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Original Article

Role of myeloid-specific G-protein coupled receptor kinase-2 in sepsis

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Abstract: Previous studies have implicated a critical role for G-protein coupled receptor kinase-2 (GRK2) in sepsis owing to its ability to regulate inflammatory response and chemotaxis of immune cells. We therefore, hypothesized that deletion of GRK2 in myeloid cells would significantly modulate the pathogenesis of polymicrobial sepsis. To test this hypothesis, we induced cecal ligation and puncture (CLP), in mice with myeloid-specific deletion of GRK2 and the corresponding GRK2 wild type littermates and determined the inflammatory response (IL-6 and IL-10), immune cell infiltration, bacterial load and survival. Six hours after surgery, plasma IL-6 and IL-6:IL-10 ratios were significantly enhanced in the GRK2 knockouts compared to the GRK2 wild type mice. Compared to these effects, IL-6was significantly elevated in the bronchoalveolar lavage but not in the peritoneal fluid of the GRK2 knockout mice. On the other hand, peritoneal IL-10 was significantly elevated in the GRK2 knockout mice compared to the GRK2 wild type. Even though GRK2 knockout mice exhibited an exaggerated cytokine response, there was no difference in immune cell infiltration into the primary site of infection or in bacterial clearance when compared between the GRK2 wild type and GRK2 knockout mice after surgery. Furthermore, in spite of the enhanced pro-inflammatory profile early after surgery, there was only a modest increase in mortality in the GRK2 knockout compared to the GRK2 wild type mice after CLP. Together, our studies demonstrate that myeloid-specific knockout of GRK2 renders the mice more susceptible to an early pro-inflammatory state. However, myeloid-specific GRK2 is not involved in immune cell infiltration to the primary site of infection or in bacterial clearance and does not significantly modulate mortality in the cecal ligation puncture model of polymicrobial sepsis.

Keywords: G-protein coupled receptor kinase-2 (GRK2), inflammation, sepsis, GRK2 knockout mice

Introduction

G-protein coupled receptor kinase-2 (GRK2) is one of the seven members of the G-protein coupled receptor kinases (GRKs) that are serine/threonine kinases and are widely distributed in different tissues [1]. GRKs in general and GRK2 in particular have been shown to play a crucial role in various cell signaling and cell biological processes, ranging from receptor phosphorylation and desensitization, to MAPK signaling, chemotaxis, and inflammatory cytokine production [2-10]. Even though GRKs were originally discovered for their role in GPCR phosphorylation, their role in receptor biology and cell signaling has considerably expanded in the last decade. Thus, while GRK2 (originally named β-adrenergic receptor kinase) was discovered for its role in phosphorylation of β-adrenergic receptor, recent studies suggest an important role for this kinase in inflammation and inflammatory diseases [11, 12].

Several studies have shown that GRK2 levels are altered in human patients suffering from different inflammatory diseases including sepsis [13-16]. Similar findings have also been reported in animal models of inflammatory disease [17, 18] and in immune cells in vitro [19, 20]. By virtue of its role in regulating GPCRs, GRK2 has been shown to be a critical modulator of chemotaxis, mediated by chemokine receptors. For example, increased expression of GRK2 in neutrophils from septic patients was

shown to correlate with significantly reduced chemotaxis [13]. In previous studies, we examined the role of GRK2 in an endotoxemia model in mice using myeloid-specific knockout of GRK2 [9]. We found myeloid-specific GRK2 to be an important negative regulator of endotoxemia in vivo, and furthermore, demonstrated that this role of GRK2 may be related to its effect on Toll-like receptor-4-induced NFκB1p105-ERK pathway in macrophages. Together, based on these studies we hypothesized that myeloid-specific knockout of GRK2 will significantly modulate the outcome of polymicrobial sepsis.

To address the role of GRK2 in a clinically relevant model of polymicrobial sepsis, in this study we used a cecal-ligation and puncture model of septic shock [21]. This model is induced by polymicrobial septic peritonitis, evoked by cecal ligation and puncture. Of the different models of sepsis, CLP has been shown to be more akin to the development of human sepsis [22]. While this model also has its drawbacks, the inflammatory response that develops has similar kinetics to that of clinical sepsis. Thus, in this study we examined the role of myeloid-specific GRK2 in cecal-ligation and puncture model in mice. We found that consistent with our results in endotoxemia model myeloid-specific GRK2 knockout mice have a slightly exaggerated inflammatory response compared to the GRK2 wild type mice. Our results however suggest that, in this model, myeloid specific-GRK2 may not play an important role in either peritoneal cell chemotaxis or in clearance of bacterial load after septic peritonitis.

