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IL-10 Modulates Host Responses and Lung Damage Induced by *Pneumocystis carinii* Infection[1]

Mahboob H. Qureshi,*‡ Allen G. Harmsen,§ and Beth A. Garvy[2†‡]

Host responses to *Pneumocystis carinii* infection mediate impairment of pulmonary function and contribute to the pathogenesis of pneumonia. IL-10 is known to inhibit inflammation and reduce the severity of pathology caused by a number of infectious organisms. In the present studies, IL-10-deficient (IL-10 knockout (KO)) mice were infected with *P. carinii* to determine whether the severity of pathogenesis and the efficiency of clearance of the organisms could be altered in the absence of IL-10. The clearance kinetics of *P. carinii* from IL-10 KO mice was significantly enhanced compared with that of wild-type (WT) mice. This corresponded to a more intense $CD4^+$ and $CD8^+$ T cell response as well as an earlier neutrophil response in the lungs of IL-10 KO mice. Furthermore, IL-12, IL-18, and IFN-γ were found in the bronchoalveolar lavage fluids at earlier time points in IL-10 KO mice suggesting that alveolar macrophages were activated earlier than in WT mice. However, when $CD4^+$ cells were depleted from *P. carinii*-infected IL-10 KO mice, the ability to enhance clearance was lost. Furthermore, CD4-depleted IL-10 KO mice had significantly more lung injury than CD4-depleted WT mice even though the intensity of the inflammatory responses was similar. This was characterized by increased vascular leakage, decreased oxygenation, and decreased arterial pH. These data indicate that IL-10 down-regulates the immune response to *P. carinii* in WT mice; however, in the absence of $CD4^+$ T cells, IL-10 plays a critical role in controlling lung damage independent of modulating the inflammatory response. *The Journal of Immunology*, 2003, 170: 1002–1009.

Pneumocystis carinii is an opportunistic fungal pathogen that causes life-threatening pneumonia in immunocompromised hosts (1–4). Despite the use of effective chemoprophylaxis, *P. carinii* pneumonia (PCP)[3] remains a major cause of death in immunocompromised patients in the United States of America (5, 6). Experimental and clinical studies indicate that hosts with compromised $CD4^+$ T cells are at risk for developing PCP (6–8). It has been recently reported in a murine model of PCP that depletion of $CD4^+$ cells also results in severe pulmonary inflammation and lung injury characterized by decreased lung compliance and arterial oxygen pressure (9). Interestingly, decreased lung compliance correlated with peak TNF-α mRNA expression in the lungs (9).

Proinflammatory cytokines, including IFN-γ and TNF-α, have been reported to be critical for the clearance of many organisms, including *Leishmania major*, *Toxoplasma gondii*, *Listeria monocytogenes*, and *Cryptococcus neoformans* (10). The role of inflammatory cytokines in the resolution of PCP is complex. In the absence of IFN-γ, mice are able to clear PCP, although they mount an exacerbated inflammatory response compared with wild-type (WT) mice (11, 12). However, administration of exogenous IFN-γ reduced the intensity of PCP in SCID mice (13). In contrast, depletion of TNF-α in *P. carinii*-infected SCID mice reconstituted with WT splenocytes resulted in the inability to clear the infection (12). Furthermore, TNF-α was critical for the early phase of the response because a single injection of anti-TNF-α on the day of reconstitution prevented resolution of PCP whereas treatment of mice with anti-TNF-α at day 6 had no effect (12). Interestingly, mice deficient in *TNFRI* genes efficiently cleared *P. carinii* organisms from the lungs; however, mice deficient in both *IFN-γ* and *TNFRI* genes were extremely susceptible to *P. carinii* infection (14). Together, these studies provide evidence that proinflammatory cytokines are important for controlling *P. carinii* infection. However, the role of anti-inflammatory cytokines has not been examined.

