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Cooperative Interactions between Toll-Like Receptor 2 and Toll-Like Receptor 4 in Murine *Klebsiella pneumoniae* Infections

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Klebsiella pneumoniae is an opportunistic and clinically significant emerging pathogen. We investigated the relative roles of Toll-like receptor (TLR) 2 and TLR4 in initiating host defenses against *K. pneumoniae*. TLR2 knockout (KO), TLR4 KO, TLR2/4 double KO (DKO), and wild-type (WT) mice were inoculated with *K. pneumoniae*. Mice in each group were sacrificed after either 12 or 24 h, and the lungs, liver, and blood were harvested to enumerate bacterial colony-forming units (CFU). Cytokine and chemokine levels were analyzed using enzyme-linked immunosorbent assay and real-time PCR, and pneumonia severity was determined by histopathological analysis. Survival was significantly shortened in TLR4 KO and TLR2/4 DKO mice compared with that of WT mice after infection with 5×10^3 CFU. TLR2 KO mice were more susceptible to infection than WT mice after exposure to a higher infectious dose. Bacterial burdens in the lungs and liver were significantly higher in TLR2/4 DKO mice than in WT mice. Serum TNF-α, MCP-1, MIP-2, and nitric oxide levels were significantly decreased in TLR2/4 DKO mice relative to those in WT mice, and TLR2/4 DKO mice showed significantly decreased levels of TNF-α, IL-6, MCP-1, and inducible nitric oxide synthase mRNA in the lung compared with those in WT mice. Collectively, these data indicate that TLR2/4 DKO mice were more susceptible to *K. pneumoniae* infection than single TLR2 KO and TLR4 KO mice. These results suggest that TLR2 and TLR4 play cooperative roles in lung innate immune responses and bacterial dissemination, resulting in systemic inflammation during *K. pneumoniae* infection.

Keywords: Chemokine, cytokine, *Klebsiella*, knockout, Toll-like receptor

Introduction

Klebsiella pneumoniae is a gram-negative bacterium that is part of the normal flora in the mouth, skin, and intestines of humans [1, 2]. *Klebsiella* infection can cause destruction in human and animal pulmonary systems when aspirated, causing a variety of effects such as pneumonia, liver abscess, and bacteremia [2, 3]. Death from pneumonia caused by *K. pneumoniae* occurs as a direct result of primary systemic bacterial dissemination resulting in sepsis and acute lung injury [3, 4].

Toll-like receptors (TLRs) are recognized as key regulators of innate immune responses that eliminate most bacterial organisms from the respiratory tract [5–7]. TLRs are expressed by numerous types of cells, including macrophages, dendritic cells, B and T lymphocytes, endothelial cells, and mucosal epithelial cells. When *K. pneumoniae* enters the lung, TLRs specific for pathogen-associated molecular patterns (PAMPs) are activated, triggering the release of chemokines and cytokines that are crucial for successful host defense against bacteria [8]. Cytokines regulate immune responses through receptors; cytokines determine the balance between humoral

and cellular immune responses and modulate the maturation, growth, and sensitivity of certain cell populations [2]. With inflammation, neutrophils and then monocytes infiltrate the site of infection or injury. Neutrophils perform functions such as bacterial killing and tissue destruction and then undergo apoptosis, whereas monocytes differentiate into macrophages at the affected site. Macrophages and other phagocytes clear apoptotic neutrophils, leading to resolution of inflammation [9]. TLR2 recognizes a wide spectrum of microbial components, including peptidoglycan and lipoteichoic acid, which are the main stimulatory components of gram-positive bacteria [10–12]. In addition, TLR2 contributes to the recognition of *Klebsiella* through interactions with bacterial lipoproteins, including outer membrane protein A [13]. Lipopolysaccharide (LPS) is part of the outer membrane of gram-negative bacteria and is in charge of stimulating innate immunity against gram-negative infections [6]. TLR4 is needed for an effective innate immune response against gram-negative bacteria, because TLR4 recognizes LPS [6, 11, 13]. TLR2 is involved in the recognition of diverse bacteria and their products, including gram-positive bacteria and peptidoglycans, whereas TLR4 is a receptor for LPS, an endotoxin of gram-negative bacteria [11]. In general, TLR2 is important for immune defense against gram-positive bacterial and fungal infections, but it has a limited role, with some exceptions, in immunity against gram-negative bacteria.

Nitric oxide, a ubiquitous biological molecule produced by various types of cell, is associated with the progression of a wide range of diseases, exerting both detrimental and beneficial effects in the lungs [14]. Inducible nitric oxide synthase (iNOS)-derived nitric oxide regulates the production of chemokines positively or negatively. Although the mechanisms underlying such dual effects of nitric oxide remain unknown, the level of nitric oxide and duration of nitric oxide exposure appear to be determining factors [9]. Nitric oxide plays a crucial role in antibacterial host defense against *K. pneumoniae* pneumonia, partially by regulating the phagocytic and microbicidal activities of macrophages [15]. TLRs recognize specific bacterial products and then activate antimicrobial mechanisms in macrophages, including the production of nitric oxide by iNOS [16].

Several earlier studies investigated the role of TLRs in host defense against *K. pneumoniae*. However, the specific TLR pathways involved in *Klebsiella* activation of an innate immune response remain controversial. In this study, to investigate the potential interacting roles of TLR2 and TLR4 in initiating an immune response against *K. pneumoniae* infection, we assessed the innate immune responses in TLR2 knockout (KO), TLR4 KO, and TLR2/4 double KO (DKO) mice that were challenged intratracheally with *K. pneumoniae*. This study also examined the effects of nitric oxide production on *K. pneumoniae* infection and the correlation between nitric oxide effects and the presence of TLRs.

