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Modulation of Immune Response by Interleukin-10 in Systemic *Corynebacterium kutscheri* Infection in Mice[§]

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Interleukin (IL)-10 is an anti-inflammatory cytokine that modulates sepsis by decreasing pro-inflammatory cytokine production and chemokine expression. In this study, IL-10-deficient and wild-type (WT) mice were infected with *Corynebacterium kutscheri* to determine if the absence of IL-10 altered the protective immunity and pathogenesis. After infection, IL-10 knockout (KO) mice had a higher survival rate than WT mice. The decrease of body weight and the increased weight of organs such as liver and spleen were greater in WT mice. Bacterial counts were significantly increased after inoculation in WT mice over those in IL-10 KO mice. WT mice had more granulomatous inflammation and coagulative necrosis in the liver and spleen, lymphocyte depletion in lymphoid follicles, and apoptosis of immune cells in the spleen. WT mice had significantly higher plasma concentrations of aspartate aminotransferase and alanine aminotransferase. Furthermore, more upregulation of tumor necrosis factor-α and IL-4 in the plasma, macrophage inflammatory protein-2, keratinocyte-derived chemokine, inducible nitric oxide synthase, and interferon-inducible protein 10 mRNA in the spleen were observed in WT mice after inoculation. These results suggest that the lack of IL-10 contributes to an increase in the systemic clearance of *C. kutscheri,* and that IL-10 plays a detrimental role in controlling systemic *C. kutscheri* infection.

Keywords: *Corynebacterium kutscheri*, inflammation, interleukin-10, knockout mouse

Introduction

Corynebacterium is a genus of Gram-positive, aerobic, non-motile, irregular, and rod-shaped bacteria. Although *Corynebacterium kutscheri* has been encountered as a primary pathogen in both mice and rats, more commonly it is the cause of inapparent infections in both host species. *C. kutscheri* infection in laboratory mice and rats is usually subclinical but stresses such as nutritional deficiencies, irradiation, experimental manipulation, and derangement of the immune system can induce clinical diseases (Amao *et al.*, 1995, 2002). Signs of clinical disease in mice infected with *C. kutscheri* are dyspnea, weight loss, humped posture, and anorexia. Natural infection of *C. kutscheri* in mice is mainly transmitted by the fecal-oral route.

A recent study reported on the isolation of *Corynebacterium* spp. from the nebulizer cup of a patient with cystic fibrosis (O'Malley *et al.*, 2007). Recently, we isolated *C. kutscheri* and *Staphylococcus aureus* from Sprague-Dawley rats with hemisected spinal cords (Won *et al.*, 2007). Also, it was reported that interleukin (IL)-10 knockout (KO) mice were much more susceptible to acute bronchopulmonary inflammation induced by an extensive *C. kutscheri* infection with 7×10^{10} colony-forming units (CFU) (Jeong *et al.*, 2009).

Inflammatory T helper 1 (Th1) cell responses successfully eradicate pathogens, but often also cause immunopathology. To minimize this deleterious side-effect, the anti-inflammatory cytokine IL-10 is produced by Th2 CD4+ T cells, macrophages, dendritic cells, B cells, human Th0, and Th1 clones, as well as a newly defined T-regulatory subpopulation of CD4+ T cells. IL-10 is capable of inhibiting synthesis of pro-inflammatory cytokines made by macrophages and regulatory T cells, and modulates inflammatory reactions and B cell function (Moore *et al.*, 1993; Qureshi *et al.*, 2003). Overproduction of inflammatory cytokines such as interferon-γ (IFN-γ), IL-12, and IL-18 have been shown in IL-10 gene-deficient mice infected with *Pneumocystis carinii* (Qureshi *et al.*, 2003). An important immunoregulatory property of IL-10 is its ability to inhibit the production of IFN-γ during an immune response. This is not due to a direct inhibitory effect on T cells but rather is due to the IL-10-mediated inhibition of accessory cell functions, including the production of cytokines such as tumor necrosis factor-α (TNF-α), IL-1, and IL-12 and expression of co-stimulatory molecules that are necessary for optimal stimulation of T cells to produce IFN-γ (Fiorentino *et al.*, 1991a, 1991b).

IL-10 is a potent regulatory component. Both human and animal studies have shown that IL-10 plays an important role in the pathogenesis of *Hypersensitivity pneumonitis* and other granulomatous lung diseases (Gudmundsson *et al.*, 1998). A recent study reported that IL-10-deficient mice were more susceptible to *P. aeruginosa* infections when compared with WT mice (Guilbault *et al.*, 2002). Also, the neutralization of IL-10 in models of sepsis inhibited pro-inflammatory cytokine expression and death (Gerard *et al.*,

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1993). However, in bacterial pneumonia, the inhibitory effect of IL-10 on this protective innate immune response may be detrimental. For example, neutralization of IL-10 improved survival in a murine model of *Klebsiella pneumoniae*, which was concluded to be a result of increased inflammation and improved bacterial killing (Greenberger *et al.*, 1995). In a model of sepsis using cecal ligation and puncture, elevated IL-10 was accompanied by increased mortality after a secondary *P. aeruginosa* challenge, whereas blockade of IL-10 improved survival as well as bacterial clearance (Steinhauser *et al.*, 1999).

On the basis of these observations, we hypothesized that IL-10 is important for the modulation of the inflammatory response in a systemic *C. kutscheri* infection. This is the first study to investigate the role of IL-10 in a systemic infection model induced by *C. kutscheri* in mice. As well, the effects of IL-10 deficiency on cytokine and chemokine responses in murine systemic *C. kutscheri* infection were assessed.

