in Fig 7, LPS-induced MEK1/2 and p105 phosphorylation were significantly enhanced in GRK2 Δ mye M ϕ compared to GRK2^{fl/fl} cells. These results suggest that GRK2 negatively regulates ERK pathway possibly at the level of p105.

Inhibition of p105-ERK pathway limits the exaggerated inflammatory response in GRK2 Δ mye macrophages

Knockout of GRK2 in primary macrophages results in the enhanced activation of p105-ERK pathway, which is associated with an enhanced inflammatory cytokine response observed in M ϕ as well as *in vivo* in mice. As noted before, activation of p105 not only stimulates the ERK pathway, it also activates p50-mediated NF κ B pathway. Thus to differentiate the downstream effects of IKK β -p105-NF κ Bp50 and IKK β -p105-ERK pathways and further to demonstrate that the enhanced cytokine responses observed in the GRK2 Δ mye cells are mediated via these pathways, we examined LPS-induced cytokine responses using pharmacological inhibitors of MEK-ERK (PD98059) (Davies et al., 2000) and IKK β (BMS345541) (Burke et al., 2003). We treated peritoneal M ϕ from GRK2^{fl/fl} and GRK2 Δ mye mice with LPS in the presence or absence of these two inhibitors. Inhibition of ERK significantly blocked the enhanced levels of (LPS-induced) IL-1 α , MCP1 and GCSF in the GRK2 Δ mye M ϕ and importantly, the levels of these factors returned to the levels observed in GRK2^{fl/fl} cells (treated with LPS) (Fig 8A). In addition, these cytokines were only modestly affected in the GRK2^{fl/fl} M ϕ by PD98059. These results demonstrate that the

enhanced secretion of IL-1 α , MCP1 and GCSF observed in GRK2 Δ mye cells is due to enhanced ERK activation. Furthermore, inhibition of IKK β (by BMS345541) significantly blocked LPS-induced IL-1 α and GCSF levels in the GRK2 Δ mye cells. Taken together these results suggest that these factors are likely regulated by GRK2 via the IKK β -p105-ERK pathway (Fig 8A).

Interestingly, inhibition of IKK β but not ERK blocked the enhanced levels of IL-1 β , KC, MIP1 α , RANTES, IL-5 and IL-6 observed in LPS treated GRK2 Δ mye cells (Fig. 8B). In the presence of the IKK β inhibitor, levels of these cytokines in the GRK2 Δ mye cells returned to levels observed in GRK2^{fl/fl} M ϕ , suggesting that this subset of cytokines is likely regulated by GRK2 exclusively via the IKK β -p105-p50 pathway. In contrast to these results, neither BMS345541 nor PD98059 inhibited IL-9 secretion in cells from both genotypes (Fig 8C) suggesting neither pathways being involved. Taken together, these data suggest that GRK2 regulation of the p105-ERK pathway negatively regulates IL-1 α , and GCSF. Furthermore, these results also suggest that regulation of the p105 (ERK-independent) pathway by GRK2 negatively modulates IL-1 β , KC, MIP1 α , RANTES, IL-5, and IL-6 in response to LPS stimulation (Fig 9).

DISCUSSION

It has been well documented that the expression levels of GRK2 are altered in specific cell types in human diseases including active relapsing-remitting multiple sclerosis (MS) or with secondary progressive MS (Vroon et al., 2005), Alzheimer's disease (Leosco et al., 2007), and rheumatoid arthritis (Lombardi et al., 1999). Related to these changes in GRK2 levels in humans, rodent models with decreased GRK2 levels exhibit altered disease pathogenesis ((Tarrant et al., 2008) (Vroon et al., 2005) (Nijboer et al., 2008). In addition to the human diseases outlined above, GRK2 levels are increased in immune cells from patients with sepsis. Consistent with this, TLR ligands enhance GRK2 levels in primary macrophages and neutrophils in culture (Alves-Filho et al., 2009; Loniewski et al., 2008). Therefore, we reasoned that GRK2 levels in myeloid cells might regulate the pathogenesis of endotoxemia and sepsis. Based on this reasoning, we generated myeloid cell-specific GRK2 knockout to determine the pathogenesis of endotoxemia in mice. Our results clearly suggest that GRK2 deficiency in the myeloid cells enhances liver and lung injury in response to high dose LPS injections.

