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Role of IL-10 Deficiency in Pneumonia Induced by *Corynebacterium kutscheri* in Mice

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IL-10 is an important anti-inflammatory cytokine that can inhibit the production of many pro-inflammatory cytokines. Both human and animal studies have shown that pro-inflammatory cytokines play an important role in pneumonia and other inflammatory lung diseases. In the present study, IL-10 knockout (KO) and wild-type mice were infected with *Corynebacterium kutscheri* to determine whether the severity of pathogenesis and whether protective immunity could be altered in the absence of IL-10. The survival rate was significantly lower in IL-10 KO mice than wild-type mice. The number of neutrophils in bronchoalveolar lavage fluid and blood were found to be higher in IL-10 KO mice than wild-type mice. IL-10 KO mice showed greater neutrophil infiltration, excessive inflammation, and weight-loss compared with wild-type mice. Furthermore, upregulation of IFN-γ in bronchoalveolar lavage fluid, and upregulation of MIP-1α and IP-10 mRNA in the lungs of IL-10 KO mice compared with wild-type mice after *C. kutscheri* infection were observed. These results suggest that IL-10 plays an important role in the anti-inflammatory properties against *C. kutscheri* infection, and that lack of IL-10 leads to a more severe pulmonary inflammatory response. This increased susceptibility to *C. kutscheri* pneumonia is at least in part caused by IL-10 deficiency and severe recruitment of neutrophils.

Keywords: *Corynebacterium kutscheri*, IL-10 KO, IFN-γ, MIP-1α, IP-10

Naturally occurring infections of *Corynebacterium kutscheri* appear mainly in the rat (*Rattus norvegicus*) and mouse (*Mus musculus*), although this organism has also been isolated from the guinea pig and hamster. Although *C. kutscheri* has been encountered as a primary pathogen in unprovoked epizootics in both rats and mice, more commonly, in both host species, inapparent infections have been unmasked by experimental modulations that lower the host resistance or impair immunocompetence [1, 2, 12].

Latent, clinically silent infections may not be associated with tissue pathology, and *C. kutscheri* may be isolated from non-lesioned tissue [1, 2]. Although the prevalence of *C. kutscheri* has declined in recent years, this organism has a long and well-deserved reputation for its emergence as a clinical entity during experimental procedures. Recent report demonstrated that *Corynebacterium* spp. were isolated from the nebulizer cup of patients with cystic fibrosis, even though most patients with cystic fibrosis were infected with a dominant organism, usually *Pseudomonas aeruginosa* [18]. Recently, we reported that *C. kutscheri* and *Staphylococcus aureus* were isolated from two Sprague-Dawley rats with a hemisected spinal cord [23].

Interleukin-10 (IL-10), a cytokine produced by Th2 CD4 T cells, macrophages, and B cells, is an important anti-inflammatory cytokine that can inhibit the production of many proinflammatory cytokines, including IFN-γ, TNF-α, IL-1β, IL-6, IL-12, inflammatory reactions, and modulation of B cell function [19, 24]. IL-10 is therefore a potent component in the regulating mechanism of these processes. Both human and animal studies have shown that IL-10 plays an important role in the pathogenesis of hypersensitivity pneumonitis (HP) and other granulomatous lung diseases [9]. Recently, IL-10-deficient male mice were found to be more susceptible to *Pseudomonas aeruginosa* infections when compared with wild-type male mice [11]. However, this study did not address whether host responses to *C. kutscheri* would be bolstered in the absence of IL-10.

On the basis of these observations, we hypothesized that IL-10 was important for the modulation of the inflammatory response in *C. kutscheri* infections and that lack of IL-10 could lead to a more severe inflammatory

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response and abscess formation. This is the first study to have investigated the role of IL-10 in pneumonia induced by *C. kutscheri* in mice. We also examined the effects of IL-10 deficiency on cytokine and chemokine responses and its cellular recruitment into the lungs in murine pneumonia.

