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G-protein coupled receptor kinase 5 mediates Lipopolysaccharide-induced NFκB activation in primary macrophages and modulates inflammation *in vivo* in mice

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Abstract

G-protein coupled receptor kinase-5 (GRK5) is a serine/threonine kinase discovered for its role in the regulation of G-protein coupled receptor signaling. Recent studies have shown that GRK5 is also an important regulator of signaling pathways stimulated by non-GPCRs. This study was undertaken to determine the physiological role of GRK5 in Toll-like receptor-4-induced inflammatory signaling pathways *in vivo* and *in vitro*. Using mice genetically deficient in GRK5 ($GRK5^{-/-}$) we demonstrate here that GRK5 is an important positive regulator of lipopolysaccharide (LPS, a TLR4 agonist)-induced inflammatory cytokine and chemokine production *in vivo*. Consistent with this role, LPS-induced neutrophil infiltration in the lungs (assessed by myeloperoxidase activity) was markedly attenuated in the $GRK5^{-/-}$ mice compared to the $GRK5^{+/+}$ mice. Similar to the *in vivo studies*, primary macrophages from $GRK5^{-/-}$ mice showed attenuated cytokine production in response to LPS. Our results also identify TLR4-induced NFκB pathway in macrophages to be selectively regulated by GRK5. LPS-induced IκBα phosphorylation, NFκB p65 nuclear translocation and NFκB binding were markedly attenuated in $GRK5^{-/-}$ macrophages. Together, our findings demonstrate that GRK5 is a positive regulator of TLR4-induced IκBα-NFκB pathway as well as a key modulator of lipopolysaccharide-induced inflammatory response.

Introduction

G-protein coupled receptor kinases (GRKs) are serine/threonine protein kinases best known for their role in phosphorylation and desensitization of G-protein coupled receptors (Johnson

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et al., 2004). GRK family of kinases is subdivided into three main groups on the basis of sequence homology: rhodopsin kinase (GRK1 and GRK7), β-adrenergic receptor kinase (GRK2 and GRK3), and GRK4 kinase (GRK4, GRK5 and GRK6) subfamilies. Although these seven members share certain characteristic features, they are distinct enzymes with specific properties. GRK5 is a well-characterized member of the GRK4 subfamily and is expressed ubiquitously in all mammalian tissues. Although originally described as membrane-associated proteins, recent studies have shown that GRK4 family of kinases have a "nuclear localization signal" in their sequence and indeed have been localized in the nucleus (Gainetdinov et al., 1999; Johnson et al., 2004; Martini et al., 2008). Apart from their role in receptor desensitization, GRKs can also perform other cell signaling functions by interacting with and phosphorylating non-receptor substrates such as tubulin, synucleins, HDACs as well as members of the IκB family including IκBα and p105 (Haga et al., 1998; Martini et al., 2008; Parameswaran et al., 2006; Patial et al., 2010a; Pronin et al., 2000).

IκBα and p105 are members of the IκB family of inhibitory proteins that "hold" NFκB transcription factors (RelA(p65), NFκBp50, NFκBp52, RelB, c-Rel) in the cytosol under unstimulated conditions (Vallabhapurapu and Karin, 2009). Upon stimulation, the canonical NFκB signaling pathway is initiated, by activation of IκB kinase (IKK) complex, comprising of IKKα, β and NEMO. Activated IKKβ phosphorylates IκBα as well as other IκBs including p105. Phosphorylated IκB then undergoes ubiquitination and proteasomal degradation releasing the NF-κB transcription factors which then translocate to the nucleus and bind onto their cognate DNA binding sites thereby initiating transcription of a wide array of genes including those of cytokines and chemokines (Vallabhapurapu and Karin, 2009). Our studies as well as that of others have shown that GRK5 can regulate NFκB signaling pathway in cell lines in response to inducers such as TNFα and lipopolysaccharide (LPS) (Parameswaran et al., 2006; Patial et al., 2010a; Sorriento et al., 2008). However, the functional outcome of this regulation to inflammation is not clear.

LPS from gram negative bacteria is a ligand for Toll-like receptor-4 (TLR4) and recent studies have implicated TLR4 in a wide variety of inflammatory diseases including endotoxemia in human and animal models (Beutler, 2009). Stimulation of TLR4 by LPS triggers the recruitment of adapter proteins such as TRIF and Myd88 as well as other TIR domain containing proteins that subsequently activate the IKK complex and NFκB signaling (O'Neill and Bowie, 2007). Although NFκB activation by TLR4 is essential for regulating both innate and adaptive immune responses, constitutive activation of NFκB is often associated with several inflammatory diseases such as sepsis, rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, and asthma (Wong and Tergaonkar, 2009). Because of this role in inflammatory diseases, IKKβ has been targeted for drug development, but has faced some pitfalls. Inhibition of IKKβ not only prevents the deleterious effects of NFκB pathway, it also blocks the protective effects of NFκB signaling, leading to harmful outcomes (Baud and Karin, 2009; Greten et al., 2007). Thus, further understanding of the regulators of the NFκB pathway is highly essential.

Even though previous studies have shown that GRK5 is an important regulator of NFκB signaling, these studies were done primarily using cell lines and *in vitro* approaches and have been controversial in terms of NFκB activation (Parameswaran et al., 2006; Patial et al., 2010a; Sorriento et al., 2008). Therefore, in this study we set out to understand the physiological role of GRK5 in TLR4 signaling and inflammation using GRK5 knockout mice. Our results demonstrate that GRK5 is a crucial regulator of TLR4-induced inflammation *in vivo* and *in vitro* and suggest that inhibition of GRK5 could be a potential strategy in treating TLR4-mediated inflammatory diseases.

Materials and methods

Materials

Protease inhibitor cocktail tablets were from Roche Applied Science (Indianapolis, IN 46250, USA); pERK, pP38, pJNK, JNK, pNFκB1p105, and pIκBα antibodies were from Cell Signaling Technology, Inc. (Danvers, MA 01923, USA). ERK2, and NFκB1p105 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA 95060, USA). Tubulin antibody was from Sigma (St. Louis, MO 63103, USA). Monoclonal GRK5 antibody was from Millipore (Billerica, MA 01821, USA). E. coli LPS (0111:B4) was from Sigma (St. Louis, MO 63103, USA) and ultra pure LPS from Invivogen (San Diego, CA 92121, USA).