Materials and methods

Animals

All animal procedures were approved by the Michigan State University Institutional Animal Care and Use Committee and conformed to National Institutes of Health guidelines. 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. Myeloid-specific GRK2 deficient mice were generated as described before [9]. Briefly, $GRK2^{fl/fl}$ mice in which exons 3-6 of GRK2 are flanked by LoxP sites (kindly provided by Dr. Gerald Dorn II, Washington University school of Medicine, St. Louis), were crossed with LysMCre mice to generate $GRK2^{fl/flLysMCre}$ mice [9, 23, 24]. 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.

Cecal ligation and puncture

Cecal ligation and puncture was performed as described before [25]. Briefly, GRK2 wild type and myeloid specific knockout mice (males, 8-12 weeks old) were anesthetized with Ketamine (80 mg/kg body weight) and Xylazine (5 mg/kg body weight). Before surgery the abdominal skin was shaved under aseptic conditions followed by a ~1.5 cm midline incision to expose the cecum. The cecum was tightly ligated with a 4.0 silk suture at the base below the ileo-cecal valve. This was followed by puncture with a 20-G needle (two punctures). The cecum was gently squeezed to extrude small fecal matter and then returned to the peritoneal cavity. The incision was then closed with a 4.0 silk. The animals were returned to their cages with adlibidum access to food and water. Sham mice underwent identical protocol except ligation and puncture. Mice were sacrificed at various time points as indicated and blood collected. Peritoneal and bronchoalveolar lavage fluids were collected as described before [9, 26]. For survival studies mice were monitored for 7 days. Differences in survival were analyzed using a log-rank test (Prism 5 software, Graph Pad Software, La Jolla, CA).

Cytokine analysis

Plasma and body fluids (peritoneal, and bronchoalveolar lavage) were used to assess the cytokine levels using Enzyme Linked Immunosorbent Assay (ELISA) kits from eBioscience, Inc. (San Diego, CA 92121, USA) as described before [27].

Flow cytometry and cell analysis

Surface staining and flow cytometry were performed as described before [10]. Briefly, cells obtained from peritoneal lavage fluid was washed and stained with antibodies for various surface markers including CD11b, Ly6G, and F4/80, to identify neutrophil and macrophage populations. Data were acquired using a LSRII (BD Biosciences) and analyzed using Flowjo

software (Tree Star) as described before [10, 27].

Statistical analysis

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 and Analysis of Variance (ANOVA) with Bonferroni Post-hoc test was used to compare more than two groups. P value of less than 0.05 was considered significant.

Results

Systemic inflammatory response after cecal ligation and puncture (CLP): In previous studies we reported the initial characterization of the myeloid-specific GRK2 knockout mice. We demonstrated that GRK2 levels are significantly reduced in the macrophages and neutrophils, but is still present at equivalent levels (compared to the GRK2 wild types) in various organ tissues examined in the GRK2 knockout mice [9]. To understand the role of myeloid-specific GRK2 in sepsis, we induced cecal ligation and puncture (CLP) (or sham surgery) in both the GRK2 wild type and the myeloid-specific GRK2 knockout mice (GRK2MyeKO) and subsequently measured various cytokines in the plasma at various time points after surgery. In preliminary 23-plex cytokine analyses, we found that plasma IL-6 and IL-10 levels were the only cytokines modulated by GRK2 (data not shown). Therefore, we focused on these two cytokines and measured their levels in the plasma using ELISA. Plasma IL-6 but not IL-10 levels were significantly enhanced in the GRK2 knockout, early after surgery (6 hours after CLP) (**Figure 1**). To determine the systemic pro- versus anti-inflammatory status of the animals [28, 29], we compared the ratios of the plasma IL-6:IL-10 between GRK2 wild type and GRK2 knockout mice. Interestingly, 6 hours after surgery, plasma IL-6:IL-10 ratio was significantly elevated in the GRK2 knockout mice (**Figure 1**). Given the important pro- and anti-inflammatory roles of IL-6 and IL-10 respectively in this model of sepsis [30], higher ratio of IL-6:IL-10 in the GRK2 knockout mice suggests that early after surgery the GRK2 knockout mice are biased towards a pro-inflammatory state compared to the GRK2 wild type mice.