MATERIALS AND METHODS

Animals

Seven-wk-old male IL-10 KO (B6.129P2-Il10^{tm1Cgn}) mice developed on a genetic background of C57BL/6J were purchased from Jackson Laboratory (Bar Harbor, ME, U.S.A.). Seven-wk-old male C57BL/6J mice were obtained from the Korea Research Institute of Bioscience and Biotechnology (KRIBB, Daejeon, Korea). The genotypes of the IL-10 KO mice were confirmed by polymerase chain reaction amplification of tail DNA. The mice were housed in polycarbonate cages with wire lids in specific, pathogen-free conditions. All experiments were approved by the animal care and use committee of KRIBB in Korea.

Bacterial Preparation and Intratracheal Inoculation

C. kutscheri (ATCC15677) was grown overnight in brain heart broth (Merck, Germany) at 37°C. The concentration of bacteria was determined by measuring the absorbance values at 600 nm and then plotting the optical density on a standard curve generated from known CFU (colony-forming units) values. The number of 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 in the dorsal recumbence. Fifty μl of the bacterial suspension (7×10^{10} CFU of *C. kutscheri*) was administered *via* sterile 31-gauge needles after exposing the trachea. The skin incision was then closed using surgical staples.

BAL Fluid Analysis

Bronchoalveolar lavage (BAL) fluid was collected as described elsewhere [10]. Briefly, mice were bled under anesthesia with ether at designated time points. After the chest was opened, the trachea and lungs were exposed. The alveoli of mice were washed three times with three 0.4-ml aliquots of sterile saline using a sterile 23-gauge needle and the retrieved BAL fluid was gently aspirated and pooled. Alveolar cells were centrifuged, and then the supernatant was removed and frozen for cytokine concentration determinations. The pellet was resuspended in 0.5 ml of sterile PBS. Total cell numbers were counted using an automatic blood cell counter (CDC Technologies Inc., CT, U.S.A.). Differential cell counts were performed with a hemocytometer.

Quantitative Histopathological Examination

Half of each lobe in the lung was 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). One section from the center of each lobe and one section halfway lateral through each lobe were examined as described previously [4]. For the quantitation of pneumonia in the lung, 20 random microscopic fields from each lung tissue section were captured through a 20× Uplan objective using the DP71 digital camera (Olympus, Tokyo, Japan). The presence or absence of penumonia was counted in each captured image using the Metaview software package (Universal Imaging Corp., West Chester, PA, U.S.A.). The percentage of pneumonia was determined by dividing the area of pneumonia by the total areas counted.

Cytokine Analysis in BAL Fluid

IFN-γ, IL-2, and IL-4 levels in BAL fluid were quantitated by using commercially available sandwich ELISA kits (Endogen, Cambridge, MA, U.S.A.), according to the manufacturer's instruction. The concentration of each cytokine present in BAL fluid was calculated with reference to a standard curve established with each recombinant cytokine. Detection limits for IFN-γ, IL-2, and IL-4 are 10 pg/ml, 3 pg/ml, and 2.5 pg/ml, respectively. Results were expressed as picograms of cytokine per milliliter.

Northern Blot Analysis

Half of each lobe of the lung 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 lung tissue by using TRIzol (Invitrogen, CA, U.S.A.) according to the manufacturer's instructions. Briefly, lung tissue was homogenized in 1 ml of TRIzol reagent, followed by chloroform extraction and ethanol precipitation. Air-dried RNA pellets were dissolved in diethylpyrocarbonate-treated water. RNA was assayed by determining the absorbance readings at OD 260 and it was further assessed by using the 260/280 OD ratios and by direct examination of 28S and 18S bands on 1% agarose gels [14]. For Northern blot analysis, equal amounts of RNA from five mice per group were pooled and 30 μg of total RNA was fractionated onto 1% agarose gels. Subsequently, the RNA was transferred to a nylon membrane by the capillary transfer method and immobilized by a UV cross-linking technique. Fixed membranes were prehybridized for 30 min at 68°C in an ExpressHyb Hybridization solution (Clontech, CA, U.S.A.). The membranes were hybridized in fresh solution for 2 h at 68°C with ^{32}P-labeled complementary DNA (cDNA) probes for murine macrophage inflammatory protein-1α (MIP-1α) and interferon-inducible protein 10 (IP-10) (for MIP-1α, forward primer 5'-ATG AAG GTC TCC ACC ACT-3' and reverse primer 3'-CTC GAA GAC TCT CAG GCA T-5'; for IP-10, forward primer 5'-ATG AAC CCA AGT GCT GCC-3' and reverse primer 3'-TGC GTG GCT TCA CTC CAG-5'; for β-actin, forward primer 5'-CTT ATA ACC TGC CTT TGC CT-3' and reverse primer 3'-GCA ACA TCA TTC CCT CTT TG-5'). After hybridization, the membranes were washed twice and then exposed to radiographic film. Hybridization with a housekeeping gene, β-actin probe, was used as a normalizing control. Relative chemokines and β-actin mRNA levels were expressed as chemokines/β-actin mRNA ratio.