IL-10 is an anti-inflammatory cytokine that maintains a crucial balance between pathology and protection (10). Reduced levels of IL-10 (from treatment with anti-IL-10 Ab or in IL-10 knockout (KO) animals) have been reported to be associated with enhanced host resistance against a number of infectious agents including *L. monocytogenes*, *L. major*, *Trypanosoma cruzi*, *Candida albicans*, *Asperigillus fumigatus*, and *C. neoformans* (10, 15–18). Elevated levels of proinflammatory cytokines, including IFN-γ, TNF-α, and IL-12, and reduced organ loads of the microorganisms characterized the increased disease resistance (10, 15–18). Very recently, it was reported that gene transfer of viral IL-10 to murine lungs resulted in suppression of inflammatory responses to *P. carinii* without altering the intensity of infection (19). However, this study did not address whether host responses to *P. carinii* would be bolstered in the absence of IL-10.

We have recently reported significant levels of IL-10 and TGF-β mRNA expression in the lungs of neonatal mice infected with *P. carinii* (20). This was associated with a delayed clearance of the organisms as compared with adults who had predominantly

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[3] Abbreviations used in this article: PCP, *Pneumocystis carinii* pneumonia; i.t., intratracheal(ly); TBLN, tracheobronchial lymph node; RPA, RNase protection assay; P_aO_2, arterial oxygen tension; P_aCO_2, arterial carbon dioxide tension; KO, knockout; WT, wild type; BALF, bronchoalveolar lavage fluid; MIP, macrophage-inflammatory protein; MCP-1, monocyte chemotactic protein-1; TCA-3, T cell activation protein-3.

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proinflammatory cytokine responses (20). These data suggested that IL-10 influences clearance of *P. carinii* by controlling a critical balance of pro- and anti-inflammatory cytokines. However, this has not been definitively demonstrated. In our present study, we examined the modulatory effects of IL-10 on the host responses to *P. carinii* infection as well as the kinetics of *P. carinii* clearance in IL-10 KO mice as compared with the WT mice. We also examined the effects of IL-10 deficiency on cytokine and chemokine responses and cellular recruitment into the lungs in response to *P. carinii* infection. Finally, using a model of CD4-depleted mice, we examined lung function as an indication of lung damage in mice deficient in IL-10. Our studies confirm that IL-10 is an important mediator of inflammation and lung injury in mice infected with *P. carinii*.

Materials and Methods

Mice

Five- to 6-wk-old homozygous IL-10 KO (B6.129P2-Il10^{tm1Cgn}) and C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or bred in our animal facilities. Colonies of C.B-17 SCID mice (originally from Taconic (Germantown, NY)), used to maintain a source of *P. carinii*, were also bred in our facilities in microisolator cages containing sterilized food and water. All mice were maintained under pathogen-free conditions.

P. carinii *infection and depletion of CD4$^+$ T cells*

Eight- to 9-wk-old IL-10 KO and WT mice were infected by inoculation of *P. carinii*. Lungs were excised from *P. carinii*-infected SCID mice and pushed through steel mesh in HBSS. *P. carinii* was isolated and enumerated by microscopy as described (8, 21). Mice were inoculated intratracheally (i.t.) with 1×10^7 *P. carinii* organisms under halothane anesthesia. CD4$^+$ cells were depleted in vivo by twice weekly i.p. injections of 0.25 mg anti-CD4 mAb (clone GK1.5; American Type Culture Collection, Manassas, VA).

Isolation of lung interstitial and tracheobronchial lymph node (TBLN) cells

Lung and TBLN cells were prepared as described elsewhere (20, 22). Briefly, right lungs were excised, minced, and enzyme treated at 37°C for 60 min in RPMI containing 3% FCS, 50 U/ml DNase (Sigma-Aldrich, St. Louis, MO), and 1 mg/ml collagenase A (Sigma-Aldrich). Digested lung tissue was pushed through mesh screens, and RBC were lysed by treatment with a hypotonic buffer. Lung cells were resuspended in HBSS for enumeration. TBLNs were collected on indicated days after infection from *P. carinii*-infected mice. Lymph node tissues were pushed through mesh screens and treated with a hypotonic lysis buffer to eliminate RBC. Cells were resuspended in HBSS for enumeration.

Lung lavage and cytokine ELISAs

Lung lavages were performed as described elsewhere (22). Briefly, lung airways were lavaged using an intratracheal cannula with five 1-ml washes of cold HBSS containing 3 mM EDTA. Cells were spun out of the first wash, and the bronchoalveolar lavage fluid (BALF) was frozen for subsequent assays. Cells were spun onto glass slides using a cytocentrifuge, and differential cell count was done by microscopy after staining with DiffQuik (Dade International, Miami, FL). IL-12, IL-18, and IFN-γ content in lavage fluids was measured by using commercially available sandwich ELISA kits (BD PharMingen, San Diego, CA), according to the manufacturer's instruction.