Animals

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

Peritoneal Macrophage isolation

To isolate peritoneal macrophages, mice were injected (intraperitoneal) with 1ml of 4% thioglycollate. Peritoneal macrophages were collected as described before (Loniewski et al., 2008). Cells were washed at least three times and plated on cell culture plates in RPMI 1640 media supplemented with 10% fetal bovine serum and penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37° C in 5% CO_2. After plating, cells were serum starved for ~ 3–4 hours and stimulated with LPS (1 μg/ml) for the indicated time points.

Cytokine analysis

A mouse 23-plex multiplex based assay (from Biorad) 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 from mice and culture supernatants from peritoneal macrophages (treated with LPS for various time points) were used to assess the cytokine/chemokine levels. Levels in the cell culture supernatants were normalized to the total cellular protein and expressed as pg/μg of total cellular protein. TNFα levels in tissues (after homogenization) were analyzed using ELISA kit from eBioscience, Inc. (San Diego, CA 92121, USA) as described before (Porter et al., 2010).

RNA isolation and PCR analysis of GRK5

RNA extraction and RT-PCR analysis were performed as described before (Porter et al., 2010). The primers used for GRK5 were as follows: Forward: 5′-AAACACTTTCCGGCAGTACC-3′ and, Reverse: 5′-GGCCATAATCATCCAGCAAG-3′ (corresponding respectively to 549–568 and 951–970 of GRK5 mRNA sequence).

Western blot analysis

Cell lysates were prepared and Western blotting performed as described previously (Loniewski et al., 2007; Patial et al., 2010a). Briefly, equal amounts of protein were run on polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were

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

Microscopy for NFκB p65 translocation

Cells grown on cover slips (coated with polyornithine) overnight, treated or not with LPS and were fixed and permeabilized as described before (Bomberger et al., 2005). Permeabilized cells were rinsed 3 times with PBS and incubated in blocking solution (1% bovine serum albumin in PBS) for 30 min at room temperature. NFκB p65 was detected using a rabbit polyclonal anti-p65 antibody (Santa Cruz Biotechnology) and a secondary goat anti-rabbit antibody conjugated to Alexa Fluor® 488 (Invitrogen). The cover slips were rinsed in PBS and mounted on the slide using Shandon mounting medium. Images were obtained using fluorescence microscope (DM1RB; Leica, Nussloch, Germany) with computer image-capture capability. For quantitation of p65 translocation, a minimum of 20 cells per field and more than 10 fields per condition were counted.

Nuclear extraction and Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared (Patial et al., 2010a) and incubated with ~9 fmol of double-stranded oligonucleotide probes (corresponding to the consensus NF-κB sequence end-labeled with IRDye® 700 (Li-cor Odyssey)) and binding reactions performed as described before (Patial et al., 2010a). Samples were then subjected to electrophoresis using 6% (w/v) non-denaturing polyacrylamide gels and then analyzed using Odyssey imaging system (LI-COR Biosciences).

Myeloperoxidase activity

Tissue myeloperoxidase (MPO) activity was performed as described before (Graff et al., 1998). Briefly, frozen tissues were homogenized in 50 mM potassium phosphate (pH 6.0) buffer. After centrifugation at 13,000 rpm for 10 min at 4°C, the pellets were incubated in 50 mM potassium phosphate (pH 6.0) buffer containing 0.5% hexadecyltrimethylammonium bromide. Samples were then centrifuged at 8000 rpm for 2 minutes. An aliquot of the supernatant was incubated at 25°C in 50 mM potassium phosphate (pH 6.0) buffer containing 0.0005% H2O2 and 167 μg/ml o-dianisidinehydrochloride. MPO activity was determined spectrophotometrically by measuring the change in absorbance at 450 nm over time using a 96-well plate reader as described before (Graff et al., 1998).

Surface expression of TLR4 by flow cytometry

Bone marrows from mice were flushed from tibiae and femurs and depleted of red blood cells with ammonium chloride. The cells were rinsed with staining buffer (1% FBS and 0.09% sodium azide in PBS) and stained with the following antibodies from BD Biosciences: anti-CD11b-FITC, anti-TLR4-PE, anti-CD3-PerCp-Cy5.5, and anti-CD19-PE-Cy7. Data were acquired using a LSRII (BD Biosciences) and analyzed using Flowjo software (Tree Star) as described before (Porter et al., 2010).

Statistical analysis

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

Results

Generation of GRK5$^{-/-}$ mice

GRK5 mutant mice mice were generated by Deltagen (on NIH-based contract) and deposited to Jackson labs. Initial phenotypic characterization by Deltagen did not reveal any significant changes in phenotype, under basal conditions. However, homozygous mice exhibited a possible increase in pain threshold, as observed by an increase in the thermal response latency during hot plate testing (http://jaxmice.jax.org/strain/005808.html). Generation of a different GRK5 mutant mice has been described before, which was also shown to have augmented and prolonged analgesic effects to muscarinic receptor stimulation compared to the wild type mice (Gainetdinov et al., 1999). The targeted sequences, however, are different between the two GRK5 mutant mice generated. In the GRK5 mutant mice described here, part of the exon 3 of GRK5 was deleted and replaced with β-galactosidase under the control of the endogenous promoter of GRK5 (Fig 1A). As shown in Fig 1A, these mice are not expected to express the N-terminal RH domain (encoded by amino acids 50–176, (Sorriento et al., 2008)). In contrast, in the GRK5 knockout mice generated by Gainetdinov et al (Gainetdinov et al., 1999), exons 7 and 8 were deleted such that exons 1–6 were still intact and therefore could potentially produce a transcript encoding the N-terminal RH domain.