Statistical Analysis

For statistical analysis, all data obtained were analyzed using the SPSS V12.0 software (SPSS Inc., Chicago, IL, U.S.A.). Statistically significant differences between studied groups were evaluated using the unpaired Student's *t* test. Results were determined to be statistically significant when $P<0.05$ was obtained. Statistical analysis of survival curves were performed by the Log-rank test using the SigmaStat V.3.5 software.

RESULTS

Body Weight and Survival Rate Changes

We compared the survival rates of IL-10 KO and wild-type mice after *C. kutscheri* infection (five mice per time point). A dose of 7×10^{10} bacteria induced no mortality over the course of four days in C57BL/6J wild-type mice. In contrast, five of ten IL-10 KO mice died three days after infection before their designated day of killing (50% survival) and the remaining five IL-10 KO mice died four days after infection (0% survival). The survival rate was significantly ($P<0.001$) higher in wild-type mice than IL-10 KO mice (Fig. 1A). Body weight decreased gradually in both IL-10 KO and wild-type mice after infection. The change in body weight was slightly higher in IL-10 KO mice compared with wild-type mice (Fig. 1B).

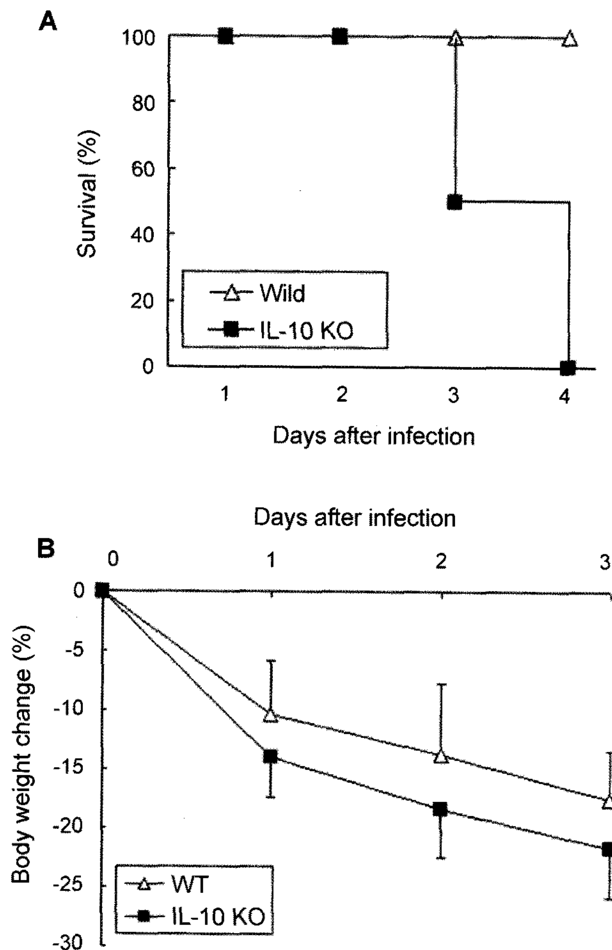

Fig. 1. Survival rates and body weight changes of IL-10 KO and wild-type mice after intratracheal *C. kutscheri* infection.
A. Fifty percent of IL-10 KO mice died after 3 days post-infection. In wild-type mice, no deaths were observed during the 4 days observation period. The survival rate was significantly ($P<0.001$) lower in IL-10 KO mice than wild-type mice. **B.** The body weight changes by *C. kutscheri* infection were decreased in IL-10 KO mice compared with wild-type mice. Data are expressed as the mean±SD of five mice per group.

