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Gprc5a-deficiency confers susceptibility to endotoxin-induced acute lung injury via NF-κB pathway

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Susceptibility to acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) varies greatly among patients in sepsis/septic shock. The genetic and biochemical reasons for the difference are not fully understood. G protein coupled receptor family C group 5 member A (GPRC5A), a retinoic acid target gene, is predominately expressed in the bronchioalveolar epithelium of lung. We hypothesized that Gprc5a is important in controlling the susceptibility to ALI or ARDS. In this study, we examined the susceptibility of wild-type and *Gprc5a*-knockout (ko) mice to induced ALI. Administration of endotoxin LPS induced an increased pulmonary edema and injury in *Gprc5a*-ko mice, compared to wild-type counterparts. Consistently, LPS administration induced higher levels of inflammatory cytokines (IL-1β and TNFα) and chemokine (KC) in *Gprc5a*-ko mouse lungs than in wild-type. The enhanced pulmonary inflammatory responses were associated with dysregulated NF-κB signaling in the bronchioalveolar epithelium of *Gprc5a*-ko mouse lungs. Importantly, selective inhibition of NF-κB through expression of the super-repressor IκBα in the bronchioalveolar epithelium of *Gprc5a*-ko mouse lungs alleviated the LPS-induced pulmonary injury, and inflammatory response. Thus, *Gprc5a* is critical for lung homeostasis, and *Gprc5a* deficiency confers the susceptibility to endotoxin-induced pulmonary edema and injury, mainly through NF-κB signaling in bronchioalveolar epithelium of lung.

Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are devastating clinical problems with a mortality as high as 40-60%.[1,2] ALI/ARDS are the result of intense pulmonary inflammation leading to respiratory failure, and ALI/ARDS occurs secondarily in a number of disease processes, most commonly sepsis, pneumonia, aspiration, trauma, pancreatitis, blood transfusions, smoke or toxic gas inhalation, and certain types of drug toxicity.[3,4] The pathogenesis of ALI/ARDS is not well understood. The disease process is characterized by diffuse damage to the alveoli resulting in disruption of the endothelium and epithelium.[5] Acute effects include fluid accumulates in the alveolar spaces, with severe inflammation and gas exchange abnormalities. These changes comprise the acute phase of ALI/ARDS. The subsequent fibrotic phase results in diffuse interstitial thickening, fibrosis, increased dead space and loss of lung compliance.

In sepsis/septic shock associated ALI/ARDS, some patients die from uncontrolled inflammation or sepsis, while others recover without major issues. The biochemical and genetic basis for the difference in susceptibility to ALI/ARDS is not fully understood.[6,7] Some proteins are used for predicting pathogenesis or as biomarkers for outcome in ALI, these include the proinflammatory cytokines TNF-α[8] and IL-6,[8] VEGF,[9] plasminogen activator inhibitor-1,[10] surfactant protein B,[11] P-selectin,[12] angiopoietin 2[13] and peptidase inhibitor 3 (PI3).[14] It has been suggested that genes involved in inflammatory and immune pathways may confer susceptibility and morbidity in lung injury,[15] in addition to gene–environmental interactions. Identification of the ALI susceptible genes that contribute to the pathogenesis of ALI /ARDS may thus provide important insight to the etiology of ALI/ARDS, and may have certain predictive value. Moreover, biomarker-based novel intervention strategies may be developed.

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G protein coupled receptor family C group 5 type A (GPRC5A), also known as RAIG1 or RAI3, is a retinoic acid-inducible gene. GPRC5A is predominately expressed in type I and type II epithelial cells of the lung.[16-19] Previous studies showed that, *Gprc5a*-knockout (ko) mice developed spontaneous lung tumors.[18] In addition, a considerable portion (40-50%) of *Gprc5a*-ko mice developed eosinophilic macrophage pneumonia.[20] These observations suggest that *Gprc5a* deficiency lead to an increased inflammatory response in lung tissues. We hypothesized that *Gprc5a* plays an important role in controlling the susceptibility to ALI or ARDS in lung. In this study, we examined the susceptibility of wild-type and *Gprc5a*-ko mice to experimentally-induced ALI. We found that *Gprc5a*-ko mice are more susceptible to endotoxin-induced pulmonary edema and injury than their wild-type counterparts. The increased susceptibility to ALI in *Gprc5a*-ko mouse lung is mainly through dysregulated NF-κB signaling since selective blockage of NF-κB in the bronchioalveolar epithelium via specific overexpression of super-repressor IκBα suppressed endotoxin-induced lung injury and inflammatory response. Thus, *Gprc5a* is important in controlling lung homeostasis, whereas *Gprc5a* deficiency confers susceptibility to endotoxin-induced ALI.

Results

Gprc5a-ko mice are susceptible to LPS-induced pulmonary edema and injury

To determine the role of *Gprc5a* in influencing the susceptibility of mouse lung to ALI, we examined the effects of experimentally-induced ALI in *Gprc5a*-ko (KO) and wild-type (WT) mice by administration of lipopolysaccharide (LPS), a component of the outer membrane of gram-negative bacteria. Following a single i.p. injection of LPS, lung tissues of KO and WT mice were harvested at different time point, 2, 6, 24, and 48 hours. One lung lobe was fixed for hematoxylin eosin (HE) staining analysis, and the remaining tissue was crushed in liquid nitrogen for protein and mRNA analysis. Pulmonary edema, a hallmark of ALI, was estimated using the ratio of wet-to-dry lung weight. Endotoxin administration resulted in decreased body weight (**Fig. 1A**), and increased wet-to-dry lung weight (**Fig. 1B**), in both WT and KO mouse groups following LPS treatment, thus validating the experimental endotoxin-induced ALI animal model. Noticeably, the ratio of wet-to-dry lung weight at 48 hour following LPS treatment was drastically higher in *Gprc5a*-ko mouse group (KO-LPS-48h) than that from the wild-type group (WT-LPS-48h) (**Fig. 1B**). This finding implies that *Gprc5a* deficiency results in increased endotoxin-induced pulmonary edema. Consistently, H&E histological analysis revealed that alveolar lesions and pulmonary edema were more obvious in *Gprc5a*-ko mouse lungs than lungs from wild-type mice (**Figs. 1B–C**). In LPS treated *Gprc5a*-ko mice, vascular wall thickness, perivascular leukocyte rich inflammation and interstitial inflammation were more sever, and the alveolar spaces were much smaller than those of wild-type mice (**Fig. 1C**). The Inflammation Score (IS), an indicator of the severity of lung injury, shows that lung injury of *Gprc5a*-ko mice were more severe than wild-type mice, especially 24/48 h post-LPS treatment (**Figs. 1C-D**). Thus, *Gprc5a*-ko mice are more susceptible to endotoxin-induced pulmonary edema and injury than wild-type mice.