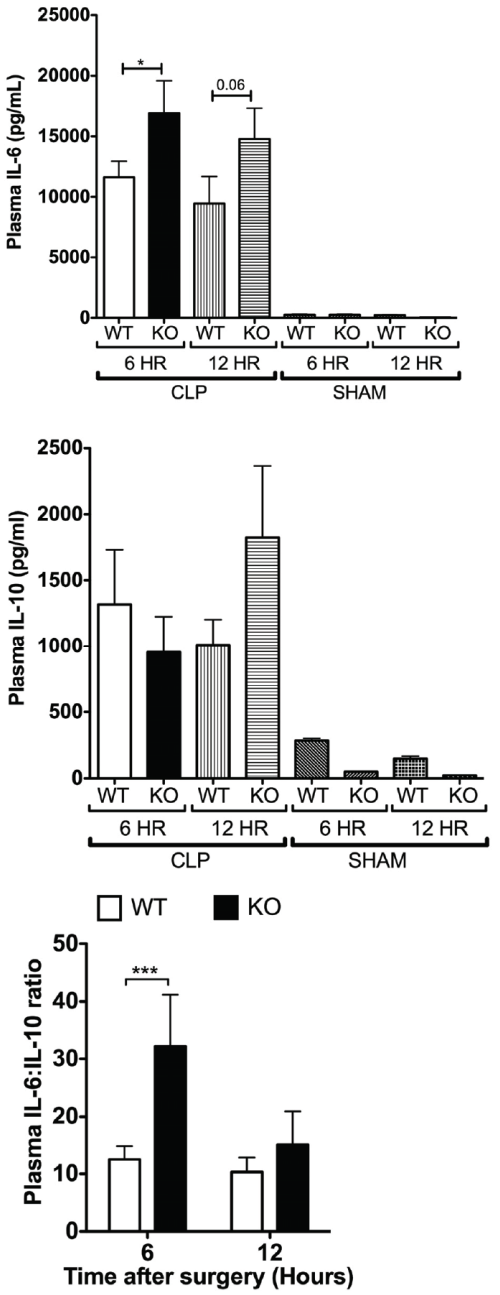

Figure 1. Plasma cytokine levels in myeloid-specific GRK2 knockout (GRK2MyeKO) and wild type littermate (GRK2 WT) mice after septic peritonitis: Mice from both genotypes were subjected to cecal ligation and puncture (CLP) or sham surgery (SHAM) and sacrificed at specific time points as shown. Plasma levels of IL-6, and IL-10 were measured using ELISA. *P<0.05 and **P<0.01 compared between the GRK2 wild type and the GRK2 knockout mice at the corresponding time point. Number of mice: CLP- 8-10 mice per genotype per time point; Sham- 3 to 5 mice per genotype per time point.

Inflammatory response in the lungs

In order to examine the role of GRK2 in tissue compartments we determined the inflammatory response in the lungs. Lungs are one of the major tissue sites affected after sepsis and therefore we examined the cytokine levels in the bronchoalveolar lavage fluid (BALF) after CLP. Twelve hours after CLP, IL-6 levels in the GRK2 wild type and GRK2 knockout mice were significantly elevated compared to the shams. Importantly, IL-6 level in the BALF was significantly enhanced in the GRK2 knockout mice compared to the GRK2 wild type mice (**Figure 2**). IL-10 levels however, did not differ between the genotypes in the BALF (**Figure 2**).

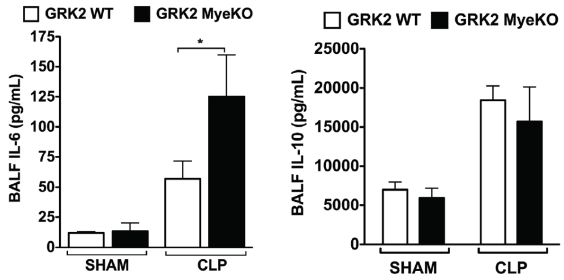

Figure 2. Cytokine levels in the Broncho-Alveolar Lavage Fluid (BALF) after septic peritonitis: Myeloid-specific GRK2 knockout and the wild type littermate mice were subjected to CLP or Sham as described in **Figure 1**. Broncho alveolar lavage fluid was collected as described before [9]. The fluid portion of the BALF was then tested for cytokines using ELISA. *P<0.05. N=9 for CLP IL-6; N=7 for CLP IL-10; N=3 for shams (IL-6 and IL-10).