Gross and Histopathological Findings in the Lung

Grossly, one to several gray-white bulging nodules were distributed throughout the parenchyma of the lung, both in the IL-10 KO and wild-type mice from 2 days infection (Fig. 2). The number of nodules in each lung increased until 3 days infection and was found to be higher in IL-10 KO mice than wild-type mice at each time point. As shown in Fig. 3, moderate bronchopneumonia was detected histopathologically in IL-10 KO mice, whereas mild bronchopneumonia manifested in wild-type mice on 1 day post-infection. After 2 days post-infection, the lungs of IL-10 mice exhibited severe bronchopneumonia with hemorrhage, whereas the lungs of wild-type mice exhibited moderate bronchopneumonia. The lungs were filled with inflammatory

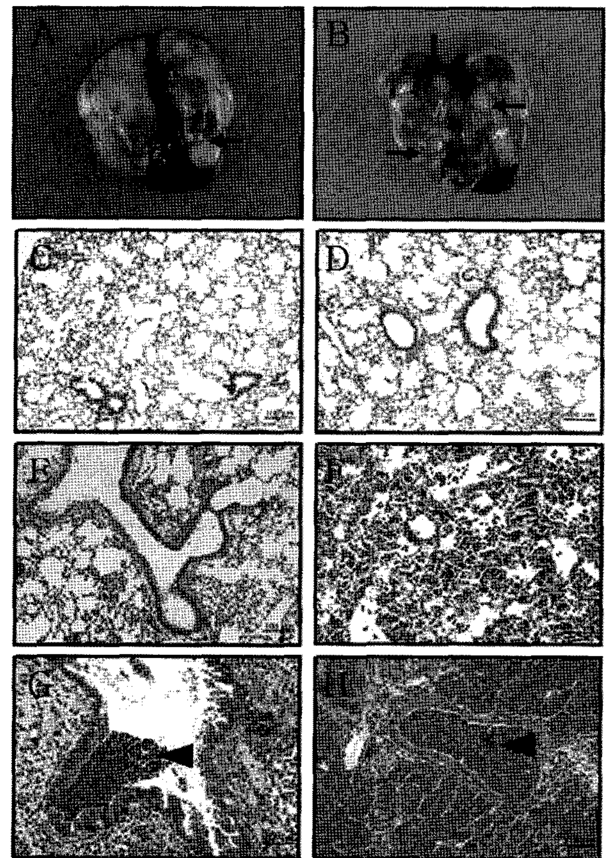

Fig. 2. Gross and histopathological findings of IL-10 KO (B, D, F, H) and wild-type (A, C, E, G) mice after intratracheal *C. kutscheri* infection.
Lung sections from both mice types were prepared and stained with H&E (C to H). One to several gray-white bulging nodules (arrows) was distributed throughout the lung. The number of nodules was higher in IL-10 KO mice than wild-type mice. C and D reveal the normal lung of uninfected control mice. Mild bronchopneumonia (E) and moderate bronchopneumonia (F) were seen in IL-10 and wild-type mice on 1 day post-infection. Severe bronchopneumonia with small abscess (G) or large abscess (H) were observed in IL-10 and wild-type mice on 2 day post-infection. Notice the large numbers of neutrophilic infiltrates in both mice types (arrowheads). Bar=100 μM.