Gprc5a-ko mouse lungs produce increased levels of proinflammatory cytokines and chemokines after LPS treatment

To characterize the biochemical features of the inflammatory response in ALI, we measured the expression of proinflammatory cytokines and chemokines from mouse lungs tissue following LPS treatment. First, we examined mRNA transcripts by RT-PCR analysis: mRNA expression levels of proinflammatory cytokines (IL1β and TNFα) and chemokine (KC) were significantly higher in *Gprc5a*-ko mouse lungs than in the wild-type following LPS treatment (**Figs. 2A-2D**). Interestingly, the relative difference of mRNA levels for IL1β, TNFα and KC between KO and WT groups peaked 48h after LPS administration (**Figs. 2A-E**). This finding suggests that the endotoxin-induced inflammatory response endured for a long time period in *Gprc5a*-ko mouse lungs, whereas the inflammatory response in wild-type mouse lungs was transient, and in recovery at 48 h.

Next, we examined protein levels of proinflammatory cytokines and chemokines by ELISA. Consistent with the mRNA results, TNFα and IL-6 were higher in KO groups than WT groups at various time points (**Figs. 2F-2G**). Interestingly, TNFα levels in serum from WT and KO groups were no difference (data not shown), suggesting that the *Gprc5a* deficiency mainly targeted TNFα production in lung tissues, not throughout the body. Il-1β protein levels were not found to be significantly different between KO and WT groups (**Fig. 2H**), the reason is unclear. Taken together, the results indicate that *Gprc5a* deficiency results in an increased and persistent inflammatory response after endotoxin treatment.

LPS induces potent and persistent NF-κB activation in the bronchioalveolar epithelium of *Gprc5a*-ko mouse lungs

NF-κB is a key intracellular signaling molecule that mediates inflammatory response. To determine if NF-κB is involved in the altered susceptibility to ALI, we examined *in vivo* NF-κB activity. We quantified *in vivo* NF-κB activity by measurement of NF-κB-dependent luminescence from NF-κB-luc mice (WT) and *Gprc5a*-ko/NF-κB-luc (KO) mice 48 h after LPS treatment. Luminescence from *Gprc5a*-ko/NF-κB-luc mice was higher than that from NF-κB-luc mice (**Fig. 3A**). To avoid any interference on luminescence from skin and hair, we also measured the luminescence of the isolated lung tissues from the same NF-kB-luc and *Gprc5a*-ko/NF-κB-luc mice (**Fig. 3A**). NF-κB-dependent luminescence from the isolated lungs of *Gprc5a*-ko/NF-κB-luc mice was much greater than that from lungs of NF-κB-luc mice (**Fig. 3B**). These results indicated that, activation of NF-κB signaling is much stronger and more persistent in *Gprc5a*-ko/NF-κB-luc mice than NF-κB-luc mice after LPS administration.

Next, we examined the downstream targets of NF-κB. RT-PCR analysis showed that LPS-induced expression of NF-κB target genes MMP9, VEGF-C, Cyclin D1 was relatively mild in wild-type mouse lungs, with increased expression that peaked at 24h. In contrast, in lungs from *Gprc5a*-ko mice, expression of those NF-κB target genes was significantly increased after LPS treatment at 6h, 24h and 48h (**Fig. 3C**). These results indicate that NF-κB activation in KO mouse lungs is more persistent than that in WT mouse lungs. Thus, increased activation of the NF-κB pathway is associated with increased susceptibility to endotoxin-induced ALI in *Gprc5a*-ko mice.

NF-κB signaling is selectively inhibited in the bronchioalveolar epithelium of *Gprc5a*-ko/SR-IκBα mouse lungs

To determine if NF-κB in lung epithelial cells is critical for the increased susceptibility to ALI in *Gprc5a*-ko mice, we generated *Gprc5a*-ko/SPC-SR-IκBα mice by cross-breeding *Gprc5a*-ko mice with SPC-SR-IκBα mice, in which super-repressor (SR) IκBα gene is driven by the type II marker surfactant protein C (SPC) promoter. Electrohoretic mobility shift assay (EMSA) showed that LPS-induced NF-κB DNA-binding activity was significantly repressed in tissue from *Gprc5a*-ko/SPC-SR-IκBα mice (**Fig. 4A**). We also examined the downstream targets of NF-κB in these mice. RT-PCR analysis demonstrated that, LPS-