In addition to liver injury, we also observed that glycogen metabolism is altered in the GRK2 Δ mye mice compared to the GRK2^{fl/fl} mice. Although the significance of this to liver injury is not clear, dysregulated glycogen and lipid metabolism are observed in human septic patients as well as in animal models of sepsis (Ali et al., 2008; Frazier et al., 2009; Hirshberg et al., 2008; Van den Berghe et al., 2006). Attenuated glycogen depletion suggests inadequate glycogenolysis in the GRK2 Δ mye mice during endotoxemia. Whether this is directly related to liver injury or the cytokine levels is not clear. However, sympathetic activation in endotoxemia plays a major role in liver carbohydrate metabolism and therefore it is possible that the adrenergic signals that regulate glycogenolysis might be dysregulated in the GRK2 Δ mye mice (Goldstein and Elwyn, 1989).

Migration of immune cells to the inflammatory sites although important for host defense, is also in part responsible for tissue damage observed in sepsis. MIP1 α , MIP1 β , MCP1, Rantes and eotaxin are members of CC chemokine family, which serve as major chemoattractants for neutrophils, mononuclear cells as well as eosinophils and therefore contribute to tissue injury observed in sepsis (Baggiolini, 1998; Jose et al., 1994; Kapp et al., 1994; Standiford et al., 1995). In this study the levels of MIP1 α and MCP1 were significantly increased in the M ϕ culture supernatants of GRK2 Δ mye mice, and interestingly MIP1 α was also elevated *in*

in vivo (in response to LPS) in the GRK2^{Δmye} mice. MIP1 α is produced by various immune cells including M ϕ and plays a crucial role in activation and chemotaxis of several populations of M ϕ . It also stimulates the proliferation of mature tissue M ϕ and has been shown to induce the secretion of TNF α , IL-6 and IL-1 α from peritoneal M ϕ (Fahey et al., 1992). Consistent with the previously reported role for MIP1 α in lung injury (Standiford et al., 1995; Standiford et al., 1993), plasma MIP1 α level was enhanced early on in the GRK2^{Δmye} mice and was associated with enhanced lung capillary damage in the GRK2^{Δmye} mice. Together these results suggest a possible role for MIP1 α in causing exaggerated tissue injury in the GRK2^{Δmye} mice. In other studies Fan and Malik (Fan and Malik, 2003) have proposed that MIP-2-CXCR2-mediated neutrophil migration is negatively regulated by GRK2, such that decrease in GRK2 levels enhances neutrophil migration. In addition, TLR4 activation was shown to enhance CXCR2-induced neutrophil migration by downregulating GRK2 levels. In the *in vivo* context, these results would suggest that reduced GRK2 levels may result in enhanced neutrophil infiltration into the organs, and therefore cause exaggerated organ damage, consistent with the results presented here.

Our studies show that in addition to MIP1 α LPS-induced IL-12p40 and IL10 are enhanced *in vivo* in the GRK2^{Δmye} mice. IL12p40, through its role in forming functional IL12, is an important mediator of cell-mediated immunity and plays an important role in inflammatory diseases including experimental autoimmune encephalitis and sepsis (Adorini, 1999; Becher et al., 2002). IL-10 is an important anti-inflammatory cytokine that prevents an over-exuberant immune response, leading to immune-suppression shifting the balance toward lethal consequences. Based on the present data, it is not clear which of the specific cytokines/chemokines regulated in the plasma or in the macrophages/neutrophils specifically contribute to the exaggerated tissue injury observed in GRK2^{Δmye} mice.

Results from signaling experiments suggest that GRK2 acts at the level of NF κ B1p105. Previous studies have shown that GRK2 and NF κ B1 p105 interact in yeast two-hybrid assays (<http://www.signaling-gateway.org/data/Y2H/cgi-bin/y2h.cgi>). Furthermore, we have shown that the RH domain of GRK2 directly interacts with the carboxy terminus of p105 in direct interaction assays, although we did not find any functional significance of that interaction in Raw264.7 macrophage cell line using RNAi (Parameswaran et al., 2006). Previous studies also demonstrated that NF- κ B1 p105 is a poor substrate for GRK2, suggesting that the regulation might be phosphorylation-independent (Parameswaran et al., 2006). This is not entirely surprising given the role of the RH domain of GRKs in mediating phosphorylation-independent cell signaling, potentially as a scaffolding protein (Ribas et al., 2007). Future studies will determine whether direct interaction of GRK2 with p105 might block IKK β phosphorylation of p105 thus preventing p105 degradation and TPL2 release (Beinke et al., 2004; Waterfield et al., 2004). Together, results from primary M ϕ demonstrate that GRK2 is a negative regulator of IKK β -induced p105-ERK pathway.