Inflammatory response at the primary site of infection (peritoneum) after surgery

Similar to the systemic inflammatory response after CLP, peritoneal cytokine levels were significantly enhanced after surgery compared to the sham operated mice in both genotypes. However, unlike plasma IL-6 levels, peritoneal lavage IL-6 level was not significantly different between the GRK2 wild type and the knockout mice (**Figure 3**). In contrast, peritoneal IL-10 levels were significantly elevated at 12 hours in the GRK2 knockout mice compared to the GRK2 wild types (**Figure 3**). In contrast to the plasma cytokine ratios, IL-6:IL-10 ratio in the peritoneal fluid was not different between the GRK2 wild type and the GRK2 knockout mice (**Figure 3**).

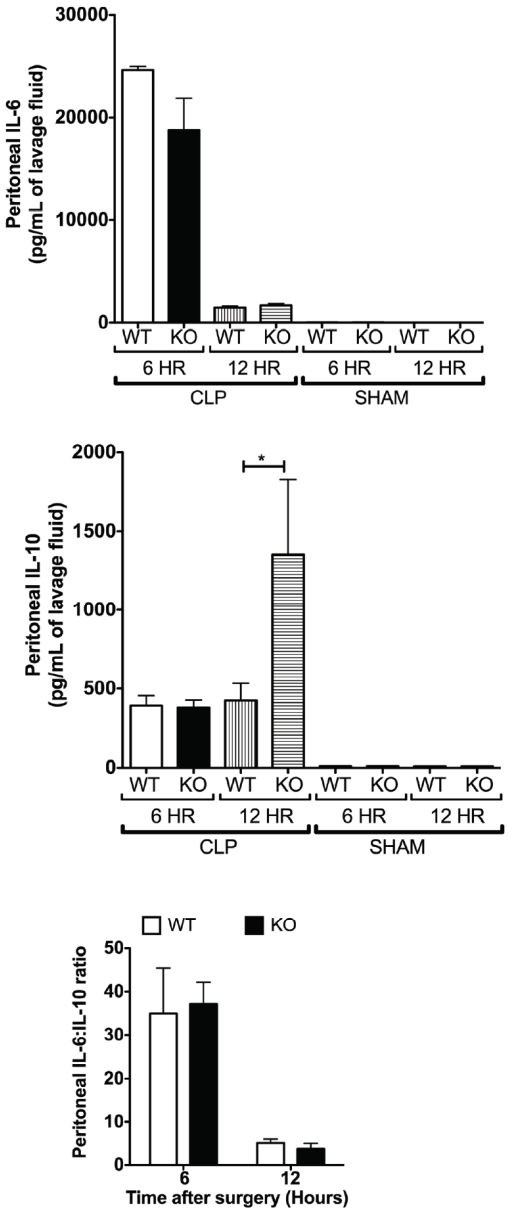

Figure 3. Peritoneal cytokine levels in myeloid-specific GRK2 knockout and wild type littermate mice after septic peritonitis: Mice from both genotypes were subjected to CLP or SHAM and sacrificed at specific time points as shown. Immediately after sacrifice, 5 ml sterile PBS was injected into the peritoneal cavity and peritoneal lavage fluid collected for various assays [26]. The fluid portion of the peritoneal lavage collection was used for cytokine assays (determined by ELISA). Number of mice: CLP- 8-10 mice per genotype per time point; Sham- 3 to 5 mice per genotype per time point.

Infiltration of immune cells into the primary site of infection (peritoneum)

Immune cell infiltration into the peritoneum after CLP surgery is an important event in the pathogenesis of sepsis [31, 32]. GRK2 has previously been shown to be important for chemotaxis in other models [7, 33]. Therefore, we assessed the total infiltration of immune cells into the peritoneum after CLP in GRK2 wild type and GRK2 knockout mice. As would be predicted, CLP induced a significant increase in peritoneal cells as early as 6 hours after surgery compared to the sham mice. The high immune cell infiltration persisted even at 18 hours after surgery (**Figure 4**). In contrast to the predicted role of GRK2 in chemotaxis, total peritoneal cell counts were similar between the GRK2 wild type and the GRK2 knockout mice at 6, 12, and 18 hour time points after surgery (**Figure 4**).