Materials and Methods

Animals

All procedures were performed according to the guidelines of and were approved by, the Institutional Animal Care and Use Committee of Konkuk University (IACUC No. KU16157). TLR2 KO and TLR4 KO mice on a C57BL/6 background were acquired from Oriental Bio-Service Inc. (Japan) and then raised in the laboratory animal breeding room of the College of Veterinary Medicine, Konkuk University. WT C57BL/6 mice were maintained in our breeding room. TLR2/4 DKO mice were generated by intercrossing TLR2 KO and TLR4 KO mice. The genotypes of the TLR2 KO, TLR4 KO, and TLR2/4 DKO mice were confirmed by polymerase chain reaction (PCR) amplification of tail DNA. All mice were housed in individually ventilated cages with sterilized bedding in specific pathogen-free conditions at a temperature of 22 ± 2°C and humidity of 50 ± 10% under a 12-h light/12-h dark cycle. Mice were fed sterilized food and water ad libitum.

Bacterial Strains and Preparation

K. pneumoniae serotype 2 (ATCC 43816), which is frequently used in mouse studies, was obtained from the American Type Culture Collection. *K. pneumoniae* was grown overnight on a 5% sheep blood agar plate at 37°C for 16 h, inoculated into tryptic soy broth (BD, USA), grown overnight with shaking, and then diluted with new tryptic soy broth and grown for 3 h to the mid-logarithmic phase at 37°C. Bacteria were pelleted by centrifugation at 1,200 ×*g* for 10 min, resuspended in sterile phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4), and quantitated by spectrophotometry. The bacterial concentration was determined by measuring the absorbance at 600 nm and then plotting the optical density on a standard curve that was previously generated from known bacterial colony-forming unit (CFU) values. The number of *K. pneumoniae* (CFU/ml) was determined by plating serial dilutions and counting the CFU.

Intratracheal Infection with *K. pneumoniae*

At 8 weeks of age, female WT, TLR2 KO, TLR4KO, and TLR2/4 DKO mice were infected with *K. pneumoniae* intratracheally. Mice were anesthetized with 40 mg/kg Zoletil (Virbac Laboratories, France) and 5 mg/kg Rompun (Bayer Korea, Korea) via intraperitoneal injection and placed recumbent on their dorsa. An inoculum of 5×10^3 CFU or 1×10^4 CFU/30 µl was administered via sterile 30-gauge needle after exposing the trachea. After inoculation, the skin incision was closed by applying surgical staples. The number

of moribund or dead mice was monitored twice daily, and the severity of disease was assessed for 7 days.

Enumeration of Survived Bacteria in Lung, Liver, and Blood

Bacterial loads in the lungs, liver, and blood of *K. pneumoniae*-infected mice were determined at different time points postinfection. Briefly, the lungs and liver were removed, maintained in sterile conditions, and weighed. For organ CFU determinations, the lungs and liver were homogenized in sterilized PBS (weight:volume, 1:4) using a tissue homogenizer. A small aliquot of tissue homogenate was serially diluted in sterilized PBS, plated on 5% sheep blood agar, and incubated at 37°C, and then colonies were counted. For blood CFU determinations, blood was collected via the caudal vena cava vein in heparin EDTA-containing tubes. The blood was serially diluted, plated on 5% sheep blood agar, and incubated at 37°C, and then colonies were counted.

Quantitative Histopathological Examination

The lungs were removed and fixed with 10% neutral formalin solution, and then each lobe was embedded in paraffin and cut into 4-μm-thick sections. The sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin, and then dehydrated, cleared, and mounted. The slides were examined under 200× objective magnification using a BX51 light microscope (Olympus, Japan), and images were captured using a DP71 digital camera and software (Olympus). To score lung inflammation, the entire lung surface was assessed for the following parameters: bronchiolitis (0: absent; 1: mild; 2: moderate; 3: severe), intra-alveolar inflammation (0: absent; 1: mild; 2: moderate; 3: severe), edema (0: absent; 1: present), and endotheliolitis (0: absent; 1: present). This scoring scheme was modified from a previously described method [4, 17]. The total lung inflammation score was expressed as the sum of the scores of each lobe after independent scoring by two pathologists.

Analysis of Cytokines and Chemokines in Serum

The levels of TNF-α, IL-10, MCP-1, and MIP-2 in the serum were quantitated using sandwich enzyme-linked immunosorbent assay kits (eBioscience, USA and R&D Systems Inc., USA) according to the company's instructions. The levels of nitric oxide in the serum were quantitated using the Griess reagent system (Promega, USA). Mouse sera were diluted 1:2 or 1:3 in diluent buffer to quantify TNF-α, IL-10, MCP-1, and MIP-2 levels. The amount of each cytokine and chemokine present in the serum was analyzed with reference to a standard curve established using recombinant cytokines and chemokines. The detection limits were 8 pg/ml for TNF-α, 30 pg/ml for IL-10, 3.91 pg/ml for MCP-1, and 15.5 pg/ml for MIP-2. The results were expressed as picograms of cytokines per milliliter (pg/ml). The detection limit for nitric oxide was 2.5 μmol.