Enumeration of P. carinii

Aliquots of digested lung tissue were diluted and spun onto glass slides, fixed in methanol, and stained with DiffQuik (Dade International). *P. carinii* nuclei were enumerated microscopically as described (8, 21). The number of *P. carinii* organisms was expressed as follows: $\log_{10}$ nuclei/right lungs. To examine induction of chemokine mRNA up-regulation in the same animals in which we are estimating the *P. carinii* lung burden, enumeration of the number of organisms was performed in the right lobes of lungs and the left lung lobes were snap frozen for RNase protection assays (RPA). The lung *P. carinii* burden determined from the right lobes is proportional to counts obtained using whole lung. The limit of detection of *P. carinii* was $\log_{10}$ 3.6.

Preparation of lung and TBLN cells for flow cytometric analysis

Cells derived from digested lung and TBLN tissue were enumerated and $\sim 5 \times 10^5$ to $\sim 1 \times 10^6$ cells were used for staining with fluorochrome-conjugated Abs specific for murine CD4, CD8, CD11b, CD11c, CD18, CD19, CD44, and CD62L. Abs were purchased from BD PharMingen. Before and after Ab staining, cells were washed once with PBS containing 0.1% BSA and 0.02% sodium azide. Finally, cells were resuspended in PBS for multiparameter analysis using a FACSCalibur cytofluorometer (BD Biosciences, Mountain View, CA). A minimum of 10,000 events were routinely collected for analysis.

Extraction of RNA and RPA

Total RNA was prepared from frozen lung tissue by using TRIzol (Life Technologies, Gaithersburg, MD) 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. Multiprobe DNA templates for chemokines (Ltn, RANTES, macrophage-inflammatory protein (MIP)-1α and -1β, monocyte chemotactic protein-1 (MCP-1), MIP-2, and T cell activation protein-3 (TCA-3)) and housekeeping genes, *L32* and *GAPDH*, were purchased from BD PharMingen. RPA was performed using the RiboQuant in vitro transcription and RPA kits (BD PharMingen) according to the manufacturer's protocol. The RNase-protected RNA duplexes were extracted with phenol/chloroform/isoamyl alcohol and resolved on 5% denaturing polyacrylamide gels. Dried gels were exposed to phosphor screens, and images were developed using a Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The intensity of each specific cytokine and chemokine band was measured by using ImageQuant software (Molecular Dynamics). The chemokine mRNA levels were corrected for RNA loaded by dividing the chemokine hybridization signal by the *L32* signal for the same sample.

Measurements of lung injury

Arterial blood gas tensions and pH were determined as we previously described (9). Briefly, mice were gently heated to enhance blood flow in the tail. The ventral artery of the tail was then nicked with a scalpel and ~100 μl of blood was collected into a heparinized capillary tube. The samples were then analyzed on a clinical blood gas machine within 45 min of drawing. Respiratory rates were measured in live mice using plethysmograph equipment (Harvard Apparatus, Holliston, MA). Albumin concentrations in BALF were determined by a colorimetric method according to the manufacturer's instructions (procedure 500; Sigma-Aldrich).

Statistical analysis

Statistical significance of results was determined by Student's *t* tests or ANOVA followed by the Student-Neuman-Keul posthoc test, where appropriate, using commercially available software (Sigmastat; SPSS, Chicago, IL). Results were determined to be statistically significant when $p < 0.05$ was obtained.

Results

Clearance of P. carinii *is enhanced in IL-10 KO mice*

To determine whether IL-10 has a role in the clearance of *P. carinii* organisms, IL-10 KO and WT mice were infected i.t. with 10^7 *P. carinii* organisms, and their lung burden was determined at various time points. As shown in Fig. 1, IL-10 KO and WT mice had similar lung *P. carinii* burdens early postinfection (day 5); however, *P. carinii* growth in the lungs was significantly slower in IL-10 KO mice compared with WT controls at later stages (days 9 and 15). Three of five IL-10 KO mice had cleared the organisms by day 15 postinfection, whereas all WT mice had $>\log_{10}$ 6.0 *P. carinii* organisms in their lungs at that time. However, by day 23 postinfection, there was no difference in *P. carinii* lung burdens between the IL-10 KO and WT mice. These data indicate that clearance of *P. carinii* in IL-10 KO mice was significantly accelerated compared with that in WT mice, suggesting that IL-10 may play a role in modulating the host responses to *P. carinii* infection.