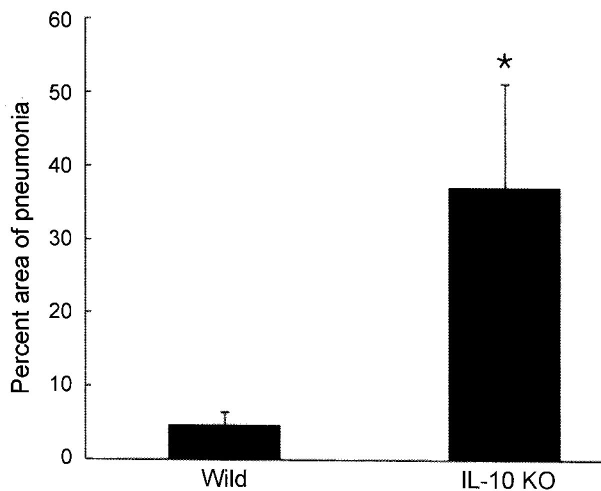

Fig. 3. Quantitation of the pneumonia of IL-10 KO and wild-type mice on 2 days post-intratracheal *C. kutscheri* infection.
The percentage area of pneumonia was significantly higher in IL-10 mice compared with wild-type mice. The percentage of pneumonia was determined by dividing the area of pneumonia by the total areas counted. Quantitation of the pneumonia was calculated as described in Materials and Methods. Data are expressed as the mean±SD of five mice per group. *, $P<0.05$ compared with wild-type mice at the same time points.

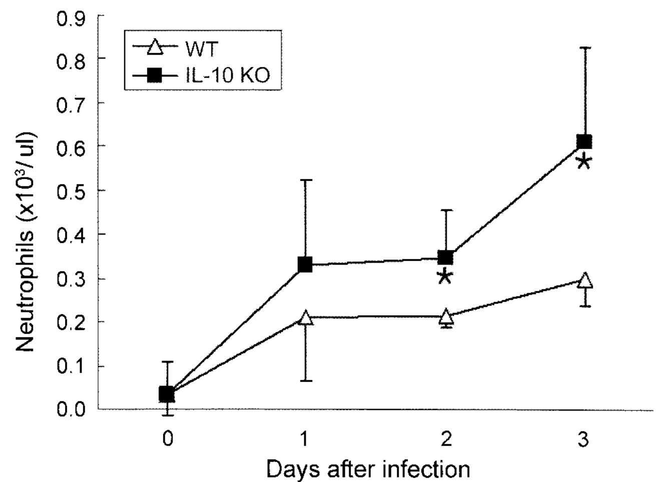

Fig. 4. The number of neutrophils in BAL fluid of IL-10 KO and wild-type mice after intratracheal *C. kutscheri* infection.
The number of neutrophils was significantly higher in IL-10 mice compared with wild-type mice at 2 and 3 days post-infection. Data are expressed as the mean±SD of five mice per group. *, $P<0.05$ compared with wild-type mice at the same time points.

cells, mainly neutrophils and blood, particularly in the IL-10 KO mice. Lung abscesses appeared in both IL-10 KO and wild-type mice from two days post-infection. The number of abscesses in the lungs increased until 3 days post-infection and was found to be higher in IL-10 KO mice than wild-type mice at each time point. The severity of the pneumonia and abscess formation was greater in the IL-10 KO mice than the wild-type mice. These observations indicated that *C. kutscheri* infection in IL-10 KO mice induced more severe pulmonary inflammation than in wild-type mice. However, the extent of pneumonia differed markedly between IL-10 KO and wild-type mice. At 2 days post-infection (Fig. 3), the percentage area of pneumonia was statistically higher in infected IL-10 KO mice (37.1±14.0) compared with infected wild-type mice (4.7±1.7).