Reverse-Transcription PCR

The lungs were harvested at 12 or 24 h after *K. pneumoniae* infection, immediately frozen in liquid nitrogen, and then stored at −70°C until further analysis. Total RNA was prepared from frozen lungs using an RNeasy Mini Prep Kit (Qiagen) according to the company's instructions. Then, using 4 μg of each lung RNA, total RNA was reverse transcribed in 20 μl of reaction mixture containing RT buffer, 1 μl of oligo-dT 12-18 (500 μg/ml), 1 μl of 10 mM dNTP mix, 4 μl of 5× first-strand buffer, 1 μl of RNaseOUT, 2 μl of 0.1 M DTT, and 1 μl of MMLV reverse transcriptase. The cDNA was used as a template for real-time PCR amplification.

Quantitative PCR

Quantitative PCR was performed with a Bio-Rad CFX96 real-time PCR detection system (Bio-Rad, ON, Canada) according to the manufacturer's recommendations. The TaqMan fluorogenic probes and PCR primers for TNF-α, MCP-1, β-actin, and GAPDH were designed by Metabion (Martinsried, The Netherlands). The sequences of primers and probes used were as follows: for TNF-α, forward 5′-CCA TCC TTT TGC CAG TTC CT-3′, reverse 5′-ATG AAC GCT ACA CAC TGC AT-3′, probe 5′-TCA CCC CGA AGT TCA GTA GAC A-3′; for MCP-1, forward 5′-CAA CAA CTT CCT CTC CTG-3′, reverse 5′-AAG GGC TTC AAT CTG TTC-3′, probe 5′-CTT CTT TGG GAC ACC TGC TGC-3′; for β-actin, forward 5′-AGC CTT CCT TCT TGG GTA-3′, reverse 5′-CAC TTG CGG TGC ACG ATG GA-3′, probe 5′-AGC CAG AGC AGT AAT CTC CTT CT-3′; and for GAPDH, forward 5′-TCC AAG GAG TAA GAA ACC-3′, reverse 5′-GGA AAT TGT GAG GGA GAT-3′, and probe 5′-CCA GCA AGG ACA CTG AGC AA-3′. Validated primers (PrimePCR Assays; Bio-Rad) for iNOS and IL-6 were used for analysis. The threshold cycle, Ct, which inversely correlates with the amount of target mRNA, was determined by the cycle number at which the reporter fluorescent emission surpassed a threshold level. The relative changes in TNF-α, IL-6, MCP-1, and iNOS mRNA levels were normalized using levels of β-actin and GAPDH mRNA in the same samples.

Statistical Analysis

The statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, Inc., USA). Survival rates were compared using a log-rank test. The results were considered statistically significant when $p < 0.05$, $p < 0.01$, or $p < 0.001$ were obtained.

Results

Survival in WT, TLR2 KO, TLR4 KO, and TLR2/4 DKO Mice after *K. pneumoniae* Intratracheal Inoculation

This study compared the survival rates of WT, TLR2 KO, TLR4 KO, and TLR2/4 DKO mice after intratracheal infection with 5×10^3 CFU of *K. pneumoniae*. Twelve to 13 mice were examined per group. The number of moribund or dead mice was recorded twice daily for 7 days. Whereas all WT ($n = 13$) and TLR2 KO ($n = 13$) mice survived 7 days after infection, 8 of 12 TLR4 KO and 11 of 13 TLR2/4 DKO mice died within that time (Fig. 1A). At 1 day after infection, one TLR2/4 DKO mice died first. At 1.5 day after infection,

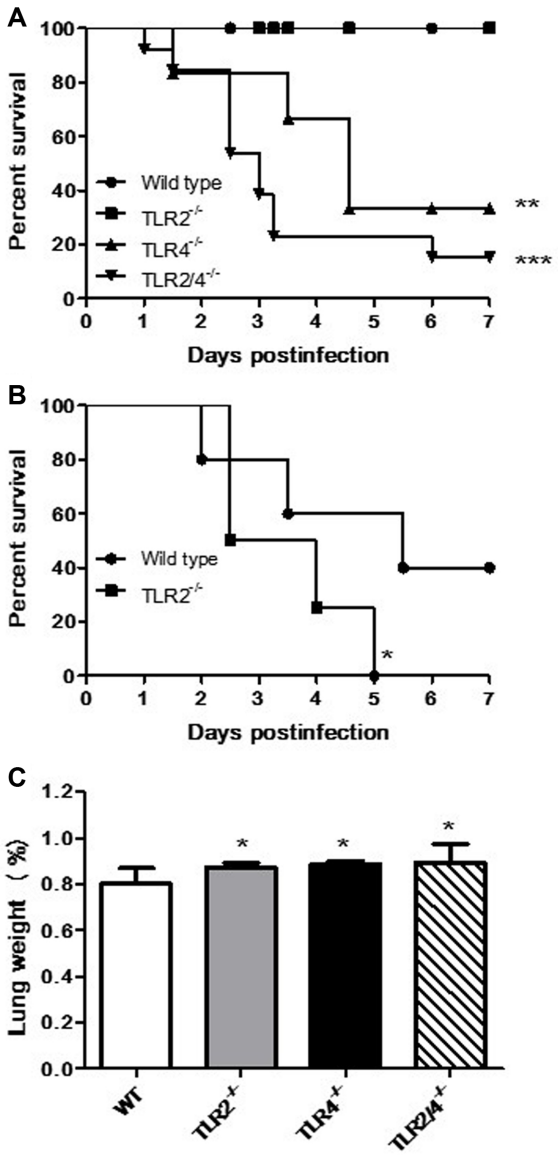

Fig. 1. Survival of mice after *K. pneumoniae* infection.
(**A**) Survival rate of wild-type (WT) (n = 13), TLR2 knockout (KO) (n = 13), TLR4 KO (n = 12), and TLR2/4 DKO (n = 13) mice. All mice were infected intratracheally with 5×10^3 CFU of *K. pneumoniae*. $^{**}p < 0.01$ and $^{***}p < 0.0001$ versus WT and TLR2 KO mouse. (**B**) Survival rate of WT (n = 10) and TLR2 KO (n = 8) mice. All mice were infected intratracheally with 1×10^4 CFU of *K. pneumoniae*. $^{*}p < 0.05$ versus WT mice. (**C**) Lung-to-body weight ratios. All mice (n = 6 in each group) were infected intratracheally with 5×10^3 CFU of *K. pneumoniae* and sacrificed at 24 h after infection. Data represent the mean ± SD. $^{*}p < 0.05$ versus WT mice.