Other studies have shown that GRK2 can regulate a number of different signaling pathways depending on the cellular context and receptor involved. Studies on the role of GRK2 in the regulation of ERK pathway however, have shown conflicting results (Jimenez-Sainz et al., 2006) (Kleibeuker et al., 2008). While these studies have focused on chemokine receptor (a GPCR)-induced ERK activation, our studies address the role of GRK2 in TLR4 signaling. Thus although GRK2 has very important roles in GPCR signaling, we demonstrate that GRK2 is equally important in regulating TLR4-induced p105 and ERK activation and the consequent cytokine/chemokine production. In contrast to the results presented here on p38, Peregrin *et al* showed that GRK2 can interact with and phosphorylate p38 MAPK and this can lead to the inactivation of p38 MAPK (Peregrin et al., 2006). In addition, Nijboer *et al* (Nijboer et al., 2009), demonstrated that ischemic injury in the brain is regulated by GRK2 via p38 MAPK. Although the reason for the differences in our results compared to the other

studies is not clear, one possible reason may be due to the different mouse models and cell types used.

In summary, mice with a deficiency of GRK2 in myeloid cells are more susceptible to LPS-mediated endotoxemia and this is associated with enhanced secretion of several cytokines/chemokines. We further demonstrate that the increased cytokine/chemokine production in the GRK2^{Δmye} Mφ is related to enhanced activation of the NFκB1p105-ERK pathways. We propose that the increase in GRK2 levels observed in immune cells under septic conditions might function as a “negative feedback regulator” of TLR4 signaling and therefore function to limit the progression of sepsis (Fig 9).

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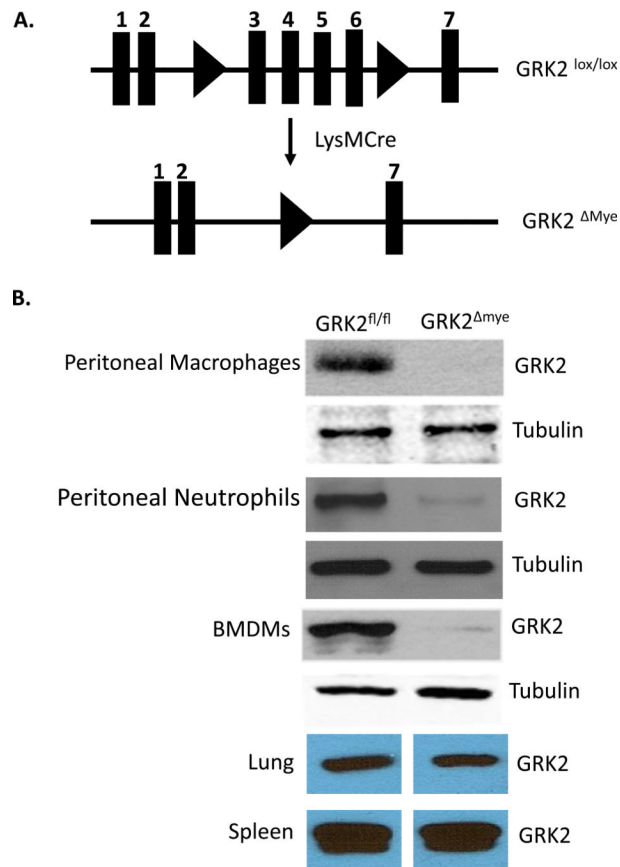


Figure 1. Generation and characterization of myeloid-cell specific GRK2 knockout

A. Schematic representation of the presence of loxP sites surrounding exons 3-6 that were targeted for deletion with Cre recombinase. Cre mediated recombination leads to the deletion of exons 3-6 of GRK2 gene.

B. Western blot showing the GRK2 protein levels in thioglycollate elicited macrophages, neutrophils and bone-marrow derived macrophages, lungs and spleen from GRK2^{ΔMye} and littermate GRK2^{fl/fl} mice. Tubulin is shown as a loading control.