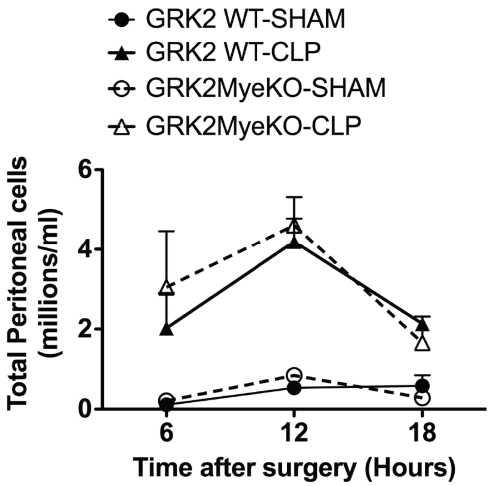

Figure 4. Effect of septic peritonitis on peritoneal cell infiltration in GRK2 wild type and myeloid-specific GRK2 knockout mice: Total cell counts were performed on peritoneal lavage from mice subjected to sham or CLP (as described in Figure 3) at the indicated time points after sacrifice. Total cell counts were performed using a Countess automated cell counter or Cytospin. For total cell counts: Number of mice: CLP- 8 mice for 6 hrs, 6 mice for 12 hrs, and 10 mice for 18 hrs for each genotype; Sham- 3-5 mice per genotype for each time point.

To examine the differential cell counts peritoneal cells (after surgery) were treated with various antibody cocktails to identify neutrophils and macrophages using flow cytometry. Cells were stained for F4/80, Ly6G and CD11b to differentiate macrophages ($F4/80^+CD11b^+Ly6G^-$) and neutrophils ($F4/80^-CD11b^+Ly6G^+$). As shown in **Figure 5**, there was no significant dif-

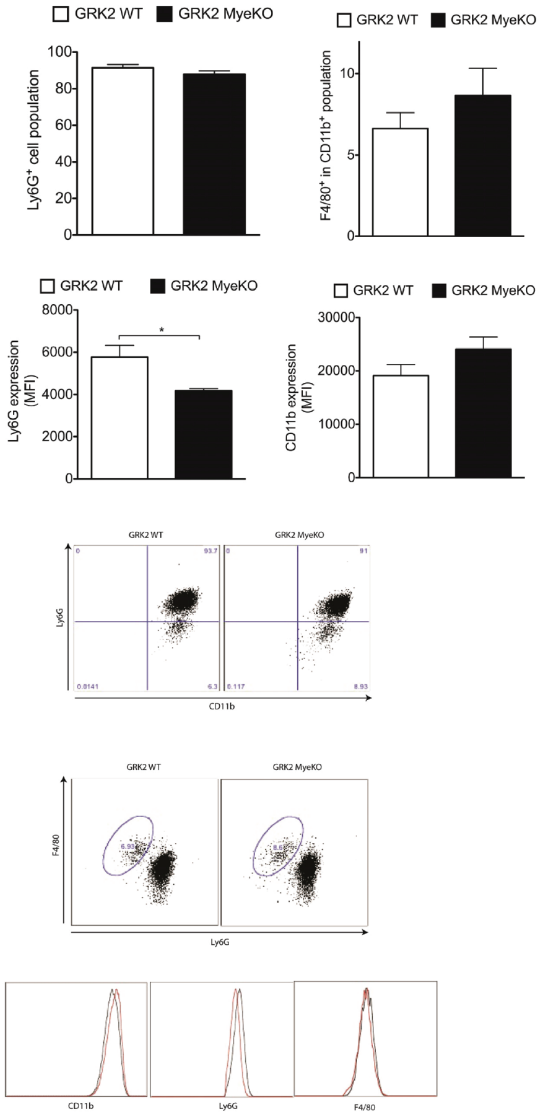

Figure 5. Effect of septic peritonitis on Ly6G+ and F4/80+ cells in the peritoneal cells from GRK2 wild type and myeloid-specific GRK2 knockout mice: Peritoneal cells from GRK2 wild type and GRK2 knockout mice were collected 6 hours after surgery (as described in **Figure 1**) and were prepared for flow cytometry as described in the methods. Stained cells were subjected to flow cytometry. N=4 for each genotype. *P<0.05. Representative dot blots and histograms are shown in the bottom three panels. For the histogram: Black line: GRK2 WT; Red line: GRK2 MyeKO. Note that the F4/80 population was assessed with in the CD11b+ population.