one TLR2/4 DKO mice and two TLR4 mice died. As shown Fig. 1A, TLR2/4 DKO mice showed a significant ($p < 0.0001$) decrease in survival relative to that of WT and TLR2 KO mice inoculated with a dose of 5×10^3 CFU of bacteria. The survival rate in TLR4 KO mice was significantly ($p < 0.01$) lower than that in WT and TLR2 KO mice, and slightly (p = 0.079) higher than that in TLR2/4 DKO mice when infected with the same dose. An inoculum with 5×10^3 CFU of bacteria induced no mortality in WT or TLR2 KO mice. However, when a dose of 1×10^4 CFU bacteria was administered, TLR2 KO mice (n = 8) all died within 5 days, and 40% of WT mice (n = 10) survived for 7 days after infection ($p < 0.05$; Fig. 1B).

Organ Weight Changes

The lung-to-body weight ratios significantly ($p < 0.05$) increased after 24 h of *K. pneumoniae* infection (5×10^3 CFU) in TLR2 KO, TLR4 KO, and TLR2/4 DKO mice relative to that in WT mice (Fig. 1C). The liver-to-body weight ratios were not different between WT and TLR2 KO, TLR4 KO, and TLR2/4 DKO mice (data not shown). The spleen-to-body weight ratio was slightly increased in TLR2/4 DKO mice relative to that in WT and TLR2 KO mice (data not shown).

Histopathological Analysis

In WT, TLR2 KO, TLR4 KO, and TLR2/4 DKO mice after intratracheal *K. pneumoniae* infection (5×10^3 CFU), inflammatory cells and exudate were present in alveolar spaces, consistent with purulent bronchopneumonia (Figs. 2A–2D). In a histopathological examination, the degree of pneumonia in each lung differed between mice. Pulmonary inflammation was expressed as a total lung inflammation score. The severity of pneumonia was significantly ($p < 0.05$) greater in the TLR4 KO and TLR2/4 DKO mice than in the WT mice at 24 h after infection (Fig. 2E).

Bacterial Clearance in *K. pneumoniae*-Infected Mice

We harvested the lungs, liver, and blood from infected mice at 12 or 24 h after intratracheal *K. pneumoniae* infection (5×10^3 CFU). The number of bacteria in WT, TLR2 KO, TLR4 KO, and TLR2/4 DKO mice increased at 24 h relative to that at 12 h after intratracheal infection (Fig. 3). In the lungs, at 12 h after *K. pneumoniae* infection, TLR4 KO and TLR2/4 DKO mice showed significantly ($p < 0.05$) higher bacterial burdens than that in the WT. At 24 h after *K. pneumoniae* infection, TLR2/4 DKO mice showed a significantly ($p < 0.05$) greater number of bacteria in the lungs than that in WT mice. In the liver, TLR2/4 DKO mice showed significantly ($p < 0.05$) higher bacterial burdens than that in WT mice at 24 h after *K. pneumoniae* infection. The number of bacteria in blood was not significantly different between WT and other mice. These data indicate