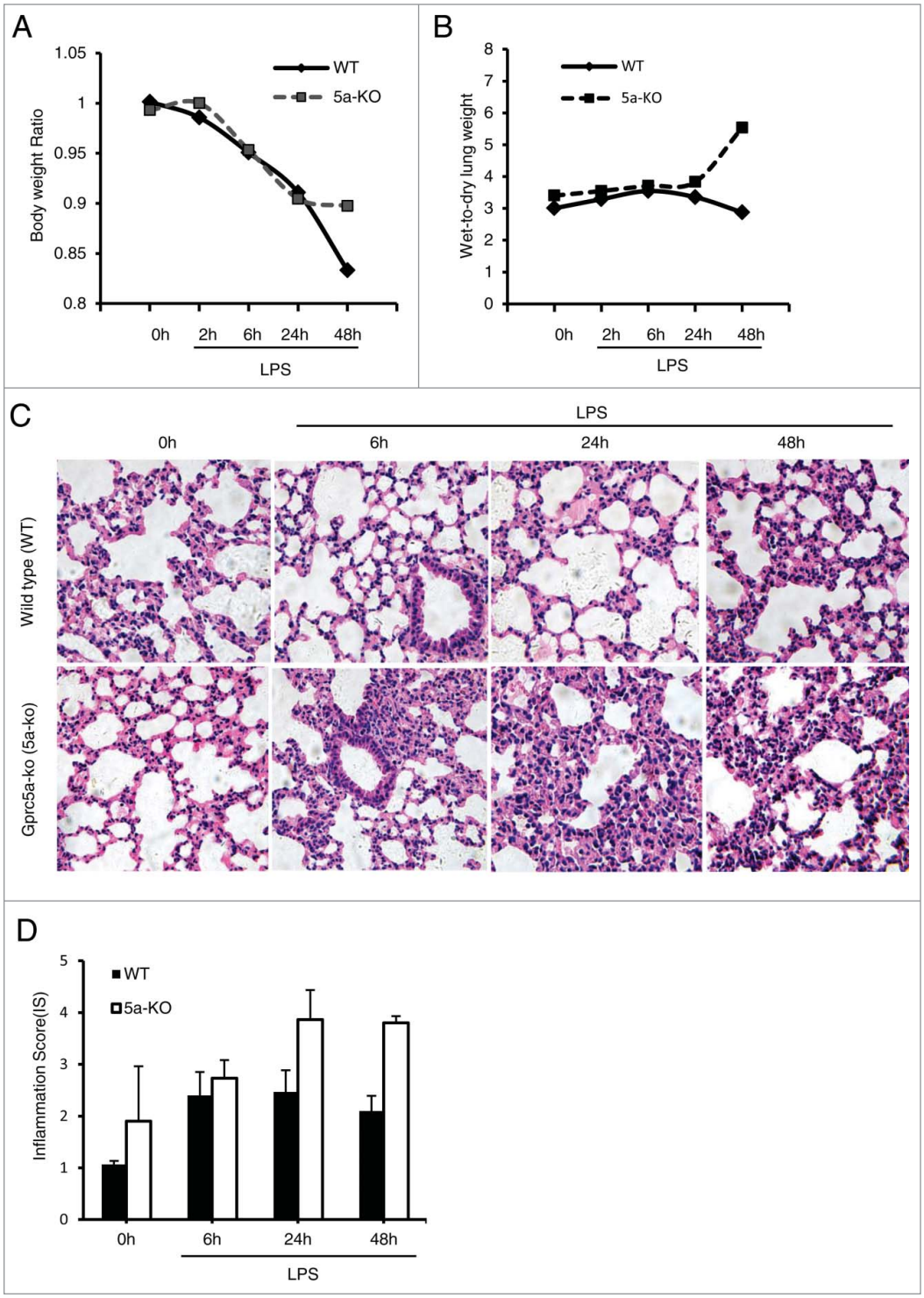

Figure 1. *Gprc5a*-ko mice are susceptible to endotoxin-induced pulmonary edema and injury. (A) Body weight of *Gprc5a*-ko (5a-KO) and wild-type (WT) mice (n = 3) treated with endotoxin over 48 h period after treatment. Weights were normalized to baseline weight in each animal. (**B**) Lung water measurements (wet:dry tissue weight) in control and endotoxin-treated mice over 48 h period after treatment. Ratio of wet-dry weights are normalized to the baseline values for each point (n = 3). (**C**) Photomicrographs are representative hematoxylin and eosin (H&E) stained sections of lung tissue from wild-type and *Gprc5a*-ko mice obtained before endotoxin (0h) and at 6 h, 24 h, and 48 h after endotoxin administration. (**D**) Inflammation score (IS) was used as a semi-quantitative assessment for pulmonary edema and injury.

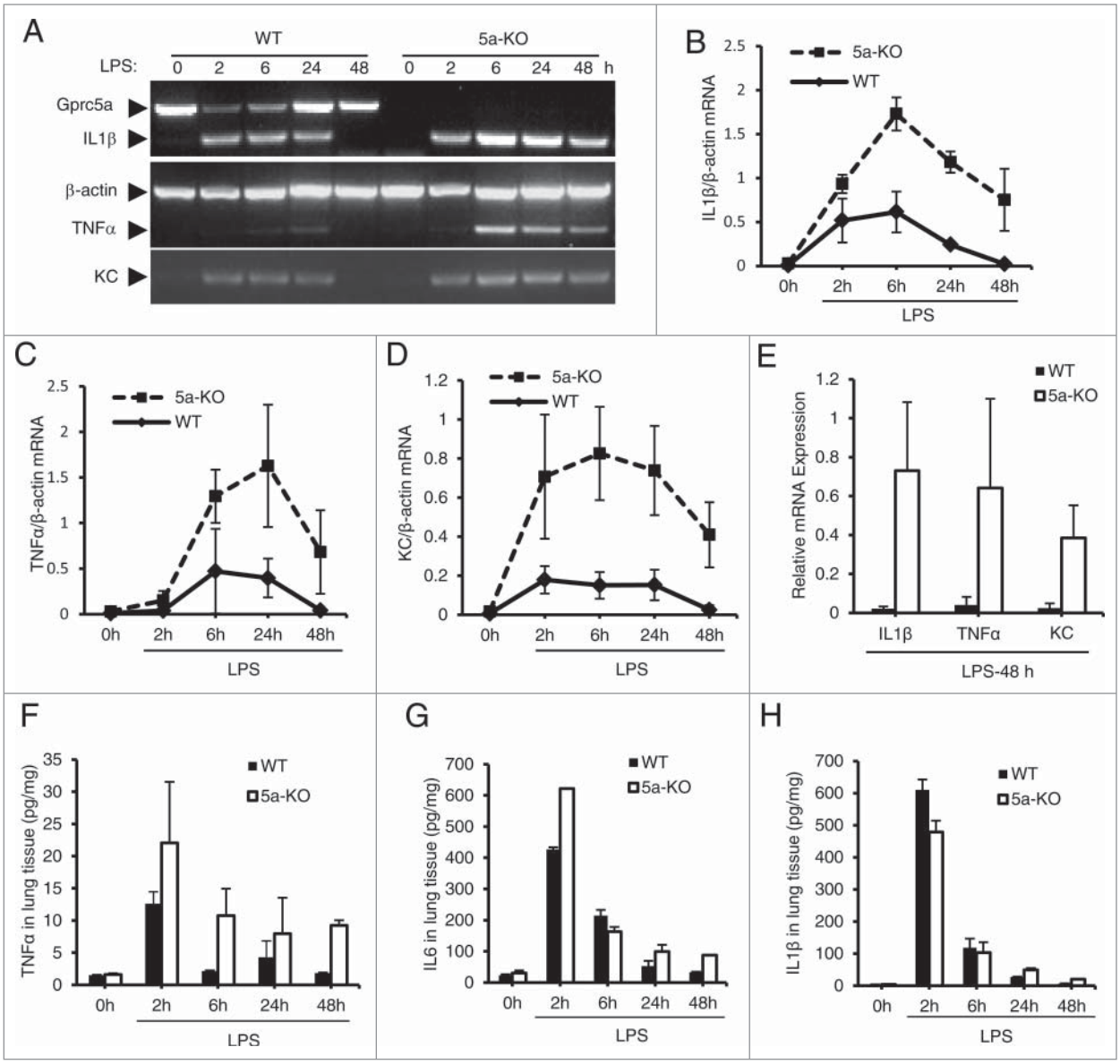

Figure 2. Lungs from *Gprc5a*-ko mouse produce increased levels of proinflammatory cytokines and chemokines after LPS treatment. (**A**) Images are representative of the RT-PCR analysis for mRNA of proinflammatory cytokines at each time point. (**B-D**) Average mRNA levels for IL-1β (B), TNFα (**C**) and KC (**D**) at each point are shown. (**E**) The average of mRNA levels of IL-1β, TNFα, and KC from wild-type and *Gprc5a*-ko mouse lungs at 48 h after endotoxin treatment. (F-H) Protein levels of cytokines, IL-1β (**F**), TNFα (**G**) and IL-6 (H), in lung tissues were measured by ELISA after LPS treatment.