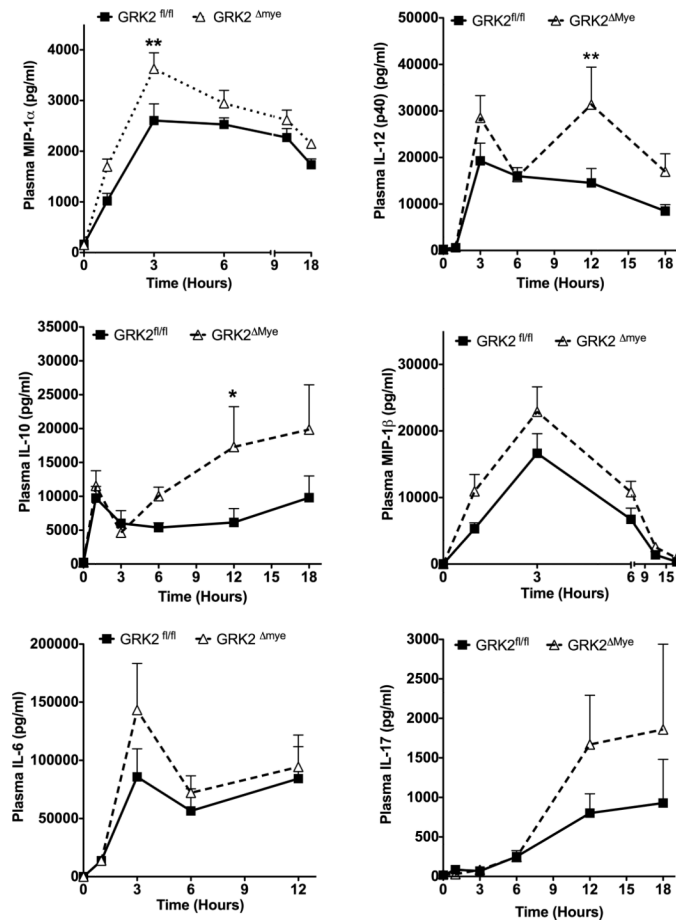


Figure 2. Enhanced plasma inflammatory cytokines in GRK2^{ΔMye} mice

Mice were injected with LPS and blood collected at the indicated time points. Levels of cytokines and chemokines were assessed using a Biorad 23-plex. N=11 mice per genotype each for 1, 3 and 12 hour time point; N=6 mice per genotype each for 0, 6 and 18 hour time points. *p<0.05; **p<0.01.