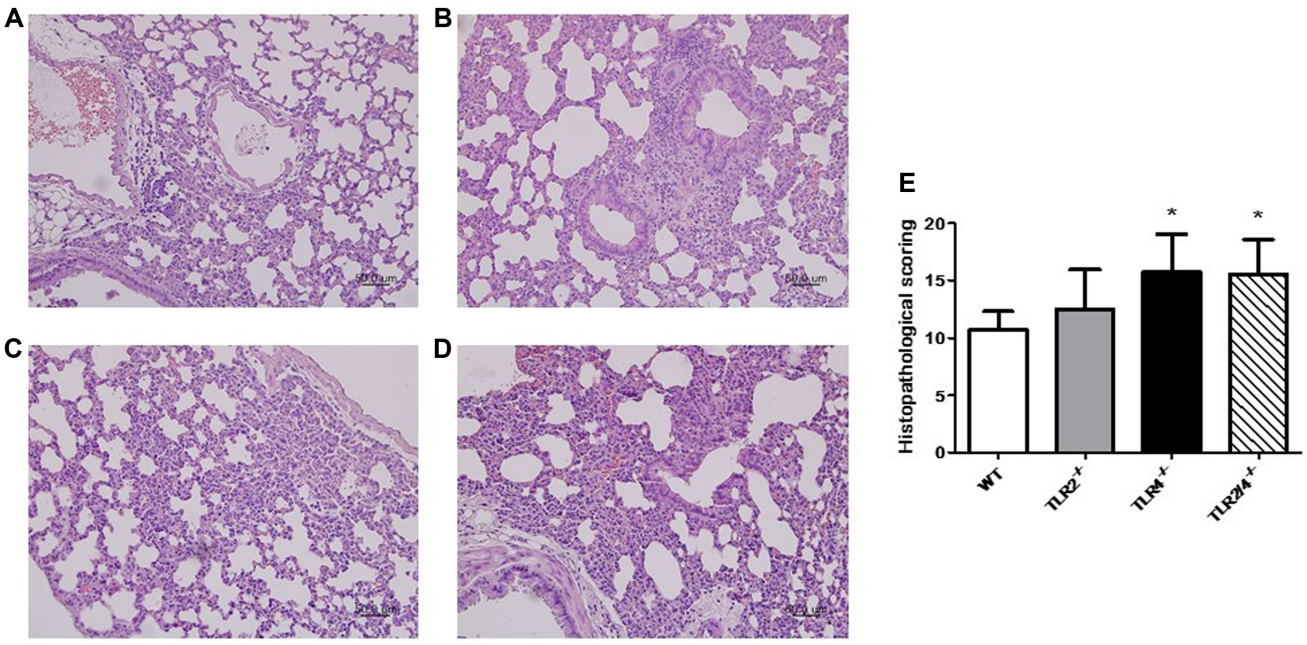

Fig. 2. Representative images of histopathological lesions (**A**–**D**) and pneumonia scores (**E**) in lungs.
Wild-type (WT) (**A**), TLR2 knockout (KO) (**B**), TLR4 KO (**C**), and TLR2/4 DKO (**D**) mice were infected intratracheally with 5×10^3 CFU of *K. pneumoniae* and sacrificed at 24 h after infection. Hematoxylin and eosin staining (200×). Data represent the mean ± SD. *$p < 0.05$ versus WT mice.

that clearance of *K. pneumoniae* in TLR4 KO and TLR2/4 DKO mice was significantly suppressed relative to that in WT mice.

Cytokine and Chemokine Levels in the Serum

We measured levels of circulating TNF-α, MCP-1, MIP-2, and nitric oxide in sera from infected mice after intratracheal *K. pneumoniae* infection (5×10^3 CFU). As shown in Fig. 4, the serum levels of TNF-α, MCP-1, and MIP-2 were significantly ($p < 0.05$) lower in TLR2/4 DKO mice than in WT mice at 12 h after infection. MCP-1 levels in the serum were significantly ($p < 0.05$) decreased in TLR2/4 DKO mice compared with those in WT mice at 24 h after infection. Finally, nitric oxide expression levels in the serum were significantly ($p < 0.05$) lower in TLR2 KO and TLR2/4 DKO mice than in WT mice at 24 h after infection.

Cytokine and Chemokine Expression

To assess the differences in immune response in WT, TLR2 KO, TLR4 KO, and TLR2/4 DKO mice, this study compared the levels of TNF-α, IL-6, MCP-1, and iNOS mRNA in lungs. Total RNA was isolated and subjected to quantitative reverse transcription PCR analysis. As shown in Fig. 5, TNF-α and IL-6 mRNA levels were significantly ($p < 0.05$) decreased in the lungs of TLR2/4 DKO mice relative to those in WT mice at both 12 and 24 h after infection with *K. pneumoniae*. MCP-1 mRNA was significantly ($p < 0.01$) decreased in the lungs of TLR2 KO, TLR4 KO, and TLR2/4 DKO mice relative to that in the lungs of WT mice at 12 h after infection. Of the three KO mice, TLR2/4 DKO mice showed the lowest levels of MCP-1 mRNA expression in the lungs at 12 h after infection. In TLR2/4 DKO mice, iNOS mRNA levels at 24 h after infection were significantly ($p < 0.05$) decreased relative to those in WT mice.

Discussion

The rapid removal of invading bacteria from the respiratory tract is important for an effective host defense mechanism against bacterial pneumonia. Apart from direct bacterial phagocytosis and killing, alveolar macrophages release diverse cytokines and chemokines that recruit blood neutrophils and monocytes into the pulmonary compartment and stimulate them [3]. To activate innate immune responses for effective lung defense against pathogens, the host recognizes conserved molecules uniquely expressed by pathogens, so-called PAMPs [18]. Outer membrane protein A from *K. pneumoniae* was reported as a new type of PAMP [18, 19]. As far as we know, it is not