Materials and Methods

Animals

Seven-week-old, male, IL-10 KO (B6.129P2-*Il10*^{tm1Cgn}) mice developed in a genetic background of C57BL/6J were purchased from Jackson Laboratory (USA), then bred at the laboratory animal breeding room, College of Veterinary Medicine, Konkuk University. Seven-week-old, male, C57BL/6J mice (wild-type, WT) were obtained from Korea Research Institute of Bioscience and Biotechnology (KRIBB, Korea). The genotypes of the IL-10 KO mice were confirmed by polymerase chain reaction amplification of tail DNA. The mice were allowed to eat and drink *ad libitum*. Survival of mice was monitored every 8 h for 6 days. Moribund mice were sacrificed at each inspection time. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Konkuk University (No. KU08077).

Bacterial preparation and intratracheal infection

C. kutscheri (ATCC15677) was grown overnight in brain heart infusion broth (Merck, Germany) at 37°C. The concentration of bacteria was determined by measuring the optical density at 600 nm and then plotting the optical density on a standard curve generated from known CFU values. The CFU/ml was determined by plating serial dilutions. Mice were anesthetized with 1.25% Avertin (2,2,2-tribromoethanol and tert-amyl alcohol in 0.9% NaCl) via intraperitoneal injections and placed dorsally recumbent. Twenty microliters of a *C. kutscheri* suspension containing 1×10^5 CFU were administered via sterile 31-gauge needles after exposing the trachea. The skin incision was then closed using surgical staples.

Bronchoalveolar lavage (BAL) and polymorphonuclear leukocyte (PMN) counts

BAL fluid was collected as described in our previous study (Jeong *et al.*, 2009). Briefly, mice were bled under anesthesia with ether. After the chest was opened, BAL was performed by instillation of three 0.4 ml aliquots of sterile saline using a sterile 23-gauge needle. The retrieved BAL fluid was gently pooled by centrifugation, and the supernatant was removed and frozen for determination of cytokine concentrations. The pellet was resuspended in 0.5 ml of sterile phosphate buffered saline (PBS). Total cell numbers were counted using an automatic blood cell counter (CDC Technologies, USA). PMN counts were performed with a haemocytometer.

Peripheral blood, lung, liver, spleen, and kidney collection and preparation for CFU analysis

Lungs, livers, spleens, and kidneys were removed and weighed in a sterile fashion at the indicated time points. One-third of each lobe of the lungs and liver, and one-third of the spleen and kidney were homogenized in PBS using a tissue homogenizer. For organ CFU determinations, a small aliquot of tissue homogenate was serially diluted, plated on 5% sheep blood agar plates, incubated at 37°C and then colonies were counted.

Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) analysis

Plasma levels of AST and ALT, as an indication of hepatic cellular injury, were determined using a Clinical Analyzer 7020 automated chemistry analyzer (Hitachi, Japan).

Quantitative histopathological examination

One-third of each lobe of the lungs and liver, and one-third of the spleen were fixed with 10% neutral formalin solution and processed routinely for paraffin sectioning. Each lobe was then embedded in paraffin and cut in 4 μm-thick sections. The specimens were stained with hematoxylin and eosin (H&E). For the quantification of the peribronchiolar pneumonia and bronchopneumonia in the lungs, granulomatous inflammation, and coagulative necrosis in the liver, and granulomatous inflammation and coagulative necrosis in spleen, 10 randomly-selected microscopic fields from each lung, liver, and spleen tissue section were examined using a 20X objective housed in a model BX51 microscope and images recorded using a DP71 digital camera (Olympus, Japan). Quantification was determined from the images using a MetaMorph 7.5 computerized image analyzer (Molecular Devices, USA).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analysis

Spleen samples were embedded in paraffin and cut in 4 μm-thick sections. The detection of apoptosis was performed using an ApopTag kit according to the instructions of the manufacturer (Chemicon International, USA). The slides were treated with 3,3′-diaminobenzidine before counterstaining with hematoxylin. After dehydration and cleaning, the sections were coverslipped and then examined using a BX51 microscope (Olympus). Images of slides processed for the apoptosis detection were quantitatively analyzed by a computerized image analyzer (MetaMorph 7.5). The immunopositive cells of spleen in three consecutive sections per five mice of each group were blindly counted with the image analyzer at a magnification of X400 by an experi-

enced pathologist. Results were expressed as the number of cells per mm^2. All values were expressed as the mean±SD.

Analysis of plasma cytokines

The plasma levels of IFN-γ, IL-12p70, TNF-α, IL-10, and IL-4 were quantified using commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, USA), according to the particular manufacturer's instructions. Plasma samples were diluted 1:3 in ELISA diluent buffer. The plasma concentration of each cytokine and chemokine was calculated with reference to a standard curve established for each recombinant cytokine. Detection limits for IFN-γ, IL-12p70, TNF-α, IL-10, and IL-4 were 15 pg/ml, 12 pg/ml, 8 pg/ml, 30 pg/ml, and 4 pg/ml, respectively.

Quantitative real-time polymerase chain reaction (PCR) and reverse transcription PCR

One-third of each spleen was harvested at the indicated time points, immediately snap-frozen in liquid nitrogen, and then stored at -70°C for further studies. Total RNA was prepared from frozen spleen tissue using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA was assayed by determining the absorbance readings at an optical density (OD) of 260 nm, and was further assessed by using the 260/280 OD ratios and by direct examination of 28S and 18S bands on 1% agarose gels. Equal amounts of RNA were reverse transcribed into cDNA using M-MLV reverse transcriptase (Invitrogen).