Expression analysis of β-Gal in the GRK5 mutant mice is provided elsewhere (http://www.informatics.jax.org/javawi2/servlet/WIFetch?page=alleleDetail&key=40140-geneExpression). Heterozygous mice (GRK5$^{+/-}$) obtained from Jackson labs were mated to generate GRK5$^{-/-}$ and GRK5$^{+/+}$ mice and in general followed Mendelian inheritance (Fig 1B). Analysis of GRK5 mRNA expression in various organs confirmed that GRK5 transcript is absent in the tested organs (Fig 1C). Furthermore, we have also confirmed the absence of GRK5 protein by Western blotting as shown in Fig 1D.

Role of GRK5 in TLR4 signaling *in vivo*

To determine the *in vivo* role of GRK5 in TLR4 signaling in mice, we tested the effect of LPS in GRK5$^{+/+}$ and GRK$^{-/-}$ mice and examined the plasma levels of various cytokines and chemokines. We injected LPS (30 μg/g body weight, intraperitoneal) for 1, 3 and 12 hours, and collected plasma samples and determined plasma cytokine/chemokine levels using a 23-cytokine multiplex assay. LPS increased the levels of all the cytokines/chemokines assayed in the GRK5$^{+/+}$ mice. However, in the GRK5$^{-/-}$ mice, levels of IFNγ, IL12p40, IL12p70, IL-5, IL-17, TNFα, IL-1β, IL-2, IL-3, IL-4, IL-13, IL-10, MCP1, Eotaxin, and RANTES, were significantly inhibited (Fig 2A). Interestingly, levels of several of these factors were markedly attenuated at the later time point (12 hours), suggesting that the initial release of these cytokines is not affected. TNFα levels in the plasma however, peaked at 1 hour post-injection in GRK5$^{+/+}$ mice and its levels were significantly inhibited in GRK5$^{-/-}$ mice at this time point. Importantly, some cytokines/chemokines including GCSF, GM-CSF, IL-1α, IL-6, KC, MIP1α, MIP1β were not affected by deficiency of GRK5 (Fig 2B), suggesting selective regulation of TLR4 signaling by GRK5.

Surface TLR4 expression in GRK5$^{-/-}$ mice

To rule out the possibility that GRK5 may regulate TLR4 cell surface expression, we analyzed CD11^{b+} cells from the bone marrow for cell surface expression of TLR4 using flow cytometry. As shown in Fig 3, surface expression of TLR4 did not differ significantly between the two genotypes, suggesting that the inhibition of cytokine/chemokine levels in the GRK5$^{-/-}$ mice is not due to a decrease in TLR4 expression.

Diminished neutrophil infiltration in GRK5$^{-/-}$ mice

Because factors that regulate neutrophil chemotaxis [such as MCP-1, RANTES, IL-17 (Ferretti et al., 2003; Pan et al., 2000; Ramnath et al., 2008)] were markedly attenuated in GRK5$^{-/-}$ mice, we hypothesized that neutrophil infiltration into lung and liver would be diminished in the GRK5$^{-/-}$ mice after LPS injection. To test this, we determined myeloperoxidase (MPO) activity as a marker for neutrophil (and macrophage) infiltration in lung and liver after various time points of LPS injection in GRK5$^{+/+}$ and GRK5$^{-/-}$ mice. As shown in Fig 4A, MPO activity increased markedly in the lung from GRK5$^{+/+}$ mice, as early as 3 hours after LPS injection, and decreased significantly at later time points. Interestingly, consistent with the decreased cytokine/chemokine levels in the GRK5$^{-/-}$ mice, MPO activity in the lung was markedly blocked, compared to the GRK5$^{+/+}$ mice. This effect was not observed in the liver (Fig 4B), suggesting that there is organ-specific regulation of neutrophil infiltration by GRK5 after LPS injection. Furthermore, there were no differences in the number of neutrophils, macrophages or dendritic cells in the spleen of GRK5$^{+/+}$ and GRK5$^{-/-}$ mice (Fig 4C), suggesting that the decrease in neutrophil infiltration in the lung is not because of lack of neutrophils (or other immune cell types) in the knockouts.

To further determine if endotoxic mortality is affected by lack of GRK5, we tested the effect of intraperitoneal injection of LPS on the survival of GRK5$^{+/+}$ and GRK5$^{-/-}$ mice. In contrast to plasma cytokine data as well as lung MPO assay, mortality was not significantly different between the two genotypes (Fig 4D). Together, these results suggest that even though LPS-induced plasma cytokine/chemokines levels and lung neutrophil infiltration are regulated by GRK5, endotoxic mortality is not affected by GRK5 deficiency.

LPS-induced cytokine production is inhibited in GRK5$^{-/-}$ macrophages

To further understand the mechanistic basis by which GRK5 regulates TLR4 signaling, we examined the effect of LPS on cytokine production in primary peritoneal macrophages from GRK5$^{+/+}$ and GRK5$^{-/-}$ mice. Thioglycollate-elicited peritoneal macrophages from GRK5$^{+/+}$ and GRK5$^{-/-}$ mice were treated with LPS (1 μg/ml) for various time points and cell culture supernatants assayed for different cytokines and chemokines using a 23-cytokine multiplex assay. Interestingly, several cytokines including IFNγ, IL-12p40, IL-12p70, IL-2, IL-3, IL-5, IL-4 and IL-17 as well as chemokines KC, GM-CSF, MCP-1 and Eotaxin (Fig 5A and C) were significantly inhibited in the GRK5$^{-/-}$ compared to GRK5$^{+/+}$ macrophages. Even though there was a broad inhibition of several cytokines, some that were regulated by GRK5 *in vivo* were not modulated significantly in the peritoneal macrophages *in vitro*. These include TNFα, IL1β, IL-10, IL-9, IL-13, and RANTES (Fig 5B). Similar to the plasma, however, levels of IL-6, IL1α, GCSF, MIP1α, and MIP1β were not significantly inhibited in the GRK5$^{-/-}$macrophages (Fig 5D).