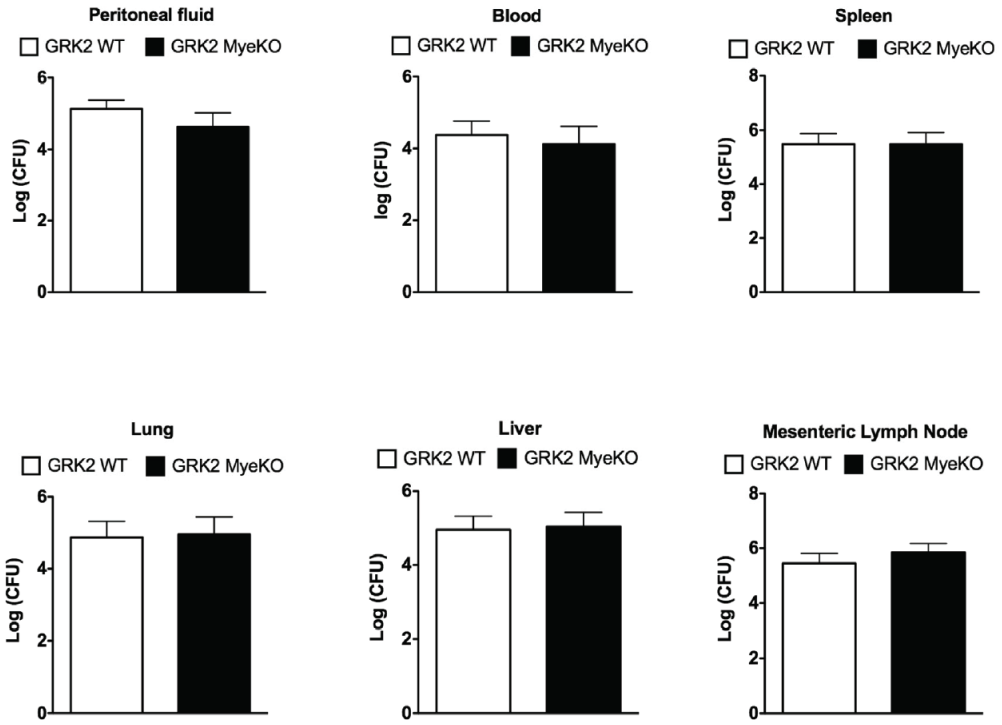

Figure 6. Bacterial load in various tissue compartments in the myeloid-specific GRK2 knockout and WT littermates after septic peritonitis: Blood, peritoneal fluid, and various tissue homogenates (collected 18 hours after CLP) were plated in blood agar plates and incubated at 37 C for 24 hours and the number of bacterial colonies were counted. N=10 for WT and N=8 for KO.

ference between the GRK2 wild type and the GRK2 knockout mice in terms of the percent of macrophages or neutrophils infiltrating into the peritoneal cavity as assessed by flow cytometry. Interestingly, however, the expression of Ly6G (as determined by mean fluorescence intensity) but not CD11b was significantly decreased in the GRK2 knockout neutrophils (**Figure 5**). While Ly6G expression has been associated with the state of neutrophil maturation [34], how this might explain the exaggerated inflammatory phenotype of the GRK2 knockout mice is not clear. Together, these results suggest that myeloid-specific knockout of GRK2 does not regulate the infiltration of immune cells (total or differential) into the peritoneal cavity in this model of sepsis.

Bacterial clearance

An important aspect to the development of sepsis in the CLP model is the clearance of bacteria [31, 32]. Dysregulated clearance of bacteria can lead to excessive bacterial load that will eventually lead to organ injury and mortality. To determine if GRK2 affects clearance of bacteria, we examined the bacterial growth from peritoneal fluid, blood, spleen, lung, liver and mesenteric lymph node, 18-hours after CLP. As shown in **Figure 6**, myeloid-specific knockout of GRK2 did not affect the clearance of bacteria compared to the GRK2 wild type mice and showed similar bacterial loads after surgery.