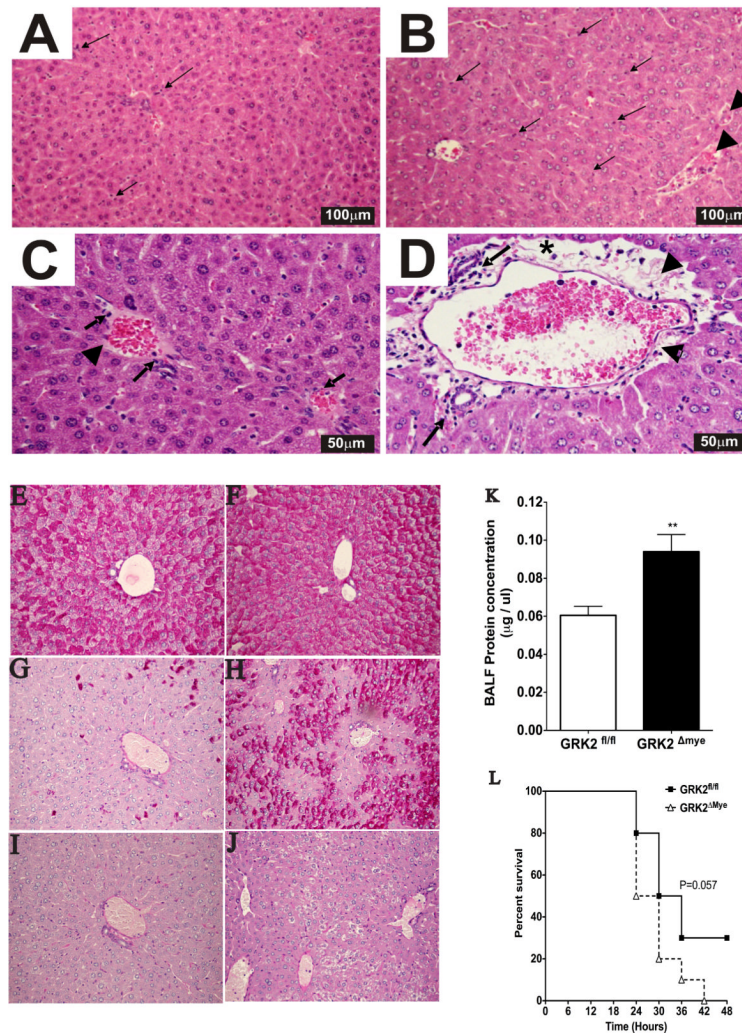


Figure 3. Enhanced tissue injury and mortality in GRK2^{Δmye} mice

A-D. Morphological changes in liver of mice (GRK2^{fl/fl} and GRK2^{Δmye}) injected with LPS (12 hours). **A.** GRK2^{fl/fl} mice. **B.** GRK2^{Δmye} mice. Arrows show examples of increased parenchymal immune cells, arrowheads show fibrin in venous spaces. (H&E, original magnification $\times 10$). **C.** GRK2^{fl/fl} mice liver. Immune cells and minimal edema present in periportal areas. **D.** GRK2^{Δmye} mice liver. Numerous immune cells; star shows edema; arrowhead shows fibrin strands in prominent periportal areas (H&E, original magnification, $\times 20$).

E-J. PAS reaction staining (for glycogen) of mouse liver section from PBS injected GRK2^{fl/fl} (E), PBS injected GRK2^{Δmye} (F), LPS injected GRK2^{fl/fl} (G), LPS-injected GRK2^{Δmye} mice (H). Specificity of cytoplasmic glycogen staining (after diastase treatment) is demonstrated in I and J, by loss of intense red staining in cell cytoplasm due to dissolution of glycogen by enzyme. Focal clusters of glycogen-rich cells show cytoplasmic clearing after diastase treatment in J but rare individual cells show clearing in I.

K. Mice were injected with LPS and broncho-alveolar lavage fluid collected after 12 hours. The cells were pelleted and the total protein content of the supernatant measured using Bradford assay (N=6 mice per genotype)

L. Survival of GRK2^{Δmye} and GRK2^{fl/fl} mice after LPS injection. N=10 mice per genotype.

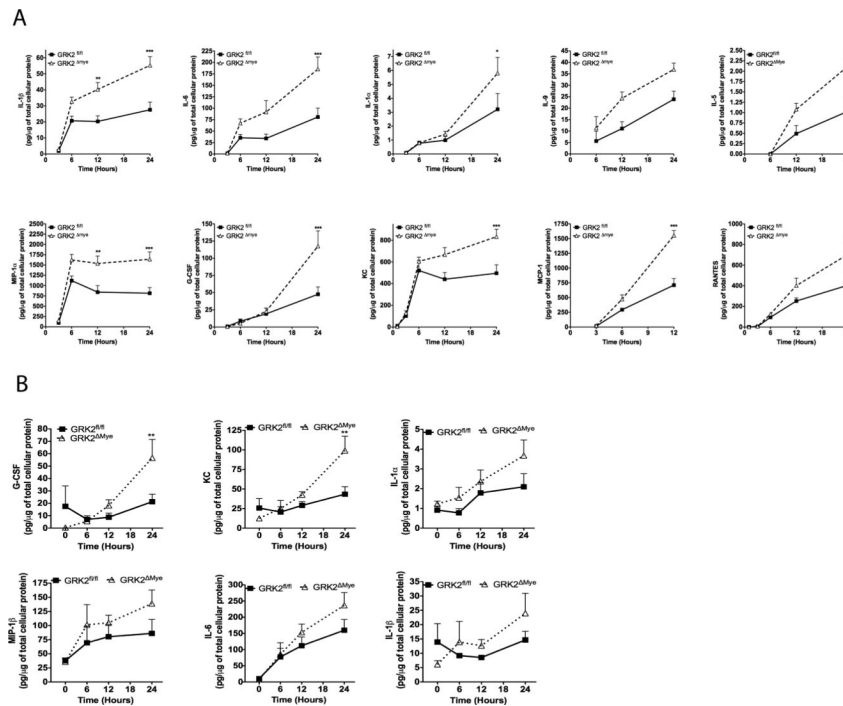


Figure 4. LPS-induced inflammatory cytokine is enhanced in GRK2^{ΔMye} macrophages and neutrophils

A. Thioglycollate-elicited peritoneal M ϕ were stimulated with LPS (1 μ g/ml) for the indicated time points. Levels of inflammatory mediators in culture supernatants were determined using Biorad-23 plex assay. Levels of cytokines and chemokines were normalized to the total cellular protein content and expressed as pg/ μ g of total cellular protein. * p <0.05; ** p <0.01; *** p <0.001. N=5 mice per genotype.