Even though GRK5 did not regulate LPS-induced TNFα production in peritoneal macrophages *in vitro*, TNFα level in the liver and lung of LPS-treated mice was indeed attenuated in the GRK5$^{-/-}$ mice compared to the GRK5$^{+/+}$ mice. As shown in Fig 6, and consistent with the plasma TNFα levels, LPS-induced TNFα in the liver and lung was significantly inhibited in the GRK5$^{-/-}$ mice. These results suggest that the profile of cytokines regulated by GRK5 in organs *in vivo* may vary from peritoneal macrophages *in vitro*. Taken together, however, our results suggest that GRK5 is an important regulator of TLR4-induced inflammatory cytokine production in macrophages.

Decreased NFκB activation in GRK5$^{-/-}$ macrophages

To begin to understand the signaling mechanisms by which GRK5 regulates TLR4-induced cytokine production in macrophages, we tested the effect of LPS (in macrophages from GRK5$^{+/+}$ and GRK5$^{-/-}$ mice) on various signaling pathways including ERK, JNK, P38 and

IκBα. Previous studies have shown that ERK activation in macrophages is dependent on NFκB1 p105 phosphorylation and that GRK5 negatively regulates p105-ERK pathway in Raw264.7 macrophage cell line (Parameswaran et al., 2006). In the present study, LPS-induced ERK and p105 phosphorylation in primary macrophages were modestly enhanced in the $GRK5^{-/-}$ macrophages, but did not reach statistical significance (Fig 7A and B). In addition, phospho-JNK and phospho-p38 levels did not differ significantly between the $GRK5^{+/+}$ and $GRK5^{-/-}$ cells (Fig 7C and D). These results suggest that the effect of GRK5 on ERK, JNK or p38 does not likely explain the attenuated inflammatory effects of LPS in $GRK5^{-/-}$ cells.

In addition to MAPK regulation of inflammatory pathways, LPS-induced inflammatory response is also mediated in large part by the IκBα-NFκB pathway (Pasparakis, 2009; Vallabhapurapu and Karin, 2009; Wong and Tergaonkar, 2009). To determine if GRK5 modulates inflammation via NFκB signaling, we tested the effect of LPS on IκBα phosphorylation in $GRK5^{+/+}$ and $GRK5^{-/-}$ macrophages. As shown in Fig 7E, LPS-induced IκBα phosphorylation was significantly inhibited in $GRK5^{-/-}$ macrophages, compared to $GRK5^{+/+}$ cells (at 60 min, $GRK5^{+/+}$=100%; $GRK5^{-/-}$ =49.7±10.9%). In the canonical NFκB signaling, IκBα phosphorylation and its subsequent degradation result in the release of NFκB subunits (e.g. p65) that translocate into the nucleus to evoke NFκB-dependent gene transcription. To test if LPS-induced p65 nuclear translocation is also affected by GRK5, we treated macrophages from $GRK5^{+/+}$ and $GRK5^{-/-}$ mice with LPS, and determined p65 translocation using immunofluorescent microscopy. As shown in Fig 8, LPS-induced p65 nuclear translocation was significantly blocked in $GRK5^{-/-}$ cells compared to $GRK5^{+/+}$ macrophages (Fig 8A&B). To further confirm whether the absence of GRK5 would also inhibit LPS-induced NFκB DNA binding, we performed electrophoretic mobility shift assays (EMSA) by incubating nuclear extracts from $GRK5^{+/+}$ and $GRK5^{-/-}$ macrophages with double stranded oligonucleotides containing NFκB consensus sequence. LPS caused a marked increase in NFκB binding in $GRK5^{+/+}$ macrophages and this was significantly attenuated in the $GRK5^{-/-}$ cells (Fig 8C).

Taken together, our results demonstrate that the absence of GRK5 leads to decreased production of inflammatory mediators both *in vivo*, and *in vitro* in macrophages, and this decrease in cytokines/chemokines is associated with a corresponding decrease in IκBα-NFκB pathway, thus implicating GRK5-regulation of the NFκB pathway as the potential mechanism in the observed diminished inflammation in the $GRK5^{-/-}$ mice.

DISCUSSION

Using cell culture models, previous studies have proposed GRK5 as an important regulator of NFκB signaling (Parameswaran et al., 2006; Patial et al., 2010a; Sorriento et al., 2008; Valanne et al., 2010). Mechanistically, this has been proposed to be largely dependent on GRK5-IκB interaction as well as potential phosphorylation of the IκB family members by GRK5. The functional consequence on NFκB regulation however, may depend on the IκB member involved, the cellular background and the stimulus. Using endothelial cells, Sorriento et al (Sorriento et al., 2008) showed that over-expression of GRK5 increases and stabilizes nuclear IκBα levels resulting in suppressed NFκB activation. Our studies in a macrophage cell line revealed that GRK5 is a negative regulator of TLR4-induced ERK activation via its effects on NFκB1 p105 (Parameswaran et al., 2006). In contrast, in a separate study we showed that GRK5 is a positive regulator of TNFα-induced IκBα-NFκB pathway in a macrophage cell line and that the kinase activity of GRK5 is important in this regulation (Patial et al., 2010a). Interestingly, a recent study has suggested that this positive regulatory role of GRK5 in NFκB activation may be an evolutionarily conserved mechanism. While this manuscript was in preparation, Valanne et al (Valanne et al., 2010)

showed using Drosophila model, that *Gprk2* (equivalent to mammalian GRK5) mediates NFκB activation in response to stimulation by bacteria. In addition, the authors demonstrated that this mechanism is also conserved in Zebra fish *in vivo* and human HeLa cell line *in vitro*. In line with our previous observations as well as these recent findings, in the present study we confirm that GRK5 is indeed an important regulator of TLR4-induced IκBα-NFκB pathway and inflammatory mediator production in mice.