Mortality after septic peritonitis

During the evolution of sepsis (after cecal ligation and puncture), an increase in cytokine and chemokine levels at the local and systemic sites trigger an increase in immune cell infiltration into the local and systemic sites to eliminate the bacteria [31, 32]. Inappropriate increases in the inflammatory cytokines however, can result in organ injury and therefore multiple organ dysfunction [31, 32]. Even though GRK2 knockout mice had similar bacterial load compared to that of the GRK2 wild type mice, their pro-inflammatory status (systemic IL-6:IL-10 ratio) was exaggerated early after surgery. Therefore, we reasoned that the multiple organ dysfunc-

tion and therefore mortality might be higher in the GRK2 knockout compared to the GRK2 wild type mice. As shown in **Figure 7**, CLP caused a marked increase in mortality in both GRK2 wild type and the GRK2 knockout mice. In the GRK2 wild type mice, CLP induced ~40% mortality within 4 days and remained at that level until day 7. In contrast, in the GRK2 knockout mice CLP caused ~40% mortality by day 2 and by day 7 the mortality increased to ~60%. Because the bacterial load was not any different between the GRK2 wild type and the GRK2 knockout mice, we then reasoned that in the presence of antibiotics, the GRK2 knockout mice would still have an exaggerated IL-6:IL10 ratio and therefore potentially higher mortality compared to the GRK2 wild type mice. When GRK2 wild type and GRK2 knockout mice were subjected to CLP and then treated with antibiotics (metronidazole 12.5 mg/kg and ceftriaxone 25 mg/kg) [35] for 7 days, GRK2 wild type mice did not significantly succumb to mortality when compared to the sham (p=0.23). In the GRK2 knockout however, there still was about 25% mortality by day 7, which was significantly different from the sham (p=0.04). The mortality in both the antibiotics and the non-antibiotics groups when compared between GRK2 wild type CLP and GRK2 knockout CLP, however, did not show any statistically significant difference.

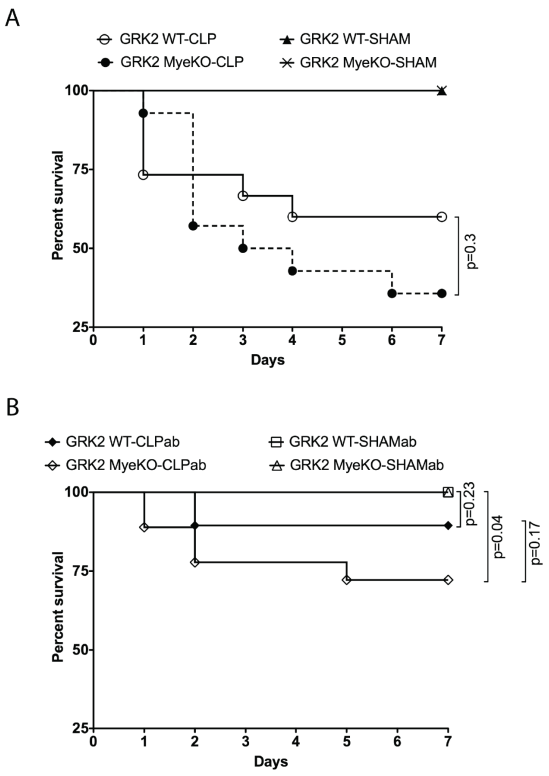

Figure 7. Mortality in myeloid-specific GRK2 knockout and GRK2 wild type littermates after septic peritonitis: Mice from both genotypes were subjected to CLP or SHAM and then monitored for mortality until day 7. In A, none of the mice received any antibiotics. (N: CLP= WT-15 mice and KO-14 mice; Sham=6 mice each genotype). In B, all the mice received antibiotics (metronidazole and ceftriaxone) once every day starting from the day of surgery until day 7 (N: CLP=WT-19 mice and KO-18 mice; Sham=13 mice each genotype).

Discussion

Since its discovery as an important regulator of G-protein coupled receptor phosphorylation and desensitization, GRK2 has been shown to have many different functions dependent and independent of its catalytic activity [1, 6, 12]. In addition, GRK2 has also been shown to regulate signaling for receptors that are not within the typical GPCR family. In this regard, multiple studies including ours demonstrated recently that myeloid-specific GRK2 is an important negative regulator of TLR4 signaling both in vitro and in vivo [9, 36]. We further showed that intraperitoneal administration of lipopolysaccharide in mice that were selectively deficient in GRK2 in the myeloid cells led to an exaggerated inflammatory response compared to the GRK2 wild type mice [9]. Based on this we predicted that knockout of GRK2 in myeloid cells would significantly modulate inflammatory response after septic peritonitis. Even though IL-6 and IL-10 were elevated (at particular time points and specific body fluids), there was no robust hyper-inflammatory phenotype. Although IL-6 and IL-10 are key cytokines in this model of sepsis, one could argue that other cytokines/chemokines may be regulated by GRK2. However, our preliminary 23-plex analysis did not reveal any other cytokines/chemokines being regulated in the GRK2 knockout mice (data not shown). Furthermore, consistent with this lack of robust hyper-inflammatory response, mortality of the GRK2 knockout mice was only modestly elevated. The difference in mortality was more evident in the antibiotic group wherein the GRK2 wild type mice did not exhibit any significant mortality after CLP in the presence of antibiotics, whereas the GRK2 knockout mice had elevated mortality compared to the shams. This