B. Thioglycollate-elicited neutrophils (collected and purified using MACS column as described before (Greten et al., 2007) were stimulated with LPS (1 μ g/ml) in 12-well cell culture plates and cell culture supernatants collected at the indicated time points. Levels of inflammatory mediators were determined using Biorad-23 plex assay and normalized to the total cellular protein and expressed as pg/ μ g of total cellular protein. ** p <0.01. N=5 mice per genotype.

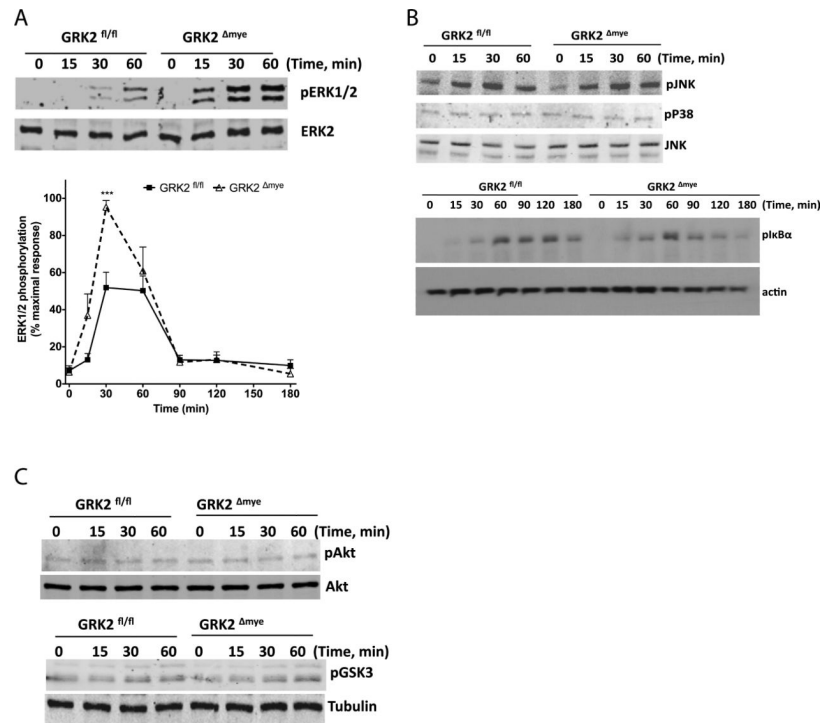


Figure 5. LPS-induced phosphorylation of ERK1/2 is selectively enhanced in GRK2^{Δmye} macrophages

GRK2^{Δmye} and GRK2^{fl/fl} Mφ were stimulated with LPS (1 μg/ml) for the indicated time points and phosphorylation of ERK1/2, JNK, p38, IκBα, Akt and GSK3 determined by western blotting. Representative blots and quantitation for p-ERK and ERK are shown in (A). Representative blots for p-p38, p-JNK, and p-IκBα, are shown in (B) along with JNK/actin for loading control. Representative blots for pAkt and p-GSK3 along with loading controls (Akt and tubulin) are shown in (C). *p<0.05 and ***p<0.001 compared to GRK2^{fl/fl}. N=6-7 mice for each genotype.

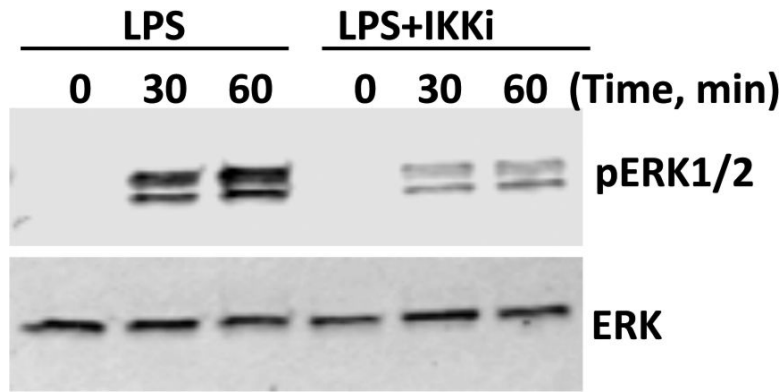


Figure 6. Existence of IKK β -ERK pathway in peritoneal macrophages

Peritoneal macrophages from GRK2^{fl/fl} mice were stimulated with either LPS alone or pretreated with BMS345541 (5 μ M, 30 minutes prior to stimulation with LPS (1 μ g/ml) for various time points as shown and immunoblotting performed as described in the methods. A representative blot from 4 such experiments is shown.