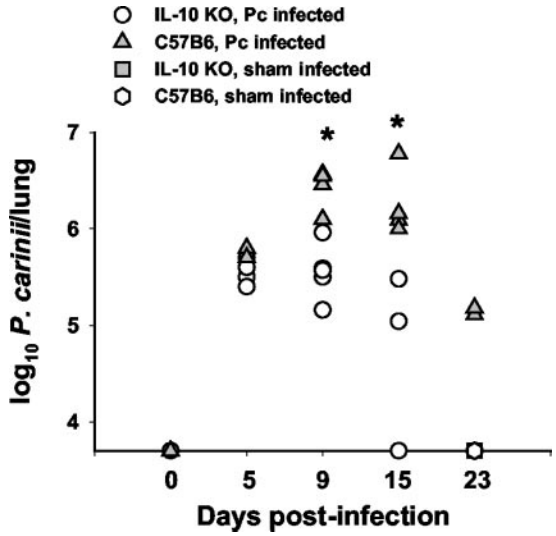

FIGURE 1. Resolution of lung *P. carinii* organisms is enhanced in IL-10 KO mice compared with that in immunocompetent WT mice. Adult WT and IL-10 KO mice were given i.t. inoculation of 10^7 *P. carinii* organisms, and lung burdens were determined at indicated time points. Note that three of five IL-10 KO animals had cleared the organisms by day 15 postinfection. Data are expressed as $\log_{10}$ *P. carinii* per right lung lobes. Data are representative of three separate experiments. $*$, $p < 0.05$ compared with WT mice at the same time points.

Differential cellular infiltration into the lungs of IL-10 KO and WT mice in response to P. carinii *infection*

Resolution of *P. carinii* is dependent on the $CD4^+$ T cell response (7, 8). Therefore, we examined whether accelerated clearance of *P. carinii* in IL-10 KO mice was associated with an enhanced cellular infiltration. $CD4^+$ and $CD8^+$ cellular infiltration into the lungs of IL-10 KO animals was enhanced compared with that of WT mice. Total numbers of activated $CD4^+$ cells ($CD44^{high}CD62^{low}$ phenotype) in the lung lavages and lung interstitium of IL-10 KO mice were significantly higher than in the WT mice on days 15 and 23 postinfection (Fig. 2*A*). Similarly, infiltration of activated $CD8^+$ T cells into the interstitium and alveolar spaces of IL-10 KO mice was also significantly elevated compared with that in the WT mice on days 15 and 23 postinfection (Fig. 2*B*). Interestingly, there was a significant difference in the *P. carinii* lung burdens between the IL-10 KO and WT mice at day 9 postinfection; however, cellular infiltration into the lungs was comparable in both groups at that time. This suggested that the accelerated clearance of *P. carinii* in IL-10 KO mice may be mediated through a different mechanism than $CD4^+$ and $CD8^+$ cellular responses.

IL-10 KO mice had significantly increased numbers of neutrophils in the alveolar spaces in response to *P. carinii* as early as day 5 postinfection (Fig. 3). Neutrophil infiltration into the lungs of IL-10 KO mice further increased and reached peak levels by day 9 postinfection, whereas neutrophil accumulation in WT mice did not peak until day 15. This kinetics is similar to that of *P. carinii* clearance in the IL-10 KO mice, suggesting that neutrophil infiltration may have influenced the resolution of *P. carinii*.