Quantitative real-time PCR was performed with an icycler iQ™ system (Bio-Rad, USA), using a TaqMan-iQ™ supermix kit (Bio-Rad) according to the manufacturer's recommendations. The TaqMan™ fluogenic probes and PCR primers for macrophage inflammatory protein-2 (CXCL2/MIP-2), keratinocyte-derived chemokine (CXCL1/KC), inducible nitric oxide synthase (iNOS) and interferon-inducible protein 10 (CXCL10/IP-10) and ß-actin were designed by Metabion (Martinsried, The Netherlands). The threshold cycle, Ct, which correlates inversely with the target mRNA levels, was measured as the cycle number at which the reporter fluorescent emission increased above a threshold level. The relative changes in MIP-2, KC, iNOS and IP-10 mRNA levels were normalized for ß-actin mRNA in the same samples.

Statistical analysis

For statistical analysis, all data obtained were analyzed using SPSS V14.0 software (SPSS, USA). Statistical significance was evaluated by one-way ANOVA analysis using SPSS for Windows. The results were determined to be statistically significant when $P<0.05$ and $P<0.01$ were obtained.

Results

Survival rate, body and organ weight changes and gross findings

Survival rates of IL-10 KO and WT mice infected with *C. kutscheri* were determined. The number of surviving mice in each group (eight per group) was recorded daily for 6 days. A bacterial dose of 1×10^5 CFU was not lethal over the 6 days in IL-10 KO mice. In contrast, 25% and 37.5% of WT mice had died by days 5 and 6, respectively, after infection. The survival rate was higher in IL-10 KO mice (100% survival) than WT mice (62.5% survival) (Fig. 1A). Body weight decreased gradually in both IL-10 KO and WT mice after infection. The decrease of body weight was significantly ($P<0.05$) higher in WT mice (Fig. 1B). Organ weight changes and gross findings of IL-10 KO and WT mice were determined following infection with *C. kutscheri*. Organ weight increased gradually in both IL-10 KO and WT mice after infection. However, the changes in organ weight were higher in WT mice. Liver and spleen weight changes were significantly greater ($P<0.05$) in WT mice

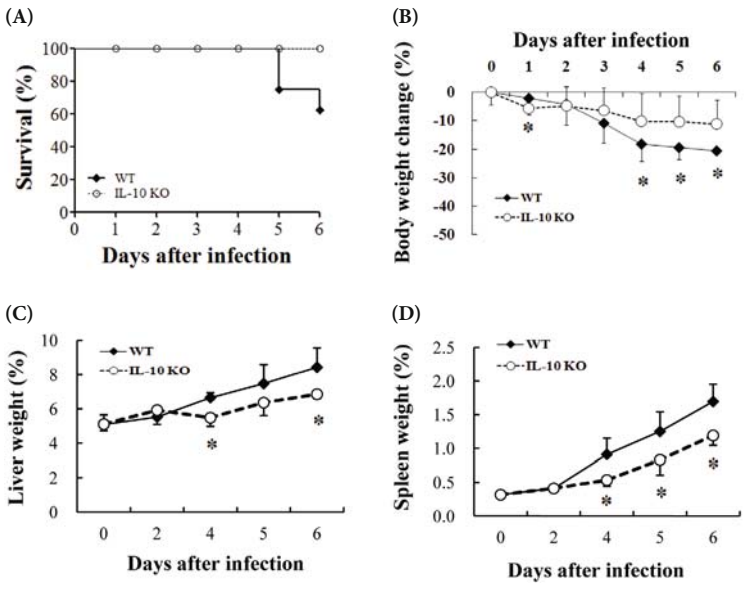

Fig. 1. Survival rate, and body and organ weight changes of IL-10 KO and WT mice after *C. kutscheri* infection. (A) The number of survivors for eight starting mice was recorded daily for 6 days after intratracheal administration of 1×10^5 CFU of *C. kutscheri*. (B) Body weights were recorded at 0, 1, 2, 3, 4, 5, and 6 days after infection. Weight increases of liver (C) and spleen (D) after infection were observed at necropsy in IL-10 KO mice and WT mice. Liver and spleen weight increases after infection were greater in WT mice as compared with IL-10 KO mice. Data are expressed as the mean±SD of eight mice per group. *P<0.05 between IL-10 KO and WT mice, calculated by one-way ANOVA analysis.