induced NF-κB target genes, MMP9, VEGF-C, Cyclin D1, were significantly suppressed, especially at 6 h after LPS treatment, in lungs from *Gprc5a*-ko/SPC-SR-IκBα mice as compared to *Gprc5a*-ko mouse lungs (**Fig. 4B**). Immunoblot analysis showed that the protein levels of the NF-κB targets, MMP9, IκBα, and cyclin D1, were relatively higher in LPS-treated lungs from *Gprc5a*-ko mice compared with those from wild-type (WT) and *Gprc5a*-ko/SR-IκBα mouse lungs (**Fig. 4C**). Taken together, these results indicate that the NF-κB pathway in the bronchioalveolar epitheliumis selectively inhibited with expression of super repressor (SR) IκBα in *Gprc5a*-ko mouse lungs.

Gprc5a-ko/SPC-SR-IκBα mouse lungs have reduced endotoxin-induced pulmonary edema and injury

To determine if NF-κB signaling in the bronchioalveolar epithelium increases susceptibility to endotoxin-induced lung injury in *Gprc5a*-ko mouse lungs, we compared the effects of LPS on lung injury in *Gprc5a*-ko/SPC-SR-IκBα mice with those in *Gprc5a*-ko mice. LPS administration decreased body weight in all mouse groups of mice, which providing supporting evidence of endotoxin-induced injury. Noticeably, the ratio of wet-to-dry lung weight of *Gprc5a*-ko/SPC-SR-IκBα mice, especially at 48 hours after LPS treatment, was significantly reduced (**Fig. 5B**). These findings suggest a reduced inflammatory

pulmonary edema and injury. Consistently, histologic analysis showed that lesions in the lungs of *Gprc5a*-ko/SR-IκBα mice were significantly reduced as compared to those in *Gprc5a*-ko mice (**Fig. 5C**). Vascular wall thickness was reduced, inflammation attenuated, and the alveolar spaces approached normal size in *Gprc5a*-ko/SR-IκBα mice, especially at 48 h following LPS treatment (**Figs. 5C-D**). These findings demonstrate that LPS-induced injury in lung morphology was alleviated in *Gprc5a*-ko/ SPC-SR-IκBα mice when compared with those of *Gprc5a*-ko mice (**Fig. 5C**). Thus, blockage of NF-κB activity in the bronchioalveolar epithelium inhibits endotoxin-induced pulmonary edema and injury in the genetic background of *Gprc5a*-ko mouse.

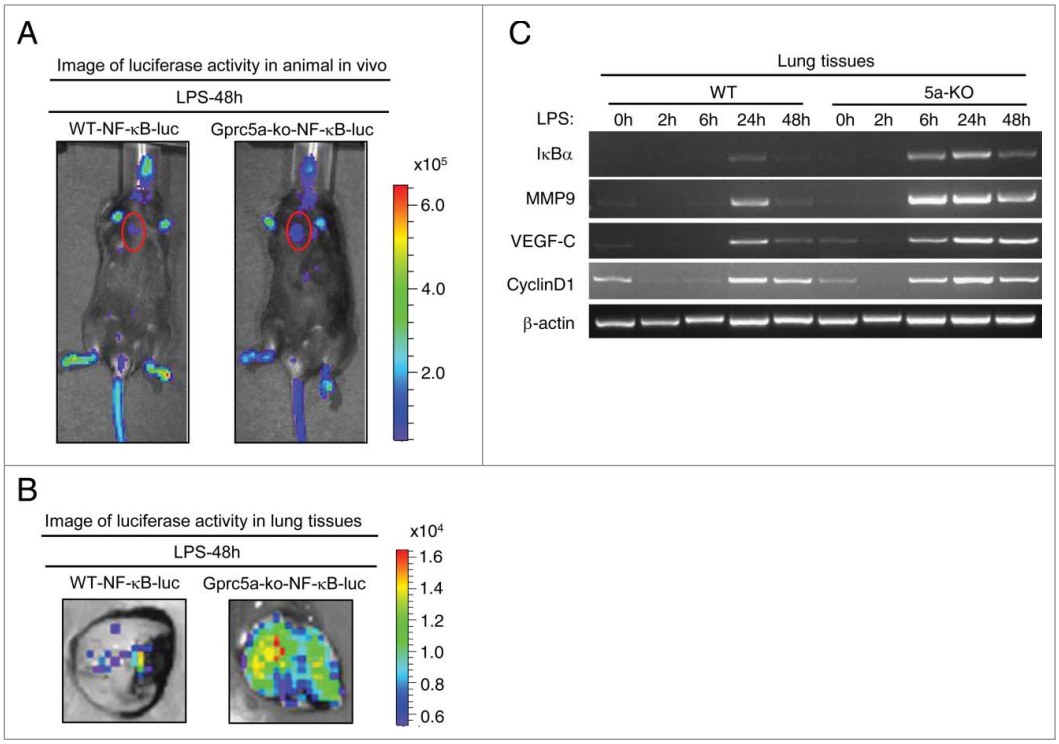

Figure 3. LPS induced potent and persistent activation of NF-κB in the bronchioalveolar epithelium of *Gprc5a*-ko mouse lungs. (**A**) Luminescence imaging from anesthetized mice of wild-type/NF-κB-luc and *Gprc5a*-ko/NF-κB-luc was performed with an ultrasensitive camera 48 h following endotoxin and luciferin administration. Tissue encircled in red is lung. (**B**) Images are of excised lung tissues from mice used above (**A**). NF-κB-dependent luminescence of lung tissue was assayed by imaging or luminometry. (**C**) RT-PCR analysis of mRNAs of NF-κB target genes, IκBα, MMP9, VEGF-C, Cyclin D1; times indicate tissue retrieval post-LPS treatment.