Enhanced cytokine production in IL-10 KO mice in response to P. carinii *infection*

Clearance of *P. carinii* infection in immunocompetent mice is associated with up-regulation of lung cytokine mRNA expression including IFN-γ, TNF-α, and IL-12 (22, 23). This led us to examine whether accelerated *P. carinii* clearance in IL-10 KO mice is associated with enhanced cytokine production. BALF concentrations of IFN-γ were significantly higher in response to *P. carinii*

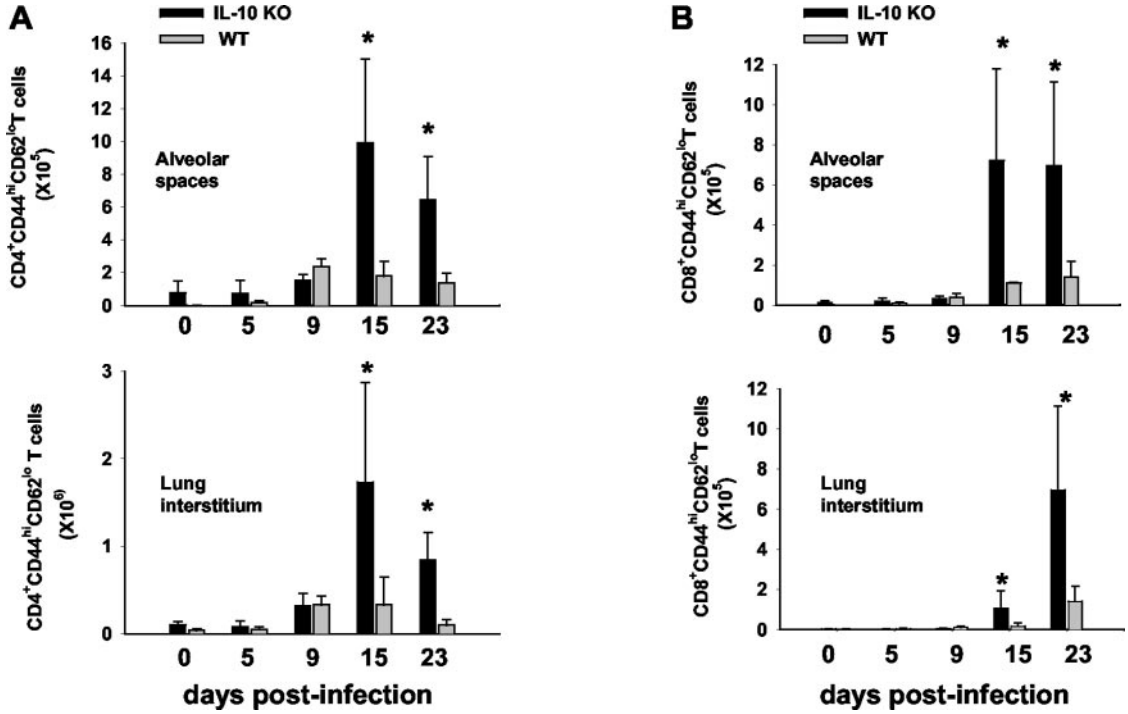

FIGURE 2. IL-10 KO animals experience an early and enhanced $CD4^+$ and $CD8^+$ pulmonary cellular response to *P. carinii* compared with that of WT mice. IL-10 KO and WT mice were given i.t. inoculations of *P. carinii.* At indicated time points postinfection, lungs were lavaged and excised. Single-cell suspensions were analyzed for the presence of activated $CD4^+CD44^{high}CD62^{low}$ cells (*A*) and $CD8^+CD44^{high}CD62^{low}$ cells (*B*) in alveolar spaces and lung interstitium by flow cytometry. Data are expressed as the mean ± SD of five mice per group and are representative of three separate experiments. $*$, $p < 0.05$ compared with WT mice at the same time points.

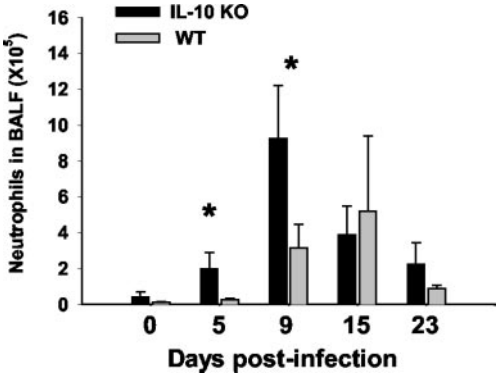

FIGURE 3. The neutrophil response to *P. carinii* infection is augmented in absence of IL-10. Mice were given i.t. inoculations of *P. carinii*, and, at indicated time points, lungs were lavaged. Cells in the BALF were spun on glass slides and stained with DiffQuik, and neutrophils were enumerated microscopically. Data are expressed as the mean ± SD of five mice per group and are representative of three separate experiments. *, $p < 0.05$ compared with WT mice at the same time points.