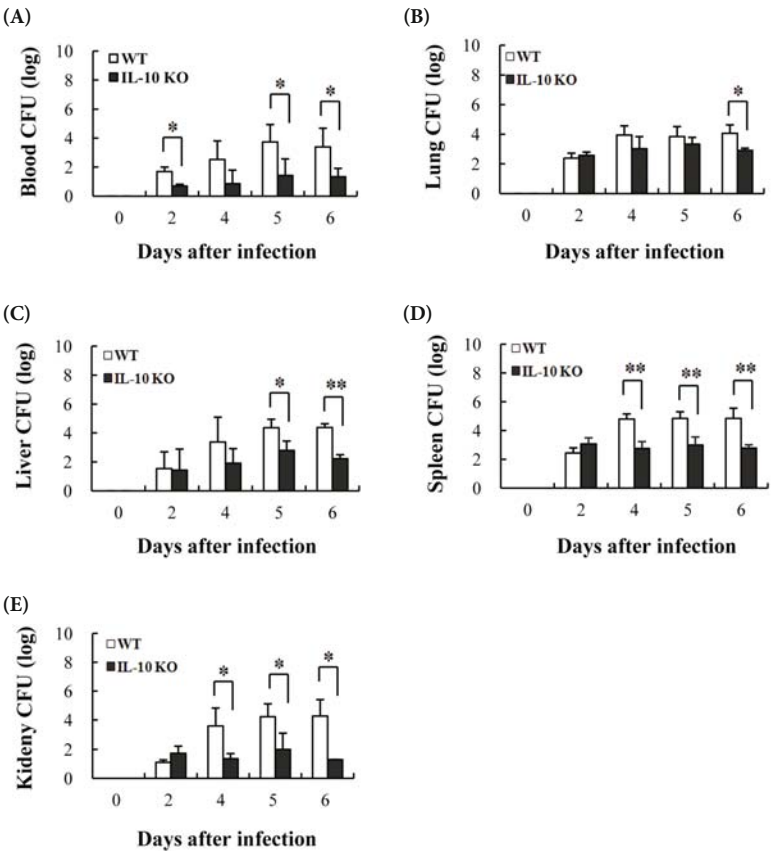

Fig. 2. Kinetics of bacterial proliferation and clearance in IL-10 KO and WT mice after *C. kutscheri* infection. Blood (A), lung (B), liver (C), spleen (D), and kidney (E) were obtained from infected mice at 2, 4, 5, and 6 days after intratracheal establishment of *C. kutscheri* infection. For CFU determinations, a small aliquot of tissue homogenate was serially diluted and plated on 5% sheep blood agar plates and incubated at 37°C, and the resulting colonies were counted. Data are expressed as the mean±SD of eight mice per group. *P<0.05 and **P<0.01 between mice, calculated by one-way ANOVA analysis.

compared with IL-10 KO mice (Figs. 1C and 1D). The lung weight was not different between IL-10 KO and WT mice (data not shown). Grossly, a reddish focus was distributed throughout the parenchyma of the lungs of both groups of mice, but was more vivid in the lungs of IL-10 KO mice. The number of white spots on the liver and spleen was prominantly augmented in WT mice as compared with IL-10 KO mice after infection (data not shown).

Bacterial proliferation and clearance

To investigate the suggestion that the substantial increase of lethality observed in *C. kutscheri* infected mice was due to impaired bacterial clearance, blood, lung, liver, spleen and kidney samples were obtained from IL-10 KO and WT mice at 2, 4, 5, and 6 days after infection. The number of bacteria in both IL-10 KO and WT groups increased after infection. The IL-10 KO mice harbored significantly fewer viable bacteria (P<0.05) than the WT mice at days 4, 5, and 6 days. The number of viable bacteria was slightly decreased in IL-10 KO mice at day 6; this decrease was not evident in WT mice at day 6 (Fig. 2).

PMN recruitment to bacterial challenge

Leukocyte recruitment plays important roles at the early stage of host innate immune response. Therefore, we examined the total WBCs and percent of lymphocytes and neutrophils after bacterial challenge in peripheral blood. The total WBCs and percent of lymphocytes in blood decreased after *C. kutscheri* administration. However, the percent of neutrophils in blood gradually increased in both IL-10 KO and WT mice after infection. There was no difference in the total WBCs, and the percent of lymphocytes and neutrophils in blood between IL-10 KO and WT mice. In both IL-10 KO and WT mice, significant PMN influx in BAL fluid was observed as early as 2 days after intratracheal challenge of *C. kutscheri* compared with uninfected mice. The total PMNs in BAL fluid gradually increased in both mice after *C. kutscheri* administration. The total PMNs in BAL fluid was not different between IL-10 KO and wild-type mice (data not shown).

Histopathological finding

In histopathological observations, the lungs had regional filling of alveoli with inflammatory cells in mice infected with *C. kutscheri*. Also, the extent of bronchopneumonia in each lung increased with time after infection and was very pronounced by day 6. Bronchopneumonia was more prevalent in IL-10 KO mice compared with WT mice at each time point. There was no statistical significance in the extent of pneumonia between the IL-10 KO and WT mice (data not shown).

Granulomatous inflammation and coagulative necrosis were observed in liver and spleen after infection, and increased with time in both IL-10 KO and WT mice, being more evident in WT mice at each time point (Fig. 3). Also, the lymphocyte depletion in the lymphoid follicles in the spleen was increased in both IL-10 KO and WT infected