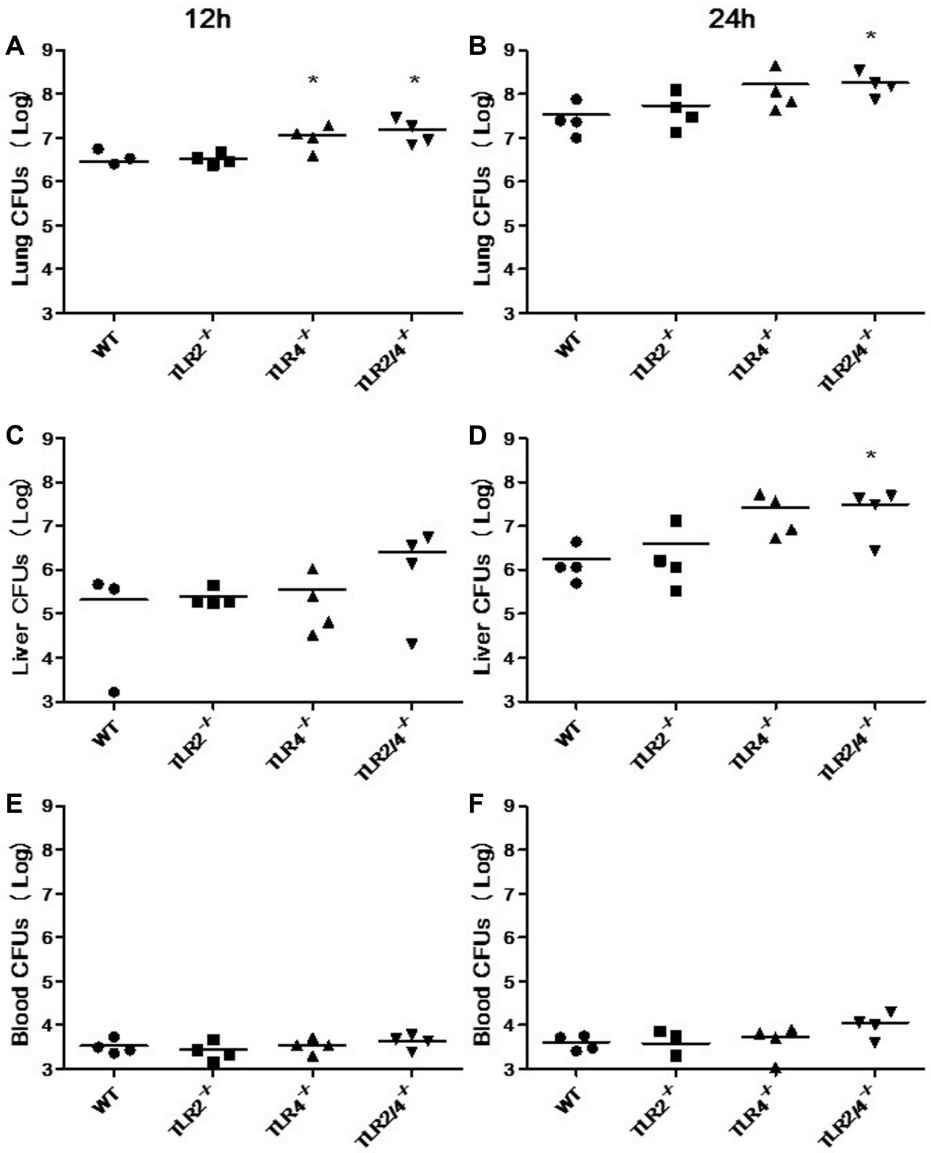

Fig. 3. Kinetics of bacterial proliferation and clearance in the lungs (**A**, **B**), liver (**C**, **D**), and blood (**E**, **F**).

All mice (n = 4 in each group) were infected intratracheally with 5×10^3 CFU of *K. pneumoniae* and sacrificed at 12 (**A**, **C**, **E**) or 24 h (**B**, **D**, **F**) after infection. TLR2/4 DKO mice showed higher bacterial burdens in the lungs and liver compared with those in wild-type (WT) mice. Data represent the mean ± SD. *$p < 0.05$ versus WT mice.

known what PAMPs of *K. pneumoniae* are recognized by TLR2 or TLR4. Several earlier studies investigated the role of TLRs in host defense against *K. pneumoniae* [6, 13]. However, the specific TLR pathways involved in *Klebsiella* activation of innate immune responses remain controversial. To determine the roles of TLR2 and TLR4 in inducing an innate host response to *K. pneumoniae* infection, we induced pneumonia in WT, TLR2 KO, TLR4 KO, and TLR2/4 DKO mice by intratracheal infection.

In the survival study with a low bacterial dose (5×10^3 CFU), higher bacterial burdens in the lungs and liver of TLR4 KO and TLR2/4 DKO mice could have contributed to their reduced survival (Figs. 1A and 3). In addition, TLR2 deficiency reduced survival after intratracheal infection with a higher bacterial dose (1×10^4 CFU, Fig. 1B). Our results indicate that both TLR2 and TLR4 are required for optimal *K. pneumoniae* clearance. Previous studies indicated that TLR2 participates in host antibacterial defenses at a late stage (48 h) of intranasal infection with a dose of 3×10^3 CFU of *K. pneumoniae* [13]. We observed the greatest impairment