infection in the absence of IL-10 as early as days 5 and 9 postinfection (Fig. 4). However, by day 15 postinfection, production of IFN-γ in WT mice was higher than that in the IL-10 KO mice indicating that IL-10 regulated production of IFN-γ during the early phase of infection. IFN-γ production by Th cells is largely

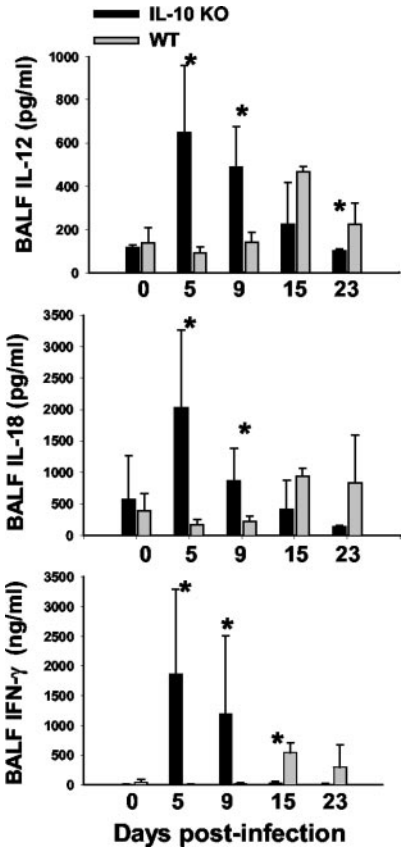

FIGURE 4. IL-10 KO mice had early and increased production of IFN-γ, IL-12, and IL-18 compared with that of the WT mice. WT and IL-10 KO mice received *P. carinii* inoculations, and lungs were lavaged at indicated time points. BALF levels of IL-12, IL-18, and IFN-γ were measured by ELISA. Data are expressed as the mean ± SD of five mice per group and are representative of three separate experiments. *, $p < 0.05$ compared with WT mice at the same time points.

regulated by the availability of IL-12 and/or IL-18 in the local environment (24, 25). To determine whether enhanced and early IFN-γ production is mediated by IL-12 and/or IL-18, we measured the BALF levels of IL-12 and IL-18 in the absence or presence of IL-10. Production of both IL-12 and IL-18 was significantly higher in the IL-10 KO mice on days 5 and 9 postinfection compared with that of WT mice (Fig. 4). Concentrations of both cytokines in IL-10 KO mice peaked at day 5 postinfection and then gradually declined over time until they were lower than in WT mice at day 15 postinfection. This kinetics corresponds with that of IFN-γ production and *P. carinii* clearance. These data indicate that production of inflammatory cytokines including IFN-γ, IL-12, and IL-18 in response to *P. carinii* is augmented in the absence of IL-10.

Up-regulated chemokine mRNA expression in the lungs of IL-10 KO mice

Cellular recruitment at the site of inflammation and/or infection is regulated by up-regulation of chemokine molecules in the local environment (26). Increased and early cellular recruitment in the lungs of mice devoid of IL-10 raised the question whether kinetics of chemokine up-regulation is different in these animals compared with that of WT mice. To address this, we examined differential chemokine mRNA expression in the lungs of *P. carinii*-infected IL-10 KO and WT mice. As shown in Fig. 5, the kinetics of chemokine up-regulation in the lungs was similar in both groups, although the magnitude of mRNA expression was higher in IL-10 KO mice. In both groups, expression of MCP-1, RANTES, and MIP-1α and -1β mRNAs were at peak levels by day 9 postinfection and gradually declined over time (Fig. 5*A* and data not shown). Expression of MCP-1 mRNA was significantly higher in the lungs of IL-10 KO mice than in those of the WT mice as early as day 5 postinfection. RANTES and MIP-1α and -1β mRNA expression was also significantly higher in the lungs of IL-10 KO mice compared with that in WT mice on day 9 postinfection (Fig. 5*A* and not shown). Up-regulated expression of all these CC chemokines in the absence of IL-10 during the early phase of infection may have modified the kinetics of cellular recruitment in the IL-10 KO mice compared with that in the WT mice. In addition, MIP-2 and TCA-3 mRNA expression was significantly increased in the IL-10 KO mice compared with that in WT mice as early as day 5 postinfection (Fig. 5*B*). This finding is consistent with the observation of increased neutrophil infiltration into the alveolar spaces of IL-10 KO mice at days 5 and 9 after infection (Fig. 3).