Neutrophil Numbers and Cytokine Production in BAL Fluid

The absolute numbers of neutrophil (PMN) in BAL fluid gradually increased in both mice types after *C. kutscheri* administration. IL-10 KO mice had significantly increased numbers of neutrophils in the alveolar spaces in response to *C. kutscheri* on days 2 and 3 post-bacterial infection (Fig. 4). To examine the host defense against *C. kutscheri* infection, we assessed the production of IFN-γ, IL-2, and IL-4 proteins in BAL fluid. As shown in Fig. 5, IFN-γ concentrations in BAL fluid of IL-10 KO mice dramatically increased after 2 days post-infection. IFN-γ concentrations were approximately 6- to 28-fold higher at 1 to 2 days post-infection compared with uninfected IL-10 KO mice. However, the production of IFN-γ in wild-type mice was unchanged during infection. The level of IFN-γ in the BAL fluid in IL-10 KO mice was significantly higher than that of wild-type mice during infection. We also observed an increase in the IL-2 levels in BAL fluid after infection in both IL-10 KO and wild-type mice, but the differences at the same time points were not statistically significant. On 1 and 2 days post-infection, the levels of IL-4 were decreased in IL-10 KO mice but remained unchanged in wild-type mice. There was no statistically significant difference in the levels of IL-4 in IL-10 KO compared with wild-type mice during infection because of the variability within each group.

Chemokine Expression in the Lung

To examine the differences between the immune responses of the two mouse strains, we compared the levels of MIP-1α and IP-10 mRNA in their lungs (Fig. 6). MIP-1α mRNA levels were not detected in uninfected lungs of IL-10 KO and wild-type mice. However, in both mice types, the expression of MIP-1α mRNA increased on days 1 and 2 post-infection. MIP-1α mRNA levels were higher in the lungs of IL-10 KO mice than in those of the wild-type mice. On day 2 post-infection, MIP-1α mRNA levels showed an increase of more than 3-fold in IL-10 KO mice compared with that of wild-type mice. The levels of IP-10 mRNA were remarkably low in the uninfected lungs of IL-10 KO and wild-type mice. However, the expression of IP-10 mRNA dramatically increased in IL-10 KO mice during infection, and remained unchanged in wild-type mice during infection. On day 2 post-infection, IP-10 mRNA

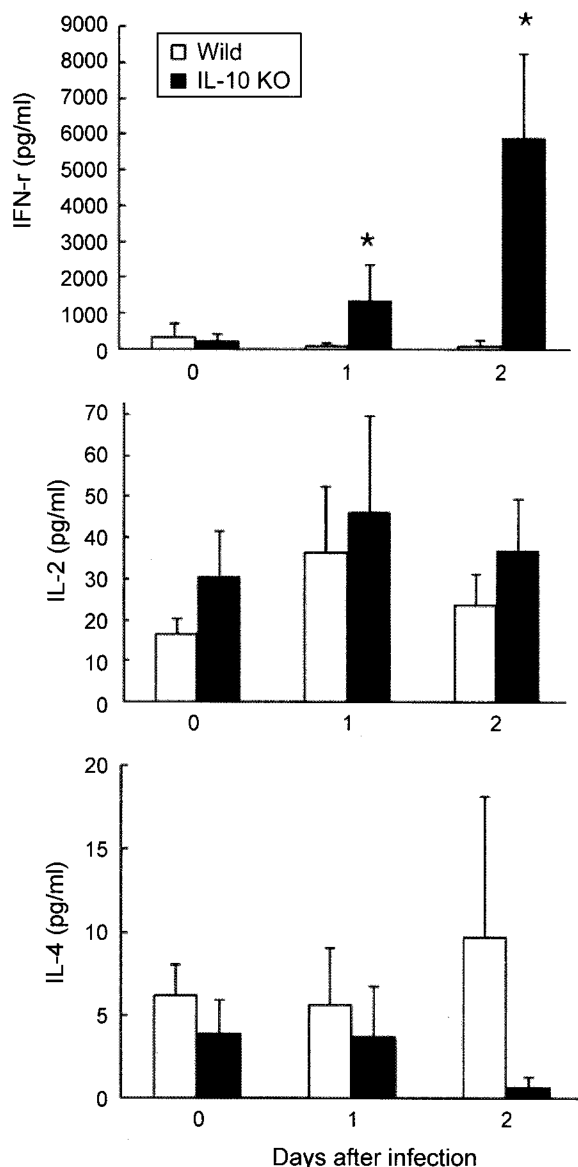

Fig. 5. Kinetics of cytokines in the BAL fluid of IL-10 KO and wild-type mice after intratracheal *C. kutscheri* infection.