In studies using Raw264.7 macrophage cell line we found that knockdown of GRK5 negatively regulates LPS-induced p105-ERK pathway (Parameswaran et al., 2006). In the present study however, $GRK5^{-/-}$ only modestly enhanced p105-ERK signaling. While the reason for this difference is not clear, we found that GRK2 (another member of the GRK family) indeed negatively regulates p105-ERK pathway in primary macrophages (Patial et al., 2010b). It is therefore, possible that in the absence of GRK5, GRK2 may compensate in the regulation of p105-ERK signaling. GRK5 regulation of IκBα-NFκB pathway however, appears to be important in primary macrophages and potentially *in vivo* in mice (present studies) as well as in Drosophila, Zebra fish and in human cells (Valanne et al., 2010). In addition, consistent with the notion that NFκB signaling regulates the expression of many cytokines and chemokines, $GRK5^{-/-}$ broadly attenuated a number of cytokines and chemokines in mice. Expression of many of these cytokines/chemokines (e.g. TNFα, IFNγ, IL-12p40, IL-17, IL-1β, IL-13, MCP-1, Eotaxin, and RANTES) has also been shown to be regulated by NFκB (Hein et al., 1997; Hinz et al., 2002; Hiscott et al., 1993; Ishikado et al., 2009; Murphy et al., 1995; Shakhov et al., 1990; Shen et al., 2006; Sica et al., 1997; Wickremasinghe et al., 2004). Other cytokines such as IL-6, and MIP1α, although reported to be regulated by NFκB, were not inhibited in the $GRK5^{-/-}$ mice (Grove and Plumb, 1993; Shimizu et al., 1990). One possibility is that GRK5-regulation of NFκB modulates a specific set of genes compared to IKKβ-NFκB-mediated gene expression. This hypothesis will be tested in future studies. Confirming our studies on cytokine production, Valanne et al also showed a similar role for GRK5 in cytokine gene expression in the Drosophila and Zebra fish model (Valanne et al., 2010). Together, these studies indeed imply an evolutionarily conserved mechanism of regulation of NFκB pathway by GRK5 as was proposed by Valanne *et al*.

Our results on cytokines and chemokines reveal that the profile of these inflammatory components is regulated by GRK5 somewhat differently in mice *in vivo* compared to peritoneal macrophages *in vitro*. For example, even though TNFα was markedly inhibited in the plasma and tissues of the $GRK5^{-/-}$ mice, *in vitro* experiments in peritoneal macrophages did not demonstrate a similar regulation. Whether this is because of *in vitro* culture conditions or due to the absence of integrative signaling (that is present *in vivo*) is not clear. It is also important to note that macrophages are heterogeneous and therefore different sources of macrophages may vary functionally in its dependence for GRK5. Previous studies have indicated that kupffer cells and alveolar macrophages are important sources of TNFα (Koo et al., 1999; Ogle et al., 1994). Consistent with that, our *in vivo* results indicate that LPS-induced TNFα levels are indeed inhibited in the liver and lungs of $GRK5^{-/-}$ mice. Whether this reflects the functional heterogeneity of GRK5 with respect to the different populations of macrophages will be the subject of future studies.

Of the cytokines that are regulated by GRK5 *in vivo*, TNFα, IL-12, and IFN-γ play a crucial role in endotoxic shock (Adorini, 1999; Akira et al., 1993; Kohler et al., 1993; Senaldi et al., 1999; Wysocka et al., 1995; Zisman et al., 1997a). Even though these cytokines were markedly decreased in the $GRK5^{-/-}$ mice, these animals did not survive better than the $GRK5^{+/+}$ mice. This suggests that there may be other factors yet identified in the $GRK5^{-/-}$ mice that may compensate for the decrease in these three major proinflammatory cytokines. It should also be noted that other proinflammatory factors such as IL-6, MIP1α, MIP1β were

not inhibited in the knockout mice and thus may contribute to the survival results observed. Whether the lack of better survival of GRK5$^{-/-}$ mice reflects their inability to inhibit some of these pro-inflammatory factors or whether other mechanisms contribute to this will be determined in future studies. Interestingly, IL-17 (known to be produced primarily by Th17 cells) was also significantly blocked in the GRK5$^{-/-}$ mice. IL-17 has been shown to be important in neutrophil infiltration and our studies show that MPO activity (a measure of neutrophil infiltration) was markedly inhibited in the lungs of LPS-treated GRK5 KO mice (Ferretti et al., 2003). It is also possible that the reduced neutrophil infiltration in the lungs is due to decrease in some of the chemokines in the GRK5$^{-/-}$ or due to differences in vascular permeability and neutrophil transmigration mediated by endotoxic shock. Further studies are certainly necessary to determine the mechanisms by which GRK5 regulates neutrophil infiltration in the lungs and whether this also occurs in other disease conditions.

Although secretion of pro-inflammatory cytokines serve as an essential prerequisite for initiating an effective innate immune response to fight infection, they are also associated with deleterious effects leading to multi organ failure and ultimately death (Adorini, 1999; Akira et al., 1993; Kohler et al., 1993; Lehmann et al., 1987; Murakami et al., 1990; Senaldi et al., 1999; Tracey and Cerami, 1994; Wysocka et al., 1995; Zisman et al., 1997a; Zisman et al., 1997b). Similarly, anti-inflammatory cytokines despite important for controlling an exaggerated inflammatory response, also lead to a suppression of the immune system (Cohen, 2002; de Waal Malefyt et al., 1991; Rittirsch et al., 2008; Weiss et al., 1989). Systemic inflammatory response syndrome (SIRS) is a consequence of an imbalance between pro and anti-inflammatory cytokines (Balk, 2000a; Balk, 2000b; Bone et al., 1997). Our studies show that, the absence of GRK5 reduces the production of several pro- and anti-inflammatory cytokines. Nevertheless, these cytokines are still produced to some extent, which may be at sufficient levels for inducing an effective immune response. Thus we propose that GRK5 may serve as an effective target in inflammatory conditions because inhibition of GRK5 will attenuate but not abolish the immune responses.

In conclusion, our results demonstrate that GRK5 is an important positive regulator of TLR4-induced inflammation *in vitro* in primary macrophages, as well as *in vivo* in mice. It remains to be seen if GRK5 mediates other inflammatory diseases and whether it might serve as a potential drug target for treating inflammatory diseases.