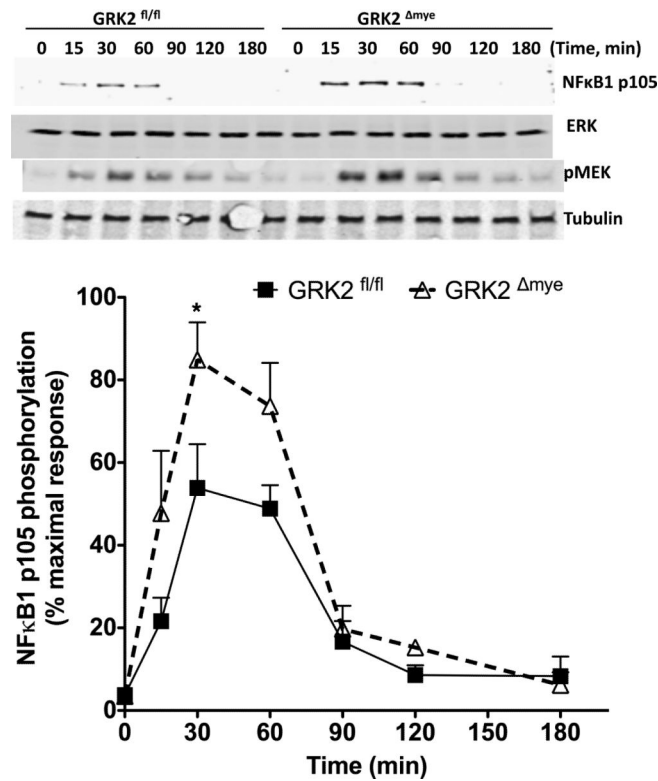


Figure 7. LPS-induced NFκB1p105-MEK pathway is enhanced in GRK2^{Δmye} macrophages GRK2^{Δmye} and GRK2^{fl/fl} Mφ were stimulated with LPS (1 μg/ml) for the indicated time points and phosphorylation of NFκB1p105 and MEK1/2 determined by western blotting. Representative blots (for pMEK and pP105) are shown in the top and quantitation for pP105 is shown in the bottom (N=7 mice for each genotype) *P<0.05 compared to GRK2^{fl/fl}.