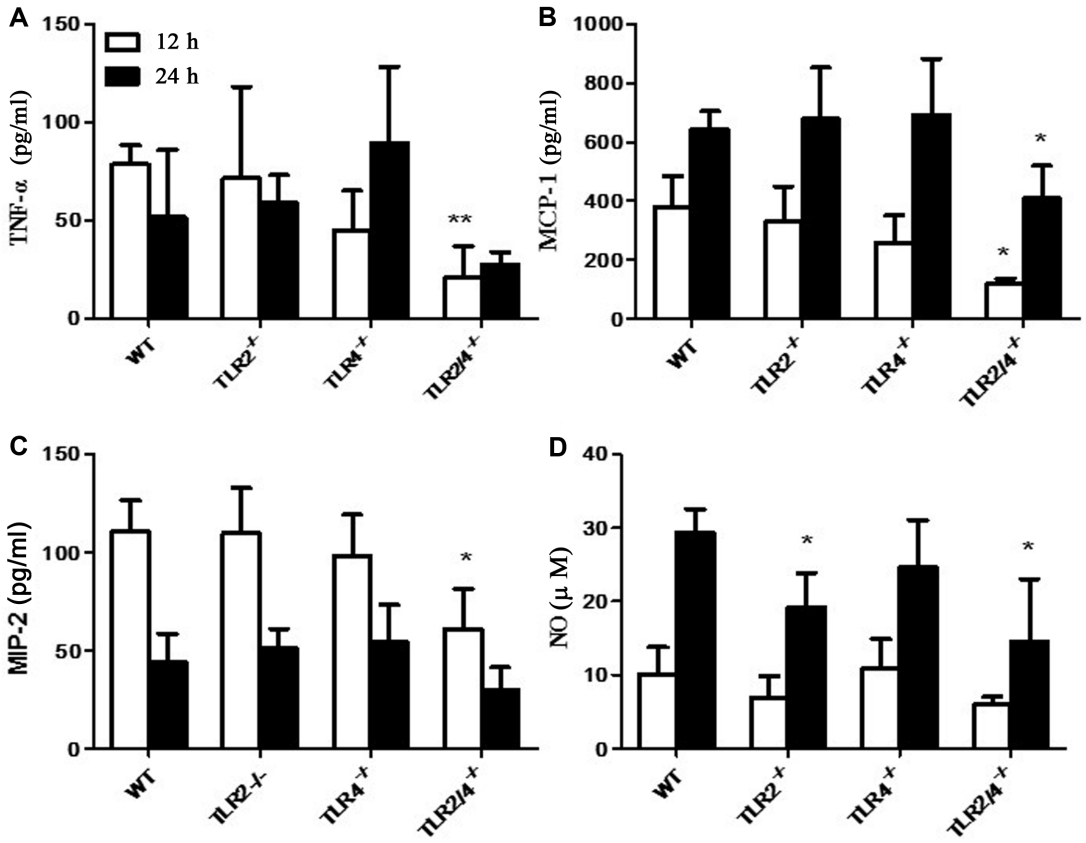

Fig. 4. Kinetics of cytokines and chemokines in the serum.

All mice (n = 4 in each group) were infected intratracheally with 5×10^3 CFU of *K. pneumoniae* and sacrificed at 12 or 24 h after infection. The levels of TNF-α (**A**), MCP-1 (**B**), MIP-2 (**C**), and NO (**D**) in the serum were measured by sandwich enzyme-linked immunosorbent assay. Data represent the mean ± SD. *$p < 0.05$ and **$p < 0.01$ versus wild-type mice.

of bacterial clearance in the lungs and liver of TLR2/4 DKO mice. The absence of TLR2 and TLR4 reduced bacterial clearance, resulting in uncontrolled bacterial growth in these organs. It was widely known that TLR2 and TLR4 can work independently and together to activate monocytes and neutrophils [20, 21]. A marked defect in clearance of, and increased mortality from, *Chlamydia pneumoniae* infection was observed in TLR2/4 DKO mice, indicating a cooperative TLR2- and TLR4-mediated response [22].

To investigate the effects of TLR2 and TLR4 deficiency on cytokines during *K. pneumoniae* infection, cytokine levels in sera and cytokine mRNA levels in the lungs were measured (Figs. 4 and 5). Changes in cytokine levels showed similar patterns in the sera and lungs in our study. The levels of TNF-α in the serum were significantly decreased in TLR2/4 DKO mice relative to those in WT mice. In addition, lung mRNA levels of TNF-α were significantly decreased in TLR2/4 DKO mice compared with those in WT mice. Pro-inflammatory cytokines, especially TNF-α, play a key role in the inflammatory response during systemic inflammation. TNF-α is a critical component of murine antibacterial host defenses against *K. pneumoniae* [23]. A previous study showed that TNF-α expression is impaired in TLR4-deficient mice during *K. pneumoniae* infection [24]. TLR4 is the main TLR involved in immune cell stimulation by gram-negative bacteria. TLR2 is also activated in the absence of TLR4 or at high concentrations of gram-negative bacteria [25]. Interestingly, in this study, IL-6 mRNA levels in the lungs were also significantly decreased in TLR2/4 DKO mice relative to those in WT mice. IL-6 is a resolving factor that maintains the balance between pro- and anti-inflammatory products to shape the immunological response. This is evident in its ability to coordinate the transition from innate to acquired immunity. IL-6 activity appears to be critical for the efficient control of acute inflammation [26, 27].

To examine the effects of TLR2 and TLR4 deficiency on chemokines during *K. pneumoniae* infection, MCP-1, MIP-2, and nitric oxide levels in the serum, and MCP-1 and iNOS