$CD4^+$ cells are required for accelerated clearance of P. carinii *in IL-10 KO mice*

IL-10 KO mice had a significantly lower *P. carinii* lung burden than that of WT mice as early as day 9 postinfection (Fig. 1); however, recruitment of $CD4^+$ cells into lungs was comparable in both groups at that time. This observation suggests that accelerated clearance of *P. carinii* organisms in the absence of IL-10 may have been mediated through a $CD4^+$ cell-independent mechanism. Establishment of the existence of such a mechanism would be of great clinical importance, particularly for HIV-infected individuals who are at risk for PCP due to decreased numbers of $CD4^+$ cells (6). To address this issue, we depleted IL-10 KO mice of $CD4^+$ T cells using specific Abs before infecting them with *P. carinii* organisms. As shown in Fig. 6, IL-10 KO mice and WT mice had comparable lung *P. carinii* burdens after depletion of $CD4^+$ cells on days 6, 11, and 34 postinfection. Clearance of *P. carinii* was not observed in these mice in the absence of $CD4^+$ T cells and lung *P. carinii* burden increased to about $\log_{10}$ 8.0 by day 34 postinfection, indicating a near terminal stage of illness. Furthermore, there were no significant differences in the total number of cells isolated from

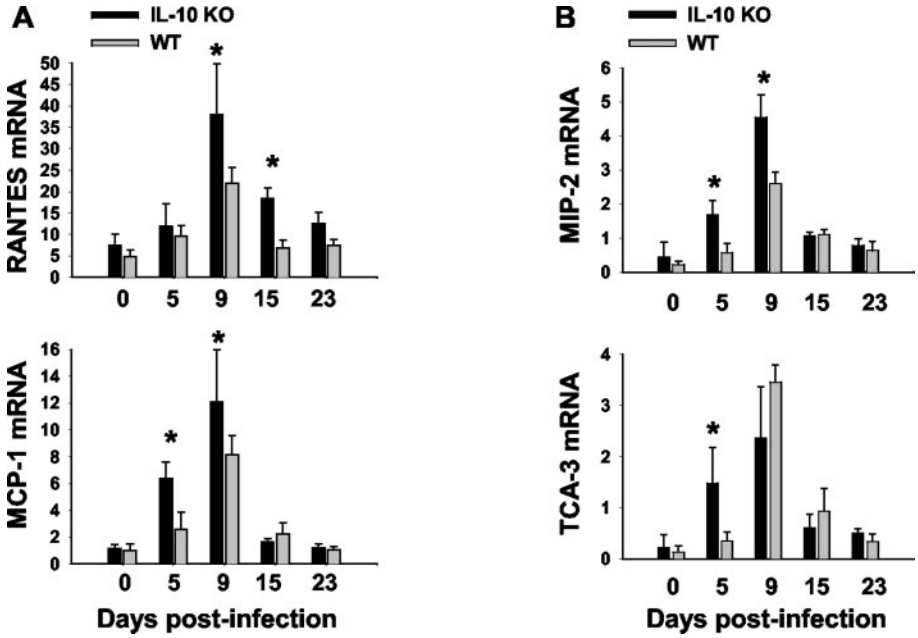

FIGURE 5. Expression of chemokine mRNA in the lungs of *P. carinii*-infected IL-10 KO mice was enhanced compared with that in WT mice. Mice were infected with *P. carinii*, and lungs were excised and snap frozen at indicated time points. RNA was extracted from frozen tissues and expression of chemokine mRNA was quantified using RPA. *A*, Expression of RANTES and MCP-1 mRNA. *B*, Expression of MIP-2 and TCA-3 mRNA. Data represent the mean ± SD of four to five mice per group and are representative of three separate experiments. ∗, $p < 0.05$ compared with WT mice at the same time points.