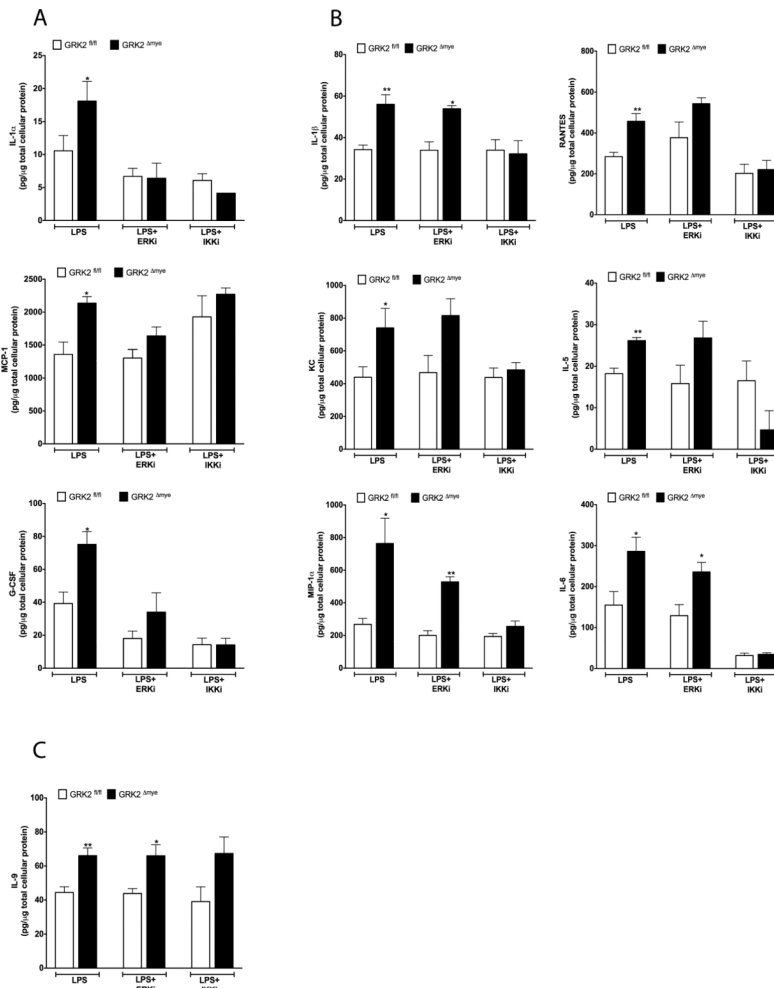


Figure 8. Inhibition of ERK and IKK β blocks the enhanced cytokine levels in GRK2 Δ Mye macrophages

Peritoneal M ϕ were stimulated or not with LPS along with PD98059 (10 μ M) or BMS345541 (5 μ M) and the cell culture supernatants collected 24 hours later. Levels of cytokines and chemokines were assessed using Biorad 23-plex assay. Cytokines blocked by PD98059 are shown in (A), cytokines blocked primarily by BMS345541 are shown in (B), and IL-9 is shown in (C). ERKi=PD98059; IKKi=BMS345541; N=5 mice per genotype each * p <0.05; ** p <0.01, compared to corresponding GRK2^{fl/fl}. PD98059 or BMS345541 alone did not affect secretion of any of the cytokine/chemokine tested (data not shown).

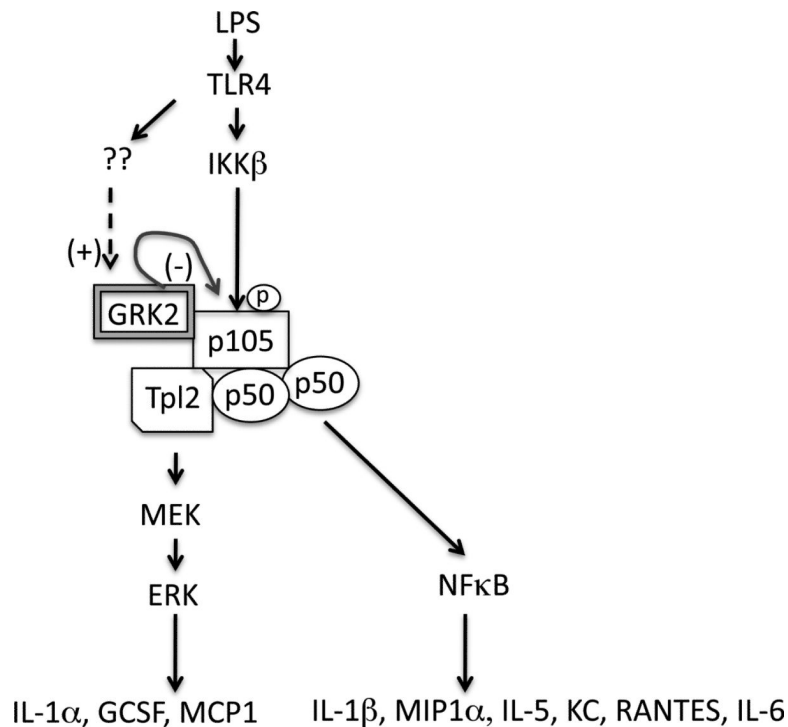


Figure 9. Proposed model of GRK2 regulation of TLR4-induced inflammatory response
 TLR4 activates ERK pathway via IKKβ-mediated phosphorylation of p105. Based on the results presented here, we propose that GRK2 negatively regulates IKKβ-induced p105 phosphorylation and therefore the ERK pathway. Because p105 phosphorylation is also essential for p50 mediated NFκB activation, negative regulation of p105 by GRK2 also modulates a subset of cytokines that are regulated by this pathway. Note that there are two exceptions: 1. Because MCP-1 was inhibited by ERK inhibitor but not by the IKKβ inhibitor, it is possible that MCP-1 is being regulated by GRK2 in an ERK-dependent but p105-independent mechanism. 2. Also, because MIP1α was modestly inhibited by PD98059, it is possible that a small component of the ERK pathway may also regulate MIP1α. Previous studies in primary macrophages and neutrophils have demonstrated that TLR4 activation increases the expression of GRK2 significantly and thus GRK2 might act as a negative feedback regulator for TLR4 signaling.