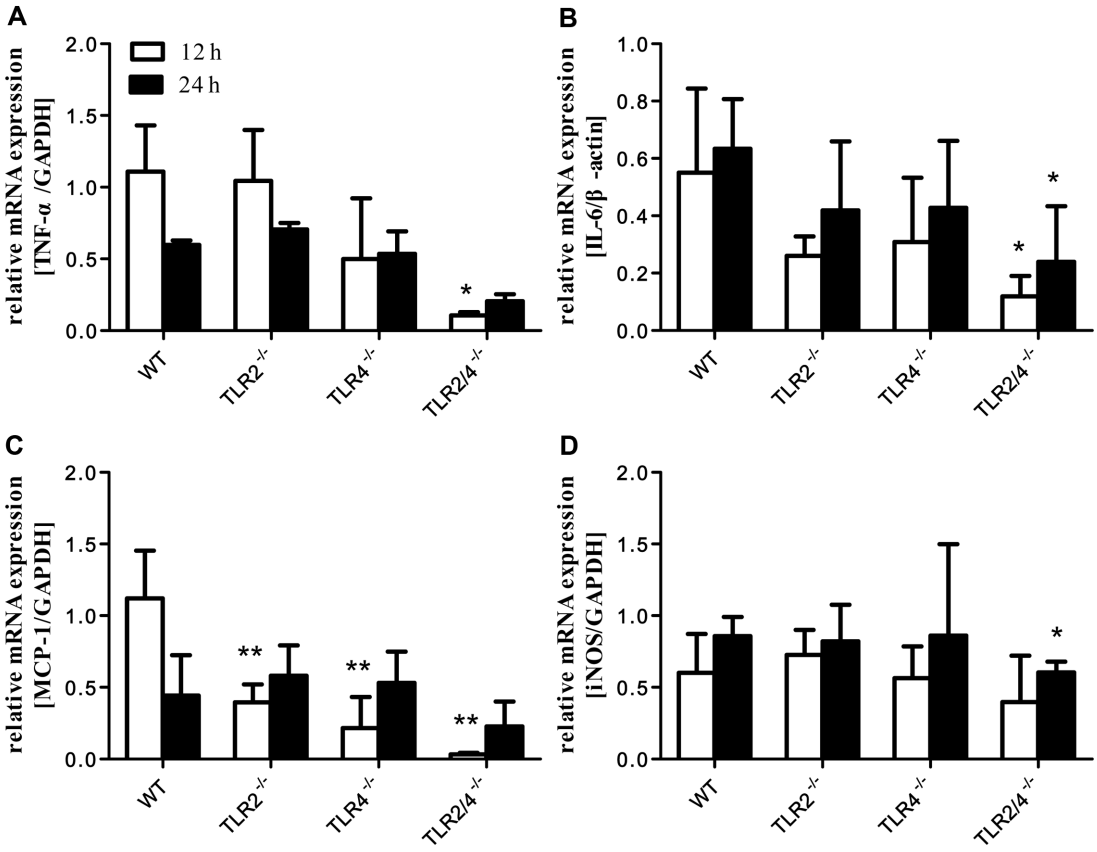

Fig. 5. Cytokine and chemokine gene expression profiles in the lungs.

All mice (n = 4 in each group) were infected intratracheally with 5×10^3 CFU of *K. pneumoniae* and sacrificed at 12 or 24 h after infection. The levels of TNF-α (**A**), IL-6 (**B**), MCP-1 (**C**), and iNOS (**D**) mRNA in the lungs were measured by reverse transcription PCR analysis. Data represent the mean ± SD. *$p < 0.05$ and **$p < 0.01$ versus wild-type mice.

mRNA levels in the lungs were measured (Figs. 4 and 5). The changes in chemokine levels in the sera and lungs showed similar tendencies to those of cytokine expression. We observed that MCP-1 levels in the sera and lungs were decreased in TLR2/4 DKO mice compared with those in WT mice. MCP-1 mRNA levels in the lungs also showed a decrease in TLR2 KO, TLR4 KO, and TLR 2/4 DKO mice compared with those in WT mice. In a previous study, MCP-1 was shown to play a role in neutrophil-mediated host defense in the lungs both directly, by attracting neutrophils, as well as indirectly, by enhancing the production of neutrophil chemoattractants during *K. pneumoniae* infection [28]. We observed that MIP-2 levels in sera had decreased in TLR2/4 DKO mice compared with those in WT mice. MIP-2 is associated with recruitment of neutrophils to the lungs. A previous study showed that MIP-2 contributes to the host defense, including neutrophil recruitment, upon challenge with *K. pneumoniae* [29]. In this study, iNOS mRNA levels in the lungs were significantly decreased in TLR2/4 DKO mice relative to those in WT mice. In addition, nitric oxide in sera was significantly decreased in TLR 2/4 DKO mice with the most severe pneumonia. Nitric oxide is a molecule that mediates the host immune response in many infections [30]. Nitric oxide depletion was shown to significantly attenuate effective bacterial clearance in the lungs of *Klebsiella*-infected mice [15]; there was an inverse correlation between susceptibility and nitric oxide secretion. Nitric oxide also plays an immunoregulatory role in the initiation and resolution of inflammation. During inflammation, nitric oxide affects the leukocyte chemotactic response through several mechanisms. It can control the production of chemokines such as MCP-1, MIP-2, and MIP-1α [5], and iNOS-derived nitric oxide enhances neutrophil infiltration [9]. Nitric oxide is an important factor in triggering host-mediated macrophage apoptosis during pneumococcal infection, indicating its importance in the resolution of inflammation following infection [31]. In this study, the greatest decreases in lung iNOS mRNA

levels and serum nitric oxide levels were observed in TLR2/4 DKO mice. TLR2 KO mice also showed decreased levels of nitric oxide in sera compared with those in WT mice. These results can be explained by results of a previous study, which showed that TLR2-triggered bacterial lipoproteins are released into circulation during gram-negative sepsis, either separately or in complexes with LPS and/or peptidoglycan [32]. Synthetic TLR2 agonists were capable of inducing excessive systemic iNOS-derived nitric oxide in vivo [32]. In patients with serious bacterial infections of the respiratory tract, modulation of TLR-mediated responses and nitric oxide could be an important therapeutic target [24].

In summary, TLR2/4 DKO mice were most susceptible to *K. pneumoniae* infection, showing reduced survival as a result of (i) high bacterial burdens in the lungs, liver, and blood, (ii) low TNF-α and IL-6 levels, (iii) low MCP-1 and MIP-2 levels, and (iv) low iNOS mRNA and serum nitric oxide levels. Survival was significantly shortened in TLR4 KO mice compared with that of WT mice after infection with 5×10^3 CFU. TLR2 KO mice were more susceptible to infection than WT mice after exposure to a higher infectious dose. Taken together, these results suggest that TLR4 is more important in the immune response against *K. pneumoniae* infection than TLR2, but TLR2 is also required for an effective innate immune response against *K. pneumoniae*. Therefore, TLR2 and TLR4 play cooperative roles in innate immune responses in the lungs and bacterial dissemination that induces systemic inflammation during *K. pneumoniae* infection. In addition, nitric oxide may play a protective role against *K. pneumoniae* infection.

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