The production level of IFN-γ was significantly increased in IL-10 KO mice compared with wild-type mice during *C. kutscheri* infection. The cytokine levels were measured by ELISA, as described in Materials and Methods. Data are expressed as the mean±SD of five mice per group. *, $P<0.05$ compared with wild-type mice at the same time points.

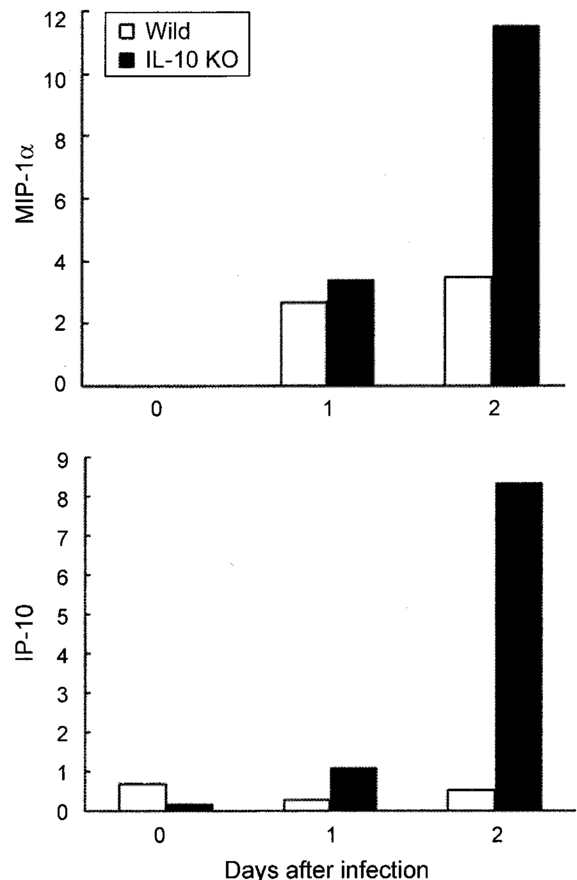

Fig. 6. Northern blot analysis for MIP-1α and IP-10 expressions in lung of IL-10 KO and wild-type mice after intratracheal *C. kutscheri* infection.

The expressions of MIP-1α and IP-10 were increased in IL-10 KO mice at 2 days post-infection compared with wild-type mice. The MIP-1α and IP-10 expressions were measured by Northern blot analysis, as described in Materials and Methods. The data are presented as fold increases versus β-actin levels.

levels had risen more than 16-fold in IL-10 KO mice compared with that of the wild-type mice.

Discussion

In the present study, we elucidated the role of IL-10 in host sensitivity to acute *C. kutscheri* infection using IL-10 KO mice. We observed that IL-10 KO mice were much more susceptible to *C. kutscheri* lung infection compared with wild-type mice. The goal of this study was to evaluate the role of IL-10 in the pathogenesis of *C. kutscheri*-infected lung. To address this question, we compared IL-10 KO mice to mice of the same genetic background (C57BL/6J), which cannot express this gene. We showed using BAL fluid analysis that there was a greater accumulation of neutrophils in alveolar spaces when IL-10 KO mice were infected with *C. kutscheri*. Furthermore, infected IL-10 KO mice displayed greater histopathological evidence of inflammation and pneumonia formation than did wild-type mice. Mice deficient in IL-10 also displayed greater weight-loss and lower survival rates. Several studies have demonstrated that IL-10 KO mice showed greater neutrophil infiltration, excessive inflammation, weight-loss, and mortality in a murine model of chronic as well as acute lung infection with *Pseudomonas aeruginosa* [3, 4]. The repeated respiratory challenge of *Pseudomonas aeruginosa* to IL-10 KO mice

was associated with increased mortality compared with wild-type mice [25]. However, conflicting reports have shown the opposite effect of IL-10 on sensitivity in different pneumonia murine models. IL-10 KO mice have been reported to be associated with enhanced host resistance against a number of fungal agents including *Pneumonocystis carinii* and *Coccidioides immitis* [13, 19].