Endotoxin-induced production of proinflammatory cytokines and chemokines is reduced in *Gprc5a*-ko/SPC-SR-IκBα mouse lungs

For comparison, we measured the expression levels of proinflammatory cytokines and chemokines in both *Gprc5a*-ko and *Gprc5a*-ko/SPC-SR-IκBα mouse lungs of different groups after LPS treatment. RT-PCR analysis showed that mRNA expressions for Il-1β, TNFα and KC were significantly reduced in *Gprc5a*-ko/SR-IκBα mouse lungs as compared to those from *Gprc5a*-ko mouse lungs (**Figs. 6A-D**). Of note, the mRNAs of IL1β, TNFα and KC in *Gprc5a*-ko/SR-IκBα mice were decreased greatly, especially at 48 h after LPS treatment, as compared to those in *Gprc5a*-ko mice (**Figs. 6A-E**). Next, we examined the protein levels of IL1β, TNFα and IL-6 by ELISA. Interestingly, the protein levels of IL-1β, TNFα and IL-6, from *Gprc5a*-ko and *Gprc5a*-ko/SR-IκBα mouse lungs, were not significantly different at 2, 6, or 24 h after LPS treatment (**Fig. 6F-6H**). However, significant differences in these protein levels were observed at 48 h after LPS treatment (**Fig. 6I**). These results indicate that, blockage of NF-κB by SR-IκBα in bronchioalveolar epithelium attenuated both the intensity and duration of endotoxin-induced inflammatory response. Thus, dysregulated NF-κB is the predominant contributor to the increased susceptibility of *Gprc5a*-ko mice to endotoxin-induced lung edema and injury.

Discussion

In this study, we performed a comprehensive characterization of the physiological, structural, and biochemical events related to lung edema and lung injury using an endotoxin-induced injury model in *Gprc5a*-ko mice. We found that *Gprc5a*-ko mice had an increased susceptibility to LPS-induced pulmonary edema, injury and an increased inflammatory response. Although endotoxin induced production of proinflammatory cytokines and chemokines in all groups of mice, the inflammatory response in *Gprc5a-ko* mouse lungs was more intense and persistent than that observed in wild-type mouse lungs. The relative nature of this finding suggests that *Gprc5a* deficiency disrupts the balance of lung homeostasis, probably through disruption of a negative feedback control mechanism in response to inflammation.

Sepsis is the leading clinical cause of ALI/ARDS. Bacterial endotoxin (LPS) induces potent biological reactions, including lung inflammation and injury. Thus, the model of experimentally-induced injury with endotoxin coupled with genetically manipulated mice are valuable tools to identify the important events surrounding the pathogenesis of endotoxin-induced lung inflammation and injury.[21-23] Previously, we found that *Gprc5a*-ko mice have an increased incidence of acidophilic macrophage pneumonia, pulmonary inflammatory disease, as well as lung tumor development.[20] In this study, we demonstrate that an

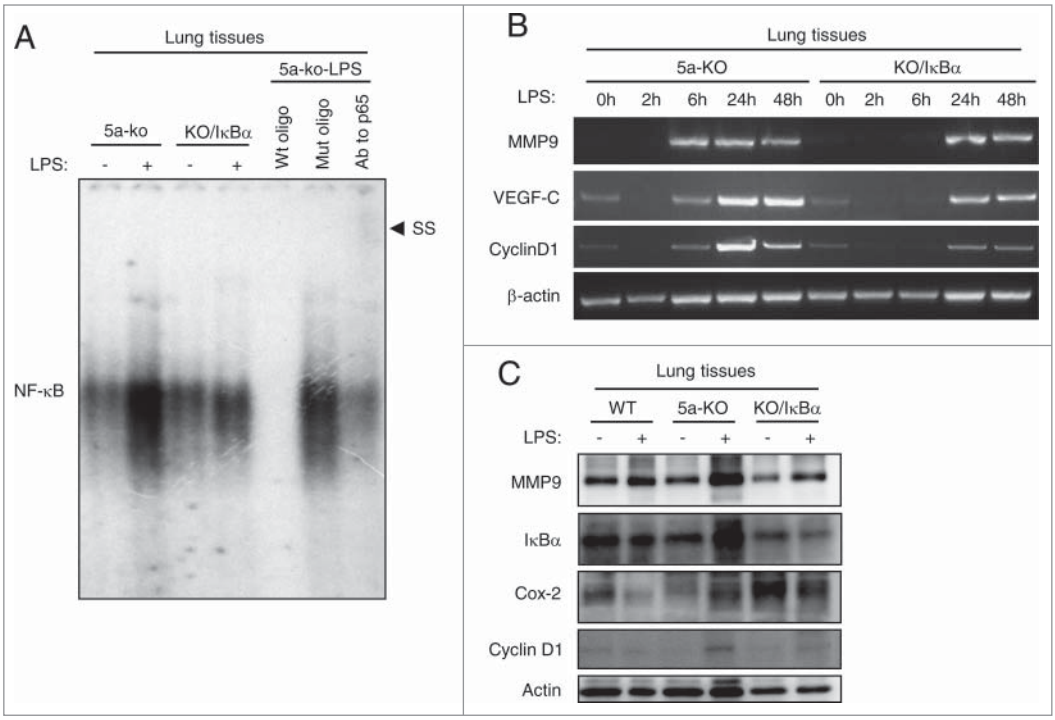

Figure 4. NF-κB signaling is selectively inhibited in the bronchioalveolar epithelium of *Gprc5a*-ko/SPC-SR-IκBα mouse lungs. (**A**) EMSA of NF-κB DNA-binding proteins in lung tissue nuclear extracts from *Gprc5a*-ko (5a-ko) and *Gprc5a*-ko/SPC-SR-IκBα(5a-ko/IκBα) mice treated with or without LPS. Specificity of NF-κB bindingconsensus site was analyzed by using (80 times) either wild-type or mutant oligonucleotides (cold) as competitors. Addition of anti-p65 antibodies to the EMSA reaction assessed the presence of p65 in the shifted complex by induction of a super-shifted complex (SS). (**B**) Representative RT-PCR analysis of NF-κB target genes MMP9, VEGF-C, Cyclin D1, from pulmonary tissues. Time points indicate retrieval of tissue post-LPS treatment. (**C**) Immunoblot analysis of NF-κB target gene products, MMP9, IκBα, Cox2, and Cyclin D1, from murine pulmonary homogenates.

upregulation in NF-κB activity in the bronchioalveolar epithelium of *Gprc5a*-ko mouse lungs is a major contributor to the increased susceptibility for lung injury in these mice. Importantly, selective inhibition of NF-κB in the bronchioalveolar epithelial cells of *Gprc5a*-ko/SPC-SR-IκBα mouse lungs resulted in suppressed activation of NF-κB after endotoxin exposure. In addition, this selective inhibition of NF-κB by specific expression of SR-IκBα in the bronchioalveolar epithelium in *Gprc5a*-ko/SPC-SR-IκBα mice greatly reduced the endotoxin-induced inflammatory response and lung injury. Therefore, these data provide compelling evidence that the susceptibility to ALI in *Gprc5a*-ko mouse lungs is mainly through the NF-κB pathway.