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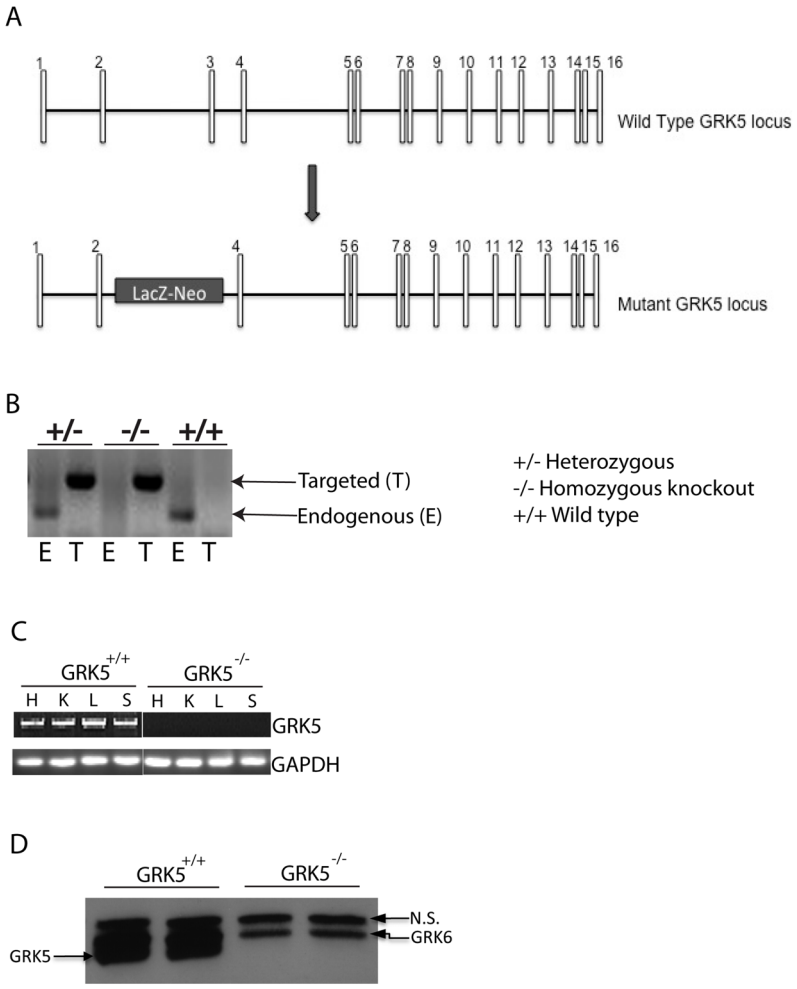

Figure 1. Generation and characterization of GRK5$^{-/-}$ mice

(A)GRK5 mutant mice were generated as described in the text. The targeted exon (exon 3) and the targeted construct (LacZ-Neo) are shown.

(B)DNA from tail snips were used for genotyping and a typical genotyping result is shown.

(C)Messenger RNA expression of GRK5 in GRK5$^{+/+}$ (wild type) and GRK5$^{-/-}$ (knockout) mice: RNA was extracted from various organs (H=Heart; K=Kidney; L=liver; S=Spleen) and subjected to RT-PCR as described in the Materials and Methods. A representative gel from the PCR reaction is shown. GRK5 mRNA is shown in the top panel and GAPDH mRNA (control) is shown in the bottom.

(D) GRK5 protein levels in the GRK5$^{+/+}$ and GRK5$^{-/-}$ macrophages: Primary peritoneal macrophages from the two genotypes were obtained as described in the methods and the lysates subjected to immunoblotting using GRK5/6 monoclonal antibody (Millipore). Note the absence of GRK5 in the GRK5$^{-/-}$ cells. N.S.=Non-specific band shown for equal loading.

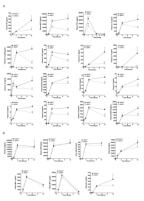

Figure 2. Effect of LPS on plasma levels of cytokines and chemokines in GRK5$^{+/+}$ and GRK5$^{-/-}$ mice

GRK5$^{+/+}$ and GRK5$^{-/-}$ mice were injected with LPS (30 μg/g body weight) and plasma collected at various time intervals as indicated. Levels of 23 cytokines/chemokines were determined using 23-cytokine multiplex assay from Biorad as described in the Materials and Methods. Cytokines and chemokines that were inhibited in the GRK5$^{-/-}$ mice are shown in (A). Cytokines/chemokines there were not affected by knockout of GRK5 are shown in (B). Levels are indicated as pg/ml of plasma. (N=6 mice per time point per genotype). *$p<0.05$; **$p<0.01$; ***$p<0.001$ compared to GRK5$^{+/+}$ at the corresponding time point.

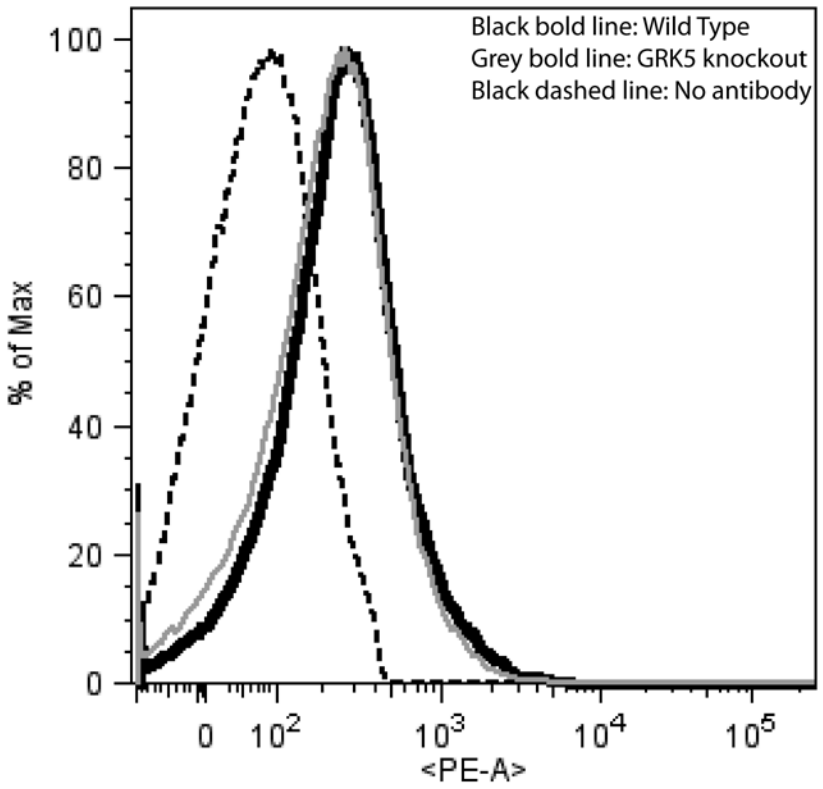

Fig 3. Surface expression of TLR4 in GRK5$^{+/+}$ and GRK5$^{-/-}$ mice

Bone marrow cells (CD11b^{+}) from GRK5$^{+/+}$ and GRK5$^{-/-}$ mice were examined for surface expression of TLR4 using flow cytometry as described in the Materials and Methods. Mean fluorescence intensity is shown from a typical experiment.