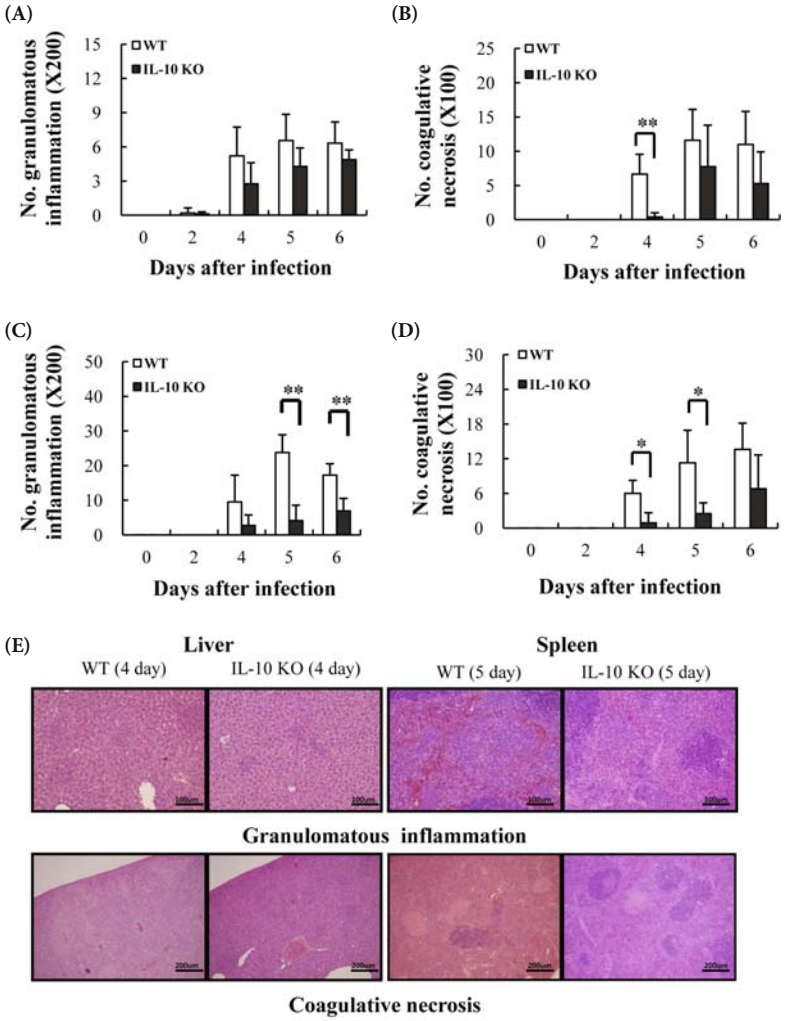

Fig. 3. Comparison of granulomatous inflammation and coagulative necrosis in liver and spleen of IL-10 KO and WT mice after *C. kutscheri* infection. Liver (A, B) and spleen (C, D) were H&E stained for granulomatous inflammation (A and C, respectively) and coagulative necrosis (B and D, respectively). For the quantification of the granulomatous inflammation and coagulative necrosis, 10 random microscopic fields from each lung and liver section were captured. Data are expressed as the mean±SD of eight mice per group. *P<0.05 and **P<0.01 between mice, calculated by one-way ANOVA analysis. (E) Representative histopathological images of liver at day 4 and spleen at day 5 after *C. kutscheri* infection.

mice. The lymphocyte depletion was significantly greater (P<0.05) in WT mice than IL-10 KO mice (data not shown).

Liver injury

To determine if excessive liver injury may also contribute to increased mortality, release of the hepatocyte associated enzymes AST and ALT into the peripheral blood was examined. Plasma levels of AST and ALT in IL-10 KO mice and WT mice were increased after infection. Especially, WT mice had higher AST and ALT concentrations in plasma at 4 and 5 days after infection. AST and ALT levels peaked 4 days after infection in WT mice. AST and ALT concentrations at 4 days were approximately 80- and 20-fold higher, respectively, in WT mice than in IL-10 KO mice (Fig. 4).

Cytokine production in plasma

Cytokines play a fundamental role in modulating inflammation, phagocytosis, tissue injury, and death. Accordingly, the plasma levels of circulating IFN-γ, IL-12p70, TNF-α, and IL-4 were ascertained. The concentrations of all the cytokines were increased in infected IL-10 KO and WT mice (Fig. 5). The increases in IFN-γ and IL-12p70 were greater

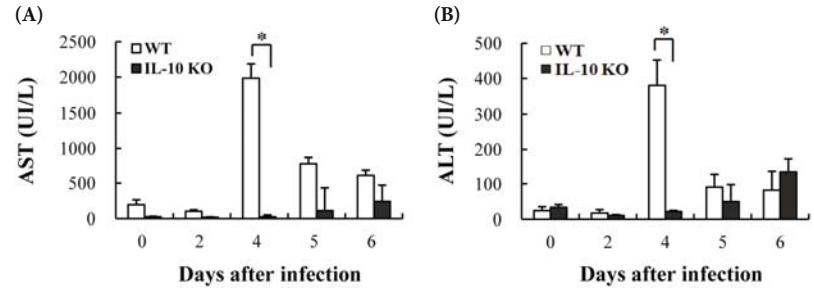

Fig. 4. Plasma AST and ALT concentrations in IL-10 KO and WT mice after *C. kutscheri* infection. Plasma levels of AST and ALT are presented in panels (A) and (B), respectively. Data are expressed as the mean±SD of eight mice per group. *P<0.01 between mice, calculated by one-way ANOVA analysis.

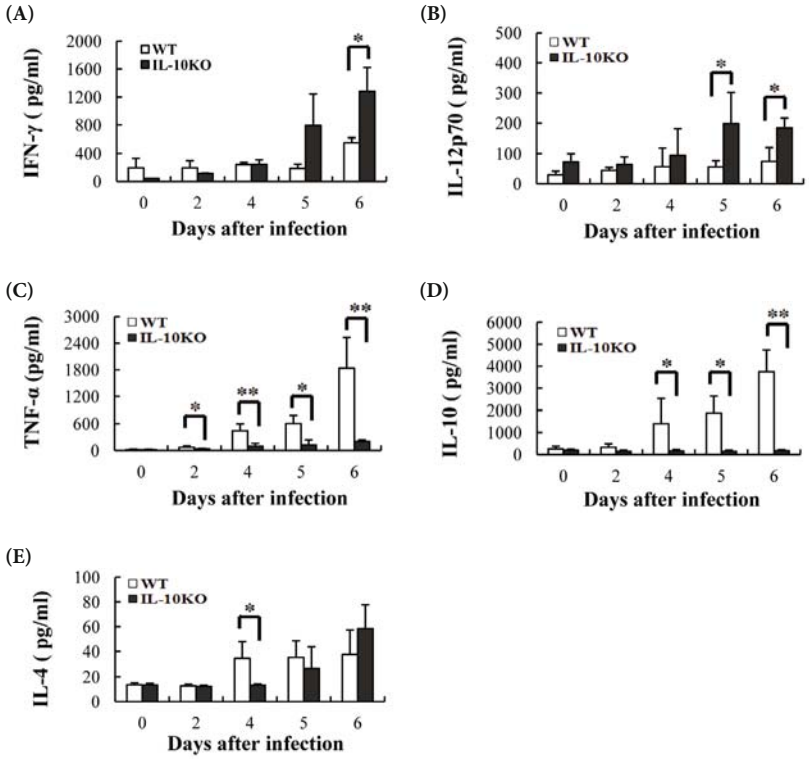

Fig. 5. Kinetics of cytokine and chemokine in plasma of IL-10 KO and WT mice after *C. kutscheri* infection. The levels of IFN-γ (A), IL-12p70 (B), TNF-α (C), IL-10 (D), and IL-4 (E) in plasma were quantified by ELISA. Data are expressed as the mean±SD of eight mice per group. *P<0.05 and **P<0.01 between mice, calculated by one-way ANOVA analysis.