There are more than 40 cell types in lung tissue. Multiple cells are implicated in initiation and regulation of pulmonary inflammation, including macrophages, epithelial cells, fibroblasts, and endothelial cells. Many studies focused on NF-κB signaling in immune cells, some suggest that alveolar macrophages are required for initiation of LPS-induced inflammatory responses in the lungs, likely through activation of NF-κB signaling.[24,25] Other studies showed that NF-κB in nonimmune cells is a critical determinant in the lung's response to injurious stimuli. For instance, some data indicate that the airway epithelium controls lung inflammation and injury through NF-κB pathway.[26] One study showed that expression of a dominant NF-κB inhibitor in airway epithelium, under the epithelial-specific CC10 promoter, reduced neutrophilic lung inflammation, and diminished lung injury induced by airway or systemic delivery of LPS.[26] Therefore, it is reasonable to focus on the bronchioalveolar epithelial cells as being critical to the regulation of acute lung inflammation and injury.[27] Respiratory epithelial cells secrete proinflammatory mediators and upregulated adhesion molecules in response to bacterial stimuli or endogenous factors such as IL-1β and TNFα[28]. *Gprc5a* is preferentially expressed in airway epithelial cells.[16] In fact, the increased levels of NF-κB in bronchioalveolar epithelial cells from *Gprc5a*-ko mouse lungs suggested that *Gprc5a* is involved in regulation of this pathway. Interestingly, the TNFα level in lung tissues of *Gprc5a*-ko mice were significantly higher than levels found in pulmonary tissue from wild-type mice, whereas TNFα levels in serum were similar between these groups. This finding supports the contention that *Gprc5a* depletion mainly affects NF-κB activation in bronchioalveolar epithelial cells.

The presence of Toll-like receptor 4 (TLR4) on lung structural cells, but not haematopoietic cells, has been reported to be critical for TLR4-mediated airway inflammation.[29] Similarly, NF-κB in bronchioalveolar epithelial cells is critical for the regulation of lung inflammation induced by aerosolized LPS. Targeted inhibition of NF-κB activation in distal airway epithelial cells impaired the inflammatory response to inhaled LPS.[30] It appears that *Gprc5a* deficiency primarily affects the persistent phase (48h after LPS treatment) of NF-κB activation, suggesting that *Gprc5a* may regulate a negative feedback mechanism. We conclude that, *Gprc5a* depletion leads to a dysregulated NF-κB activation in bronchioalveolar epithelial cells, which results in the increased susceptibility to LPS-induced ALI.

Previous reports showed that genes involved in inflammatory and the immune pathways play a role in conferring susceptibility to lung injury. Candidate genes associated with susceptibility, based on clinical course and outcomes in ALI/ARDS, are ACE, EGF, GSTM1, IL-6, IL-10, MIF, NRF2, NFKB1, TLR1, TNF, PLAU, and VEGFA. The role of these genes are varied and related to inflammation, immune response, vascular

permeability, vascular tonus, repair, chemotaxis, cell motility and coagulation.[6] It appears that *Gprc5a* fits this scenario since *Gprc5a* deficiency results in a dysregulated NF-κB pathway with increased pulmonary injury and inflammation.[20]

The results of this study allow us to make the following conclusions: First, the *Gprc5a* gene is important in controlling the susceptibility to endotoxin-induced lung injury. Second, NF-κB in bronchioalveolar epithelial cells is the major pathway responsible for the increased susceptibility to endotoxin-induced lung injury in *Gprc5a* knockout mice since selective inhibition of NF-κB by super-repressor IκBα in bronchioalveolar epithelial cells diminished the inflammatory response, and alleviated lung injury. In the clinical setting, if a genetic or molecular marker of the susceptibility to ALI/ARDS were known, an altered treatment plan may be employed. Our study demonstrates that *Gprc5a* deficiency confers susceptibility to endotoxin-induced pulmonary edema, injury and inflammatory response, mainly through NF-κB signaling in bronchioalveolar epithelium of lung.

Materials and Methods

Experimental animal and LPS administration

Gprc5a-knockout (ko) mice were generated in a mixed background of 129sv × C57BL/6 as