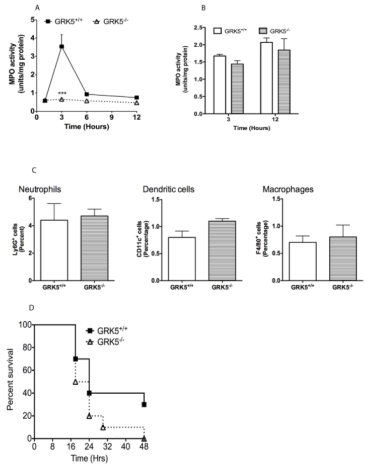

Figure 4. Effect of LPS on lung myeloperoxidase (MPO) activity and mortality in GRK5$^{+/+}$ and GRK5$^{-/-}$ mice

A and B. GRK5$^{+/+}$ and GRK5$^{-/-}$ mice were injected with LPS for the indicated time points, and lung (A) and liver (B) tissue collected and MPO activity determined as described in the Materials and Methods. N=6 mice for each time point/genotype. ***p<0.001 compared to GRK5$^{+/+}$ at the corresponding time point.

C. Spleens from GRK5$^{+/+}$ and GRK5$^{-/-}$ mice were collected, splenocytes prepared (Porter et al., 2010), fixed and stained for markers of neutrophils (Ly6G^{+}), dendritic cells (CD11c^{+}) and macrophages (F4/80^{+}). Cells were then analyzed using flow cytometry. N=4 for each genotype.

D. GRK5$^{+/+}$ and GRK5$^{-/-}$ mice were injected with LPS and survival of mice monitored for various time points up to 48 hours. Survival curves were analyzed using a Kaplan-Meier test (Prism 5 software, Graph Pad Software, La Jolla, CA). N=10 mice per genotype; p=0.0814.

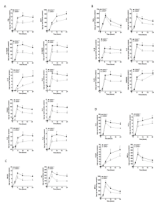

Figure 5. Effect of LPS on cytokine/chemokine production in GRK5$^{+/+}$ and GRK5$^{-/-}$ mice macrophages

Thioglycollate-elicited peritoneal macrophages were stimulated with 1 μg/ml LPS and cell culture supernatants collected at various time intervals and levels of cytokines/chemokines determined using Biorad's 23-cytokine multiplex assay. Levels of cytokines and chemokines were normalized to total protein concentration and results shown as picograms/μg of total cellular protein. Cytokines/chemokines that were regulated by GRK5 in both macrophages and plasma are shown in (A). Cytokines/chemokines that were regulated by GRK5 in the plasma but not in the macrophages are shown in (B). GM-CSF and KC were both regulated by GRK5 only in the macrophages as shown in (C). Cytokines/Chemokines that were regulated by GRK5 neither in the plasma nor in the macrophages are shown in (D). N=6 mice per genotype. *$p<0.05$; **$p<0.01$; ***$p<0.001$ compared to GRK5$^{+/+}$ at the corresponding time points.

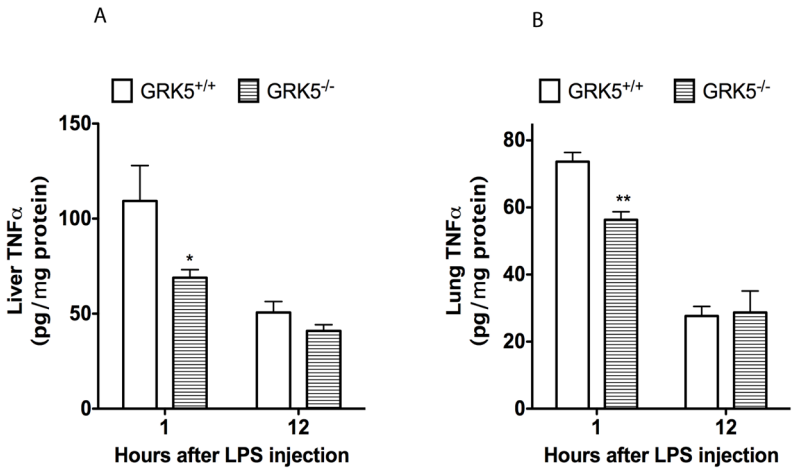

Figure 6. TNFα levels in liver and lung from GRK5$^{+/+}$ and GRK5$^{-/-}$ mice

GRK5$^{+/+}$ and GRK5$^{-/-}$ mice were injected with LPS for the indicated time points. Liver and lung were collected, homogenized and TNFα levels determined by ELISA (e-Biosciences). TNFα levels in the tissues were normalized to mg of protein. N=6 mice per time point/genotype. *$p<0.05$ and **$p<0.01$ compared to GRK5$^{+/+}$ at the corresponding time point.