in IL-10 KO mice than in WT mice. The production of IFN-γ was significantly increased (P<0.05) in IL-10 KO mice at 6 days. Also, the level of IL-12p70 was significantly increased (P<0.05) in IL-10 KO mice at days 5 and 6 during infection (Figs. 5A and 5B).

In contrast, the production of TNF-α and IL-10 were significantly increased in WT mice compared with IL-10 KO mice. The production of TNF-α was significantly increased (P<0.05) in WT mice over the 6-day period, and was approximately 8-fold higher than that of IL-10 KO mice at day 6 after infection (Fig. 5C). Also, the production of IL-10 was significantly increased (P<0.05) in WT mice compared with IL-10 KO mice at days 4–6 (Fig. 5D). The level of IL-4 at day 4 after infection was increased in WT mice compared with IL-10 KO mice, but at day 6, the level was greater in IL-10 KO mice (Fig. 5E).

Chemokine expression

To examine the differences of the immune response between the IL-10 KO and WT mice, the levels of MIP-2, KC,

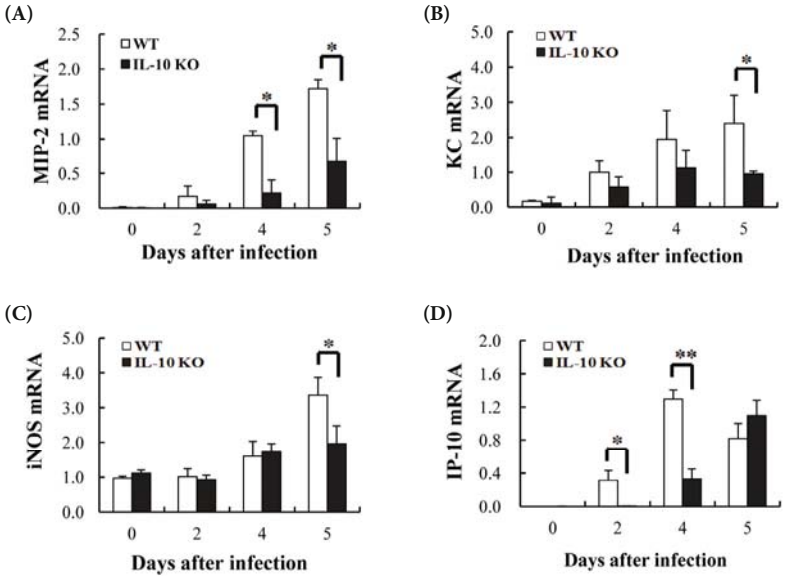

Fig. 6. Chemokine gene expression profiles in spleen of IL-10 KO and WT mice after *C. kutscheri* infection. MIP-2 (A), KC (B), iNOS (C), and IP-10 (D) mRNA levels were quantified by quantitative real-time PCR. The relative changes in MIP-2, KC, iNOS, and IP-10 mRNA levels were normalized to β-actin mRNA in the same samples. Data are expressed as the mean±SD of eight mice per group. *P<0.05 and **P<0.01 between mice, calculated by one-way ANOVA analysis.

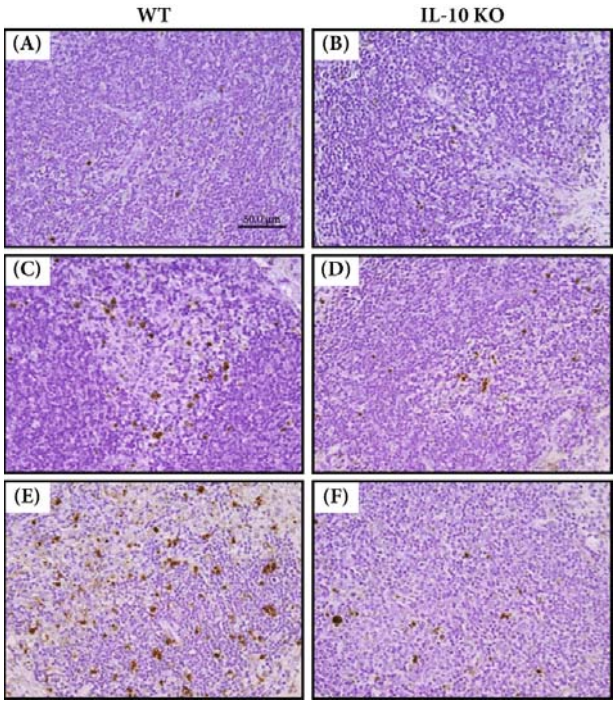

Fig. 7. Apoptosis in spleen of IL-10 KO and WT mice after *C. kutscheri* infection. Induction of apoptosis was evaluated by the TUNEL method. The immunopositive cells of the spleen in three inconsecutive sections per five mice of each group were blindly counted with the image analyzer at a magnification of ×400 by an experienced pathologist and are expressed as the number of cells/mm^2. (A and B) represent spleen tissue from uninfected mice. (C and D) represent spleen tissue collected 4 days after infection. (E and F) represent spleen tissue collected 5 days after infection.

iNOS and IP-10 mRNA in the spleen were quantified. MIP-2, KC, iNOS and IP-10 mRNA were not detected in uninfected spleens. However, the expression of MIP-2, KC, iNOS, and IP-10 mRNA increased on days 2–5 following infection in both IL-10 KO and WT mice (Fig. 6). MIP-2, KC and iNOS mRNA levels were higher in the spleens of WT mice than in those of IL-10 KO mice (Figs. 6A, 6B, and 6C).