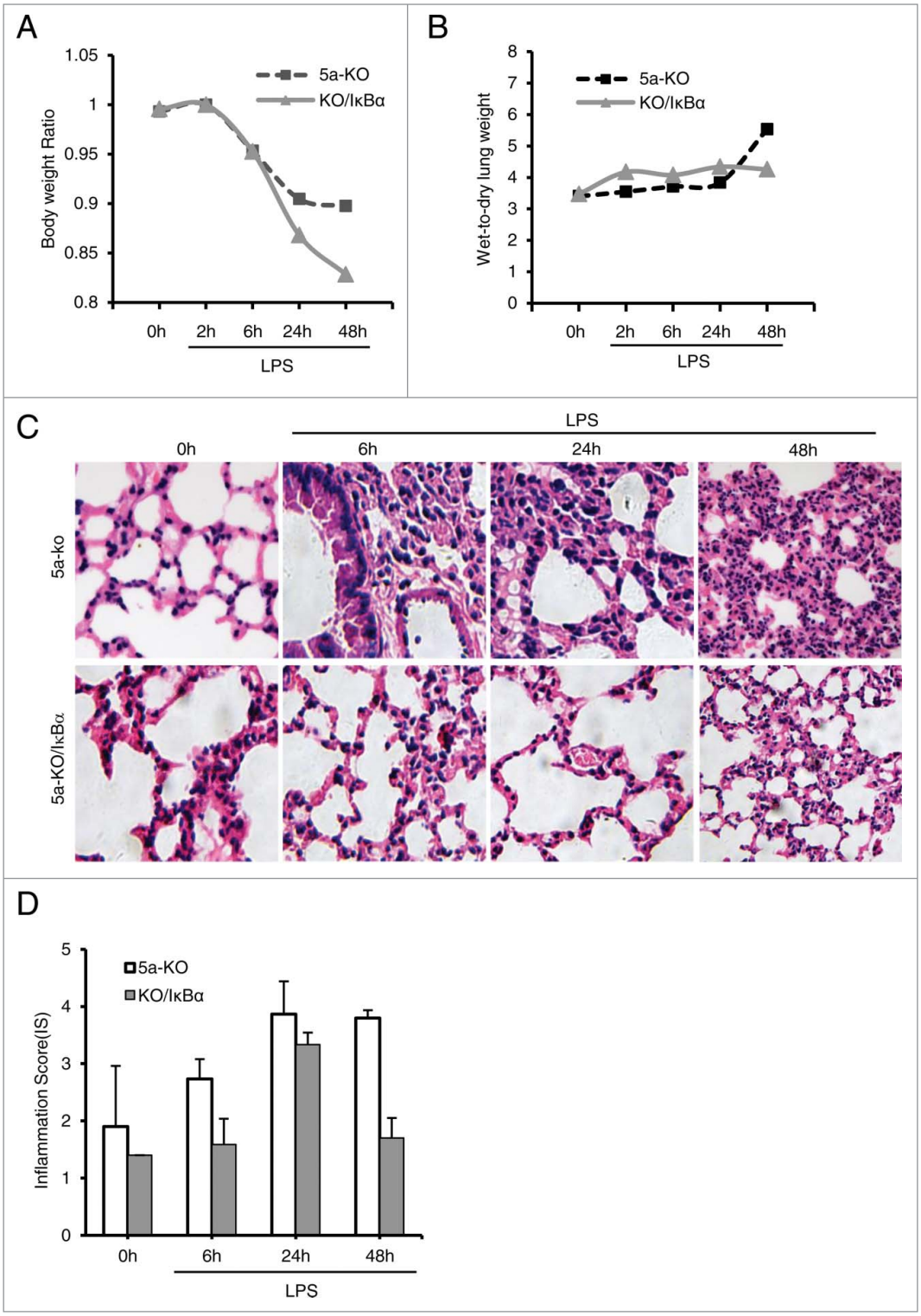

Figure 5. *Gprc5a*-ko/SPC-SR-IκBα mice are resistant to endotoxin-induced pulmonary edema and injury. (**A**) Body weight of *Gprc5a*-ko (5a-KO) and *Gprc5a*-ko/SPC-SR-IκBα (KO/IκBα) mice (n = 3) treated with endotoxin over 48 h period. Present data are weights normalized to baseline for each animal. (**B**) Lung water measurements (wet:dry tissue weight ratio) in mice treated with endotoxin and in control animals over 48 h period after treatment. Wet-dry weights are normalized to the baseline values (n = 3 for each time point). (**C**) H&E-stained sections of lung tissue from 5a-KO and 5a-KO/IκBα mice taken before endotoxin administration (0h) and at 6 h, 24 h, and 48 h after endotoxin administration. (**D**) Inflammation score (IS) was used as a semi-quantitative assessment for pulmonary edema and injury.

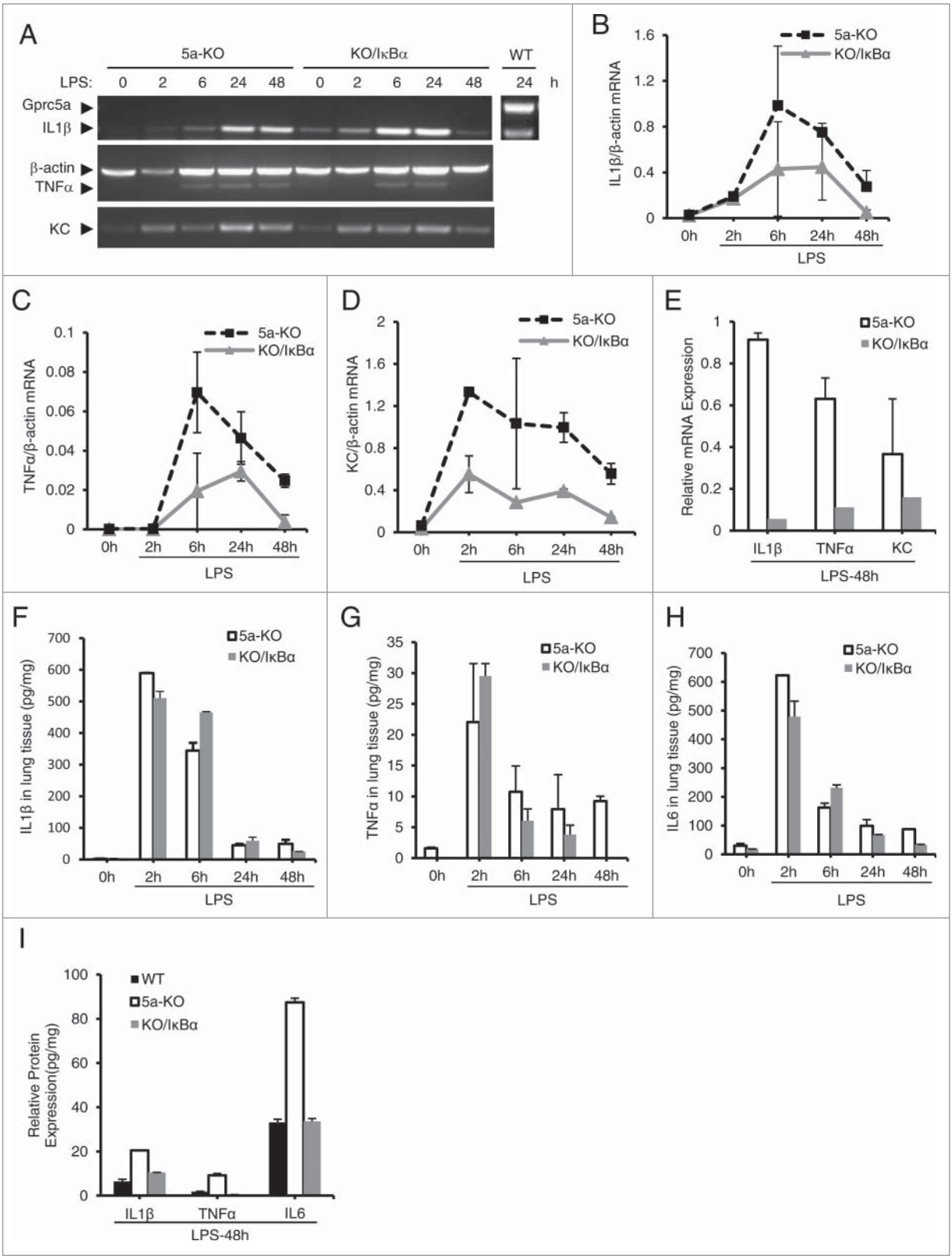

Figure 6. Production of proinflammatory cytokines and chemokines is reduced in *Gprc5a*-ko/SPC-SR-IκBα mouse lungs after endotoxin treatment. (**A**) Images are representative of RT-PCR analysis for proinflammatory cytokines at each point. The averages of mRNA, IL-1β (**B**), TNFα (**C**) and KC (**D**), at different time point were shown. (**E**) The average of mRNA expression levels for IL-1β, TNFα, and KC from wild-type and *Gprc5a*-ko mouse lungs at 48 h after endotoxin treatment. (**F-I**) Cytokine proteins, IL-1β (F), TNFα (**G**) and IL-6 (**H**), in lung tissues were measured after LPS treatment. (**I**) Protein levels for IL-1β, TNFα, and IL-6 from WT, KO and KO/IκBα mouse lungs 48 h after LPS treatment using ELISA.