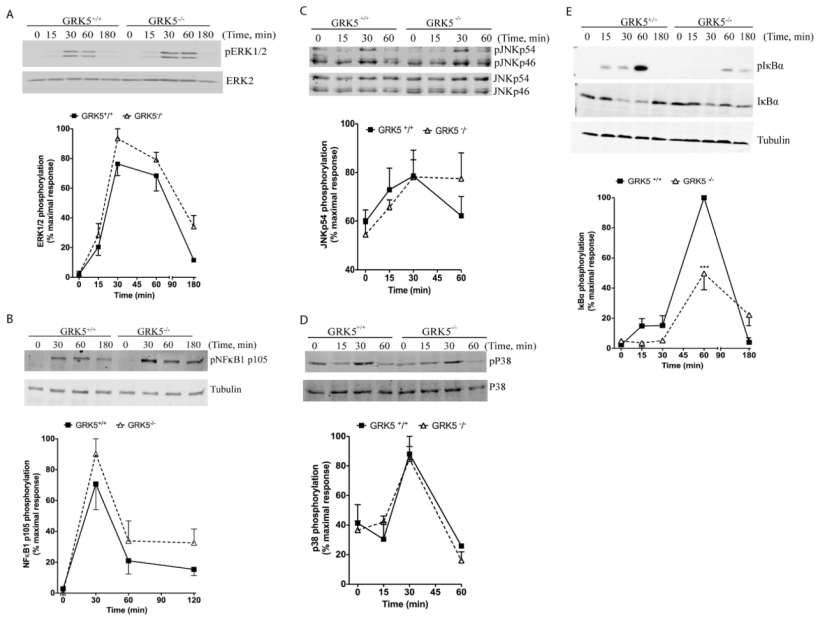

Figure 7. Effect of LPS on various signaling pathways in GRK5$^{+/+}$ and GRK5$^{-/-}$ macrophages

Thioglycollate-elicited peritoneal macrophages from GRK5$^{+/+}$ and GRK5$^{-/-}$ mice were stimulated with LPS (1 μg/ml) for various time points as indicated. Cell lysates were immunoblotted with primary antibodies against p-ERK1/2/ERK2, pP105/tubulin, pJNK/JNK, pP38/tubulin, and pIκBα/IκBα/tubulin. Secondary antibodies were fluorescent tagged and the blots were developed using LI-COR Biosciences Odyssey system. Representative blots are shown in the top and quantitation in the bottom. N=4 mice per genotype for pERK (A) and pP105 (B). N=3 mice per genotype for pJNK (C) and pP38 (D). N=4 mice per genotype for pIκBα (E). ***p<0.001 compared to GRK5$^{+/+}$ cells at the corresponding time point.

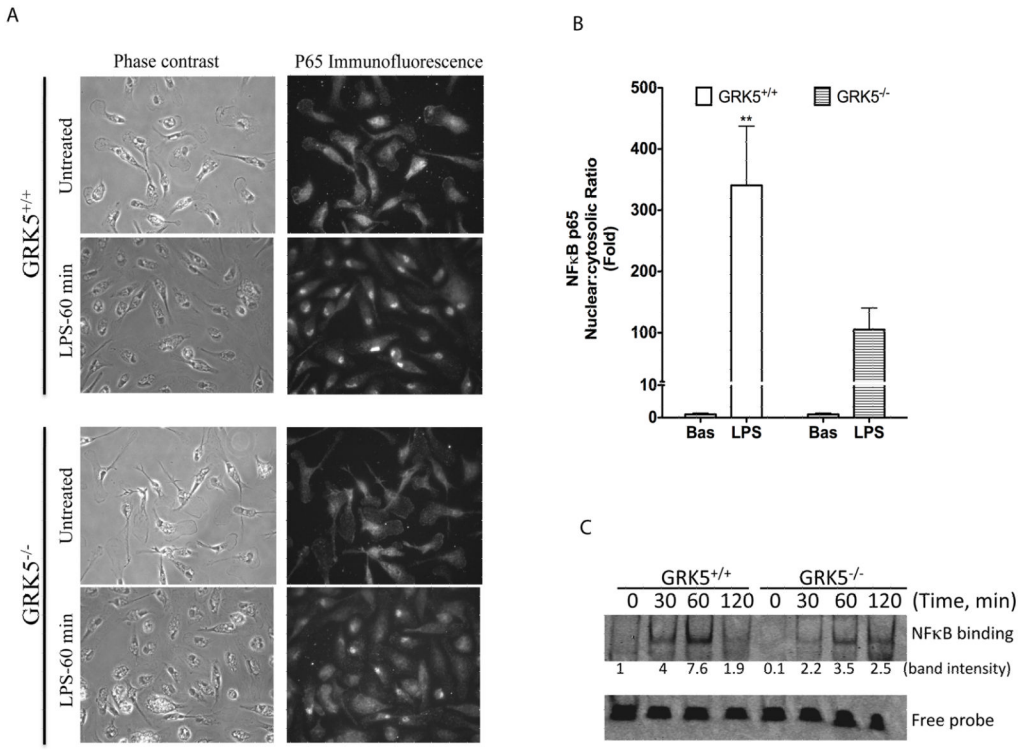

Figure 8. NFκBp65 nuclear translocation and DNA binding activity in GRK5$^{+/+}$ and GRK5$^{-/-}$ macrophages

(A&B): Primary macrophages from GRK5$^{+/+}$ and GRK5$^{-/-}$ mice were plated on cover slips (as described in the Materials and Methods) and treated or not with LPS for 60 minutes. Cells were then subjected to immunofluorescent microscopy for cellular p65 levels as described in the Methods. In Fig 8A, Phase contrast is shown on the left panel and the corresponding microscopic field for p65 immunofluorescence is shown on the right panel. Number of cells that showed p65 in the nucleus v/s the cytosol in the untreated and treated cells from both genotypes were counted from at least 10 different fields per N. Experiment was repeated in macrophages from 4 different mice for each genotype. Representative immunofluorescent microscopy images are shown in (A) and quantitation is shown in (B). **p<0.01 compared to knockout at the corresponding time point.

(C): Primary peritoneal macrophages from GRK5$^{+/+}$ and GRK5$^{-/-}$ mice were treated with LPS for the indicated time points. Nuclear extracts [obtained as described before (Patial et al., 2010a)] were incubated with IRDye® 700-labelled NFκB oligonucleotide probes (Licor) and EMSA performed as described before (Patial et al., 2010a). Representative gels from three such experiments are shown. NFκB binding is shown in the top and free probe in the bottom panels. Fluorescent intensity of the bands for NFκB binding is shown below the top panel.