Apoptosis of immune cells in spleens

To investigate the apoptosis of innate immune cells in the spleen, apoptotic cells were measured using a TUNEL method in the spleen from *C. kutscheri*-infected IL-10 KO and WT mice. On days 4 and 5 the infected WT mice harbored more spleen apoptotic cells (90±60 and 121±41, respectively) than did IL-10 KO mice (35±11 and 39±9, respectively). Uninfected mice had a very low level of apoptosis in the spleen (Fig. 7). Interestingly, apoptotic cells in the spleen were significantly higher ($P<0.05$) at 5 days after infection in WT mice compared with IL-10 KO mice (Fig. 7).

Discussion

Bacteremia leading to sepsis is an important cause of morbidity and mortality. Sepsis has been characterized as "dysregulation of inflammation" in response to infection. Key determinants in sepsis are pathogen control and the host's inflammatory response to the infection (Londoño *et al.*, 2008).

Pulmonary inflammation is a major hallmark of acute lung injury produced by a variety of pro- and anti-inflammatory cytokines during the initial stages of the disorder (Bhatia and Moochhala, 2004). As lung injury progresses, the compartmentalization of pulmonary inflammation is lost and mediators can be released and/or secreted from the lung into the systemic circulation. Clinical studies have shown that the same markers of inflammation observed in the bronchoalveolar lavage of patients with acute lung injury are also elevated in serum, indicating a potential link between the initial pulmonary inflammatory response and subsequent systemic inflammation (Meier *et al.*, 2008).

IL-10 is an anti-inflammatory cytokine that has immunosuppressive functions, which, *in vitro*, are exerted through the down-regulation of IFN-γ production and macrophage activation (Fiorentino *et al.*, 1991b). IL-10 is one of the most important anti-inflammatory cytokines, playing a key role in sepsis and acute lung injury by modulating both the systemic and pulmonary inflammatory response, respectively. In various model systems, IL-10 has been shown to inhibit production of numerous pro-inflammatory cytokines and chemokines that are known to contribute to the development of acute inflammatory states (Gudmundsson *et al.*, 1998).

The present study was undertaken to clarify the immune response generated by IL-10 during systemic *C. kutscheri* infection induced by a lower number (1×10^5 CFU) of *C. kutscheri*, as compared with our previous study (Jeong *et al.*, 2009). The role of IL-10 was ascertained for systemic infections established in IL-10 KO and WT mice. To address this question, IL-10 KO mice and C57BL/6J mice of the same genetic background were used. The survival rate was higher in IL-10 KO mice than WT mice after infection. Also, the changes in body weight and tissue weight such as liver and spleen were higher in WT mice compared with IL-10 KO mice after infection. WT mice presented a significantly higher number of the bacteria after infection. Neutrophils play a crucial role in the early control of acute pulmonary bacterial infection, especially local infection (Mayer-Scholl *et al.*, 2004; Sun *et al.*, 2009). However, their contributions might not be the same for systemic infection. This possibility is supported by our finding that total PMNs in BAL fluid and the percent of neutrophils in blood at each time point were not different between IL-10 KO and wild-type mice (data not shown).

The role of IL-10 becomes more complicated when pathogen clearance is involved. Experiments have shown that IL-10 neutralization or deficiency improves the clearance of bacteria, parasites, and fungi (Gudmundsson *et al.*, 1998; Steinhauser *et al.*, 1999). Neutralization of IL-10 in *Klebsiella pneumoniae*-infected animals results in enhanced expression of pro-inflammatory cytokines, enhanced bacterial clearance and prolonged survival (Greenberger *et al.*, 1995). However, transgenic mice overexpressing IL-10, infected by *P. aeruginosa,* display greater weight loss and lower survival rates (Sun *et al.*, 2009). Our previous study also demonstrated

that IL-10 KO mice exhibit greater neutrophil infiltration and acute pulmonary inflammation in the lung, without systemic infection, after they received a massive (7×10^{10} CFU) dose of *C. kutscheri* via the trachea (Jeong *et al*., 2009). Also, the IL-10 KO mice showed a lower survival rate than WT mice after both mice were infected intratracheally with high infection doses, 1×10^{8} and 1×10^{11} CFU, in our other study (Supplementary data Fig. S1). But, there was significant difference between the two kinds of mice infected with *C. kutscheri* at a dose of 1×10^{11} CFU only. It seems plausible that an infectious dose of 1×10^{8} is the point where the susceptibilities of WT and IL-10 KO mice become identical. Results obtained in the present study and our previous study (Jeong *et al*., 2009) indicate that the bacterial dose used in the infection is one of the most important factors in determining whether immune response by IL-10 will result in detrimental effects on the outcome in C. *kutscheri* disease. In a study conducted over 40 years ago, C57BL/6 mice were found to be resistant to *C. kutscheri* infection compared with Swiss mice after they were injected intravenously with 10^{4} CFU (Pierce-Chase *et al*., 1964). These old data are not comparable with our observations due to differences in the infection route, infection dose, and bacterial strain of *C. kutscheri*.