described previously.[18] INS-NF-κB-luc transgenic mice (C57 BL/6J) were obtained from Cgene (Oslo, Norway). *Gprc5a*-ko/ NF-κB-luc mice were obtained by cross-breeding *Gprc5a*-ko mice with INS-NF-κB-luc mice. SPC-SR-IκBα mice were kindly provided by Dr. Christopher B. Wilson (University of Washington School of Medicine, Seattle).[30] *Gprc5a*-ko/SPC-SR-IκBα mice were obtained by cross-breeding *Gprc5a*-ko mice with SPC-SR-IκBα mice. INS-NF-κB-luc transgenic mice (C57BL/6J-background) were purchased from Cgene AS (Oslo Innovation Center, Norway). *Gprc5a*-ko/ NF-κB-luc mice were obtained by cross-breeding *Gprc5a*-ko mice with INS-NF-κB-luc transgenic mice. The genotype of mouse progeny was performed as described previously[18] or by the manufacturer. Mice were maintained according to a protocol approved by Shanghai Jiao Tong University School of Medicine Animal Care and Use Committee [experimental animal use permission No: SYXK (Shanghai) 2008-0050] in the specific pathogen-free animal facility in the university. Mice were monitored daily for evidence of disease or death. Eight-week-old wild-type, *Gprc5a*-ko and *Gprc5a*-ko/SPC-SR-IκBα mice were received an i.p. injection of LPS (*Escherichia coli* 0111:B4 LPS, from Sigma, St. Louis, MO) at 150 μg/25g of body weight, dissolved in saline solution (0.9% NaCl) or saline alone (n=3). Groups of animals were killed at 2, 6, 24 and 48 h following LPS administration. Lung tissues were collected for different analysis and determination of lung wet weight. The left lobe of lung was fixed with 4% paraformaldehyde in phosphate-buffered saline (pH 7.2–7.4) for 24 h. The remainder of lung tissues wshomogenated in liquid nitrogen for protein or RNA extraction. Lung and body weight of each mouse were obtained. Blood samples, collected before death,were used to quantify cytokine and growth factors production. Control groups of animals received no LPS treatment.

Hematoxylin-Eosin (H&E) staining and IS calculation

Fixed lungs were embedded in paraffin, and sequential 5μm sections were stained with hematoxylin eosin (H&E). Each lung section was systematically scanned with a magnification of x 100; 5 successive fields were graded according to the degree of inflammatory infiltration and the area of involved lesions: grade 0, normal tissue; grades 1–3, the presence of pulmonary inflammation with the extent of pathology graded as 1 (<20% of the slide), 2 (20% to 50% of the slide) or 3 (>50% of the slide). After examination of the entire section, the mean score from all examined fields was calculated as the inflammation score (IS).

Lung cytokines mRNA expression analysis by RT-PCR

Total RNA samples from lung tissues were extracted with TriPure Isolation Reagent (Roche, Switzerland) and cDNA prepared from 1 μg of total RNA using the SuperScript III System (Invitrogen Life Technologies). mRNAs levels were determined by RT-PCR, using the following primers (mouse orgin):

Gprc5a (F: 5'-GACACACTCTATGCACCTTATTC-3' and R: 5'-ACAGACCTTGTCTACTCCAG-3'); IL1β (F: 5'-GAAATGCCACCTTTTGACAGTG-3' and R: 5'-CTGGATG CTCTCATCAGGACA-3'); TNFα (F: 5'-CCTGTAGCC-CACGTCGTAG-3' and R: 5'-GGGAGTAGACAAGGTACA ACCC-3'); KC (F: 5'-ACTGCACCCAAACCGAAGTC-3'; R: 5'-TGGGGACACCTTTTAGCATCTT-3'); internal standard β-actin (F: 5'-AACAGTCCGCCTAGAAGCAC-3' and R: 5'-CGTTGACATCCGTAAAGACC-3'); CyclinD1 (F: 5'-ACACGGACTACAGGGGAGTT-3'; R: 5'- CTCACA-GACCTCCAGCATCC-3'); VEGFC: (F: 5'-GCTTCTTGT CTCTGGCGTGT-3'; R: 5'-TGCCGTCCTTATCGTAGT CA-3'), MMP9: (F: 5'-TGTCATCCAGTTTGGTGTCG-3'; R: 5'-TGCCGTCCTTATCGTAGTCA-3'); IκBα (F: 5'-AAAT CCCCTGCCAGCGTTTA-3'; R: 5'-CCAAGTGCAGGAAC-GAGTCT-3').

Lung cytokines detected by ELISA assay

Mice were sacrificed at predetermined time points, and lungs homogenized in RIPA buffer (Millipore,Billerica, MA,USA) containing complete protease inhibitors (Roche, BasalSwitzerland). Cytokine protein levels were measured by ELISA (R&D Systems, Minneapolis, MN).

***In vivo* and *ex vivo* imaging of luciferase activitiy**

Mice anesthetized with isoflurane were injected i.p. with 150 mg luciferin/kg body weight for *in vivo* imaging. Five minutes later, mice were placed face up in the chamber and imaged for 5 min with the camera set at the highest sensitivity by IVIS Imaging System 100 Series (Xenogen, UK). Photons emitted from tissues were quantified using living image software (Xenogen).

For *ex vivo* imaging, mice were anesthetized and i.p. injected with luciferin. Five minutes later, mice were sacrificed and lung tissues were rapidly removed. Isolated tissues were placed in the IVIS system and imaged with the same setting used for *in vivo* studies. Signal intensity was quantified as the sum of all detected photon counts from tissues and presented as photons/sec.

Electrophoretic mobility shift assay (EMSA)

NF-κB DNA-binding activity in nuclear extracts from lung tissues (4 mice per group) was examined as described.[20,31] The following oligonucleotides were used for the analysis: wild-type NF-κB–binding oligonucleotide,5'-CGGAAAGTCCCCAGCG GAAAGTCCCTGAT-3'; mutant NF-κB-binding oligonucleotide, 5'-CGGAAAGTGAGCAGCGGAAAGTGAGTGAT-3'.

Statistical analyses

Expressed data are the mean ± SEM. Statistical analysis utilized the statistics package SPSS19.0 (SPSS Inc.., Chicago, USA). Comparisons were made using unpaired t-test assuming unequal distribution. Multiple group comparisons used one-way ANOVA. Statistical significance was set at $P < 0.05$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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