increase in mortality may be related to the enhanced IL-6 observed in the GRK2 knockout mice early after sepsis. It is also possible that this may be due to difference in antibiotic resistance. Even though previous studies including ours predicted an important role for GRK2 in sepsis, our results in this model of polymicrobial sepsis suggest that it may not be the case. It is also possible that while GRK2 may be critical, genetic deletion of GRK2 may result in compensation by other GRKs including GRK6, which has been shown before to regulate immune cell function [42]. In addition, it is possible that deletion of GRK2 in the myeloid compartment alone may not be sufficient to alter the phenotype in this model significantly. These ideas will be tested in future studies.

In addition to regulation of TLR4, GRK2 has also been shown to play a key role in the phosphorylation and desensitization of chemokine receptors [37-40]. Based on this, increased GRK2 expression in neutrophils from human septic patients and septic mice has been associated with a decrease in chemotaxis in vitro [13, 20]. Furthermore, TLRs have been shown to increase expression of GRK2 in neutrophils and this also has been associated with downregulation of CXCR2 [20]. A recent study has proposed that TLR ligand induced GRK2 expression can be negatively regulated by Interleukin-33 [41]. Thus, IL-33 could indirectly then increase the surface levels of CXCR2, thereby mediating enhanced neutrophil chemotaxis in sepsis, and this could result in a beneficial outcome. In the present study however, we did not observe any difference in chemotaxis to the primary site of infection even though GRK2 is deficient in both macrophages and neutrophils in the GRK2 knockout mice. Consistent with the lack of enhanced neutrophil infiltration in our model, we did not observe any difference in the bacterial load in the peritoneum or in any other body compartments examined. While an increase in neutrophil infiltration early after sepsis could enhance bacterial clearance and therefore protect mice from lethality [43], in our experiments lack of either enhanced chemotaxis or bacterial clearance was in fact associated with a modestly elevated lethality in the GRK2 knockout mice. Together, these results suggest that knockout of GRK2 in the myeloid compartment alone may not be sufficient to either prevent or enhance lethality in a significant way after polymicrobial sepsis.

Even though GRK2 plays an important role in GPCR-mediated signaling and biology, recent studies have clearly demonstrated the importance of GRK2 in non-GPCR signaling [6]. In particular, studies from our laboratory have shown that GRK2 can negatively regulate TLR4-induced signaling and cytokine/chemokine (including IL-6 and IL-10) production in peritoneal macrophages [9]. The question still remains whether the early-enhanced pro-inflammatory phase (systemic IL-6 and IL-6:IL-10 ratio) observed in this model of sepsis is because of GRK2's role in GPCR or non-GPCR (such as TLR4) signaling. One of the primary classes of GPCRs that GRK2 regulates in the immune system is the chemokine receptor. If the observed phenotype in the GRK2 knockout is due to altered signaling of the chemokine receptors, one would expect differences in migration of the immune cells at least to the peritoneum (the initial site of infection). Since that was not the case, it would argue against a role for excessive chemokine receptor signaling. Other GPCRs such as C3aR and C5aR (receptors for complements C3a and C5a) have also been shown to play a crucial role in the pathogenesis of sepsis [31]. C5aR especially has been shown to be an important regulator of IL-6 production in vivo in the CLP model of sepsis [44, 45]. Even though in vitro studies have shown an important role for GRK2 in the regulation of complement receptors [46-48], the in vivo role of GRK2 in C5aR signaling especially in the context of sepsis is unknown and will be the subject of future studies.

Although other studies prior to ours have implicated a role for GRK2 in the pathogenesis of sepsis [13, 41], this is the first study to directly examine the role of myeloid-specific GRK2 in a clinically relevant model of polymicrobial sepsis. Our results demonstrate that knockout of GRK2 in macrophages and neutrophils may not be sufficient to modulate the outcome of sepsis. Future studies will determine if whole body knockout of GRK2 or knockout of GRK2 in tissue compartments other than myeloid cells might be important in the pathogenesis of sepsis.

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