Bacterial proliferation and clearance data in this study were consistent with the view that clearance of *C. kutscheri* in IL-10 KO mice was accelerated compared with that in WT mice. The results also indicated that the presence of IL-10 impaired bacterial clearance, resulting in uncontrolled growth of bacteria in the blood and tissues such as lung, liver, spleen and kidney, with modest spillover into the circulation, which was associated with the increased mortality of the infected WT mice. The high mortality rate of the WT mice was attributed to tissue injury and organ dysfunction. These data also indicate that IL-10 is important for combating bacterial infection and that the inhibition of phagocytosis by IL-10 resulted in insufficient bacterial clearance in the liver and spleen, which led to excessive development of inflammation, increased tissue injury, increased organ dysfunction and increased mouse mortality.

Cytokines play a fundamental role in modulating inflammation, phagocytosis, tissue injury and death. To examine the effect of IL-10 deficiency on the systemic *C. kutscheri* infection, the levels of IFN-γ, IL-12p70, TNF-α, IL-10, and IL-4 cytokines in plasma were measured. The plasma levels of all these cytokines except TNF-α and IL-10 increased in IL-10 KO mice and WT mice after infection. Also, a significantly higher plasma concentration was observed in the levels of IFN-γ and IL-12p70 in IL-10 KO mice compared with WT mice during the course of infection. These data indicate that IL-10 plays a role in the regulation of IFN-γ and IL-12p70 production during the infection. This finding agrees with a previous report that demonstrated that IL-10 is a potent negative regulator of IFN-γ and IL-12 production (Sapru *et al*., 1999).

In our study, a significantly higher concentration of TNF-α was evident in plasma of the WT mice, as compared with IL-10 KO mice, during the course of infection. This is consistent with the view that IL-10 plays a role in the regulation of TNF-α production during infection, and indicates that the presence of IL-10 compromises the ability of the innate immune system to control the pathogen load in WT mice. Pro-inflammatory cytokines, in particular TNF-α, play a key role in the amplification of the inflammatory response in the course of sepsis. Although a pro-inflammatory response can be beneficial for bacterial clearance, too much activation can lead to cell injury and even shock. IL-10 is essential to counterbalance TNF-α production, allowing the host to tolerate a high pathogen load. The majority of murine models have reported a correlation between improved pathogen control and increased production of TNF-α and IFN-γ (Deckert *et al*., 2001; Latifi *et al*., 2002). Presently, the high pathogen load in WT mice resulted in increased production of TNF-α, which led to prominent leukocyte apoptosis resulting in a further increase in the pathogen load. Local or systemic production of TNF-α has been implicated in the induction of apoptosis in several types of eukaryotic cells, including leukocytes (Sewnath *et al*., 2001). In sepsis caused by Gram-negative bacteria, also characterized by high production of TNF-α and persistent bacteremia, several studies have evaluated the importance of apoptosis of immune cells in contributing to the high mortality (Groesdonk *et al*., 2007; Wesche-Soldato *et al*., 2007a). Results in this study provide novel insights into the role of IL-10 and TNF-α in the control of the pathogen load during high bacteremia. Some studies have reported that persistent bacteremia is the result of the loss of pathogen clearance due to apoptosis of spleen and liver macrophages and lymphocytes (Wesche-Soldato *et al*., 2007b).

There is evidence that patients dying from sepsis have markedly increased lymphocyte apoptosis in the spleen. Increase of apoptosis in spleen lymphocytes has been shown to reduce survival in experimental animals with sepsis. The immune cells most affected by dysregulated apoptotic cell death in sepsis appear to be lymphocytes (Tinsley *et al*., 2003; Wesche-Soldato *et al*., 2007b). Thus, it appears that the excessive levels of TNF-α led to marked apoptosis of immune cells in WT mice in this study. Results in this study indicate that, at times of high levels of bacteremia, IL-10 deficiency helps to control infection by protecting innate immune cells from apoptosis via the regulation of TNF-α.

The present study examined the histopathology, and cytokine and chemokine mRNA expression in tissues such as liver and spleen of *C. kutscheri*-infected IL-10 KO and WT mice. The lungs were filled with inflammatory cells in both IL-10 KO and WT mice after infection. Also, the extent of pneumonia in each lung was increased by the infection. However, *C. kutscheri*-infected WT mice displayed greater histopathological evidence of granulomatous inflammation and coagulative necrosis in the liver, and granulomatous inflammation, coagulative necrosis and apoptosis of immune cell in the spleen compared with IL-10 KO mice. Also, WT mice had significantly higher AST and ALT plasma concentrations compared with IL-10 KO mice. Furthermore, the up-regulation of MIP-2, KC, iNOS, and IP-10 mRNA in the spleen of WT mice was evident. These observations support the view that the production of these pro-inflammatory factors and induced hepatic damage may influence the modulation of the mortality due to systemic infection in

mice. Local production of CXC chemokines, such as MIP-2 and KC, is essential for the recruitment of neutrophils to the lung during a bacterial infection (Kobayashi, 2008). IP-10, MIP-1α, and monokines induced by IFN-γ play a key role in promoting type 1 immune responses.

Taken together, the present data demonstrate that WT mice are much more susceptible to systemic *C. kutscheri* infection compared with IL-10 KO mice. This suggests that IL-10 is involved in the regulation of tissue damage induced by systemic *C. kutscheri* infection. Anti-inflammatory mediators, especially IL-10, are essential to counterbalance the pro-inflammatory response. In sepsis, the concentration of IL-10 is often indicative of the magnitude of the inflammatory stress, being the highest in patients with the most severe disease (Gudmundsson *et al*., 1998; Steinhauser *et al*., 1999). The present results show that at times of high bacteremia, IL-10 deficiency can inhibit TNF-α production and prevent apoptosis of innate immune cells.

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