The PMNs release mediators that damage tissues, result in deleterious functional alterations, and interfere with local defense mechanisms [16]. PMN-dominated pulmonary inflammation is known to be the major cause of loss of lung function and mortality of patients with cystic fibrosis. The high mortality rate of the IL-10 KO mice in this study was attributed to inflammation-induced tissue injury and organ dysfunction. Previous reports demonstrated that an exaggerated inflammatory response dominated by PMNs correlates with susceptibility to infection [5]. Our results also showed that the recruitment of PMNs was significantly higher in the lungs of IL-10 KO mice compared with that of wild-type mice on days 2 and 3 post-*C. kutscheri* infection. In the acute model used in this study, 50% of IL-10 KO mice died within three days after infection, but all wild-type mice stayed alive during the course of infection. Our data clearly indicated that the IL-10 KO mice were highly susceptible to *C. kutscheri* infection.

In order to examine the effect of IL-10 deficiency on the *C. kutscheri* infection, the cytokine levels in BAL fluid were measured. We observed a significantly higher concentration of IFN-γ in BAL fluid from the IL-10 KO mice compared with wild-type mice during the course of infection. These data indicated that IL-10 played a role in the regulation of production of IFN-γ during the infection. This finding is in agreement with previous reports that show that IL-10 is a potent negative regulator of IL-12 and IFN-γ production [8, 21]. As a component of the early host defense against infection, early expression of IFN-γ in the lung during pneumonia is derived from several cellular sources, including NK cells, γδ-T cells, and alveolar macrophages [6, 7, 17]. IFN-γ is a pivotal cytokine involved in host defense against a variety of pulmonary pathogens, and regulates inflammatory responses with adverse or beneficial consequences depending on the pathogens. Systemic infections with *Staphylococcus aureus* and *Escherichia coli* have indicated that IFN-γ plays a detrimental role in the resolution of these pathogens [15, 22]. In contrast, IFN-γ appears to play a more protective role in defense against bacteria with *Streptococcus pneumoniae* [20]. Our current study further supports the concept that upregulation of IFN-γ expression in IL-10 KO mice has the potential to cause lung injury. IFN-γ also recruits circulating PMNs and lymphocytes to the sites of infection. Upregulation of IFN-γ expression in IL-10 KO mice in our study enhanced PMNs recruitment into lung tissue in response to the *C. kutscheri* infection.

Cellular recruitment at the site of inflammation and/or infection is regulated by the upregulation of chemokine molecules in the local environment. Increased and early cellular recruitment in the lungs of mice devoid of IL-10 raised the question as to whether the kinetics of chemokine upregulation was different in these animals compared with that of wild-type mice. To address this, we examined the differential chemokine mRNA expression in the lungs of *C. kutscheri*-infected IL-10 KO and wild-type mice. IP-10 (CXCL10), MIP-1α, and monokines induced by gamma interferon (MIG) play a key role in promoting type 1 immune responses. Our study indicates that intrapulmonary challenges with *C. kutscheri* results in a marked induction of IP-10 and MIP-1α expressions. Previous observations indicated that the expression of IP-10 in the lungs of *Klebsiella pneumoniae*-infected mice was largely dependent upon IFN-γ, although other signals could serve to induce IP-10 levels in pneumonia independently of IFN-γ [26]. We report here that upregulation of IP-10 mRNA levels in the lung was detected in IL-10 KO mice along with high levels of IFN-γ protein.

Taken together, we demonstrated in this study that IL-10 KO mice were much more susceptible to *C. kutscheri* lung infection compared with wild-type mice. This suggests that IL-10 is involved in the regulation of lung damage induced by intratracheal *C. kutscheri* infection. Further experiments are warranted to confirm these findings and to determine whether other pro-inflammatory cytokines are differentially controlled in IL-10 and wild-type mice.

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