the BALF, nor in the number of activated $CD8^+$ cells nor neutrophils at any of the time points examined (Fig. 7). These data are consistent with previous observations demonstrating that $CD4^+$ cells are essential for *P. carinii* clearance (8). Notably, unlike mice with intact $CD4^+$ cell populations, the IL-10 KO mice depleted of $CD4^+$ cells had no early protection against *P. carinii*. Consistent with this finding, the concentrations of IFN-γ, IL-12p40, and IL-18 in the BALF were not different between the CD4-depleted IL-10 KO and WT mice at any of the time points (data not shown). This suggests that the mechanism responsible for accelerated *P. carinii* clearance and activation of alveolar macrophages in IL-10 KO mice is dependent on $CD4^+$ cells.

The absence of IL-10 exacerbates lung injury in $CD4^+$ cell-depleted mice

Even though lung *P. carinii* burden was comparable in both IL-10 KO and WT mice after $CD4^+$ T cell depletion, IL-10 KO mice appeared sicker and suffered from loss of body weight compared with WT mice (Fig. 8). Fig. 8 shows that, on average, the CD4-depleted IL-10 KO mice lost weight over the 34 days of the experiment, whereas

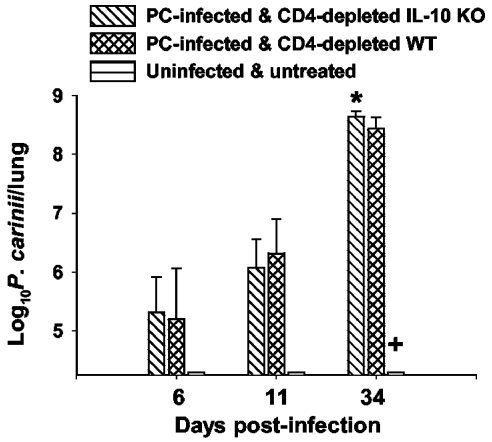

FIGURE 6. IL-10 KO and WT mice lacking $CD4^+$ cells are unable to control *P. carinii* growth. $CD4^+$ T cells were depleted in vivo using neutralizing anti-CD4 mAb, and mice were infected with *P. carinii*. Lung burdens of *P. carinii* organisms were determined at indicated time points postinfection. Controls were uninfected and untreated WT mice. Data are the mean ± SD of five to six mice per group. ∗, $p < 0.05$ compared with the CD4-depleted WT mice at the same time points. +, $p < 0.05$ compared with the *P. carinii*-infected groups at the same time points.

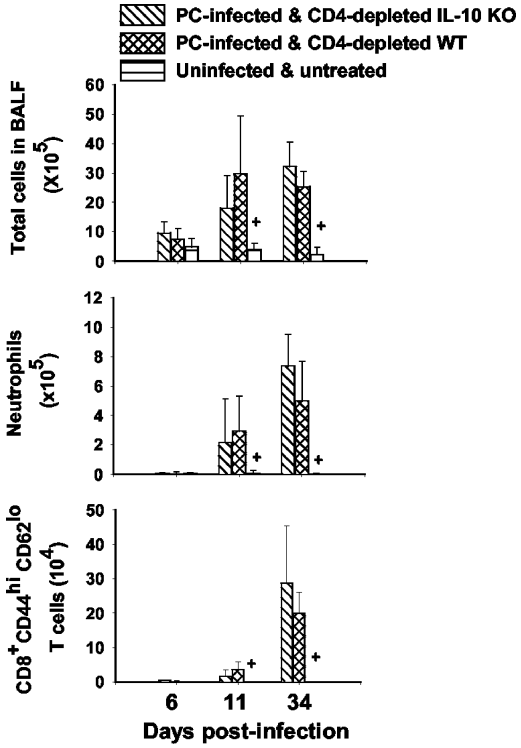

FIGURE 7. Pulmonary cellular responses to *P. carinii* were not different in IL-10 KO and WT mice in the absence of $CD4^+$ cells. Mice were depleted of $CD4^+$ cells and infected as described in Fig. 6. At indicated time points postinfection, lungs were lavaged, and the total number of cells and neutrophils present in BALF was enumerated microscopically. Infiltration of activated $CD8^+CD44^{high}CD62^{low}$ cells into the alveolar spaces was determined by flow cytometry. Controls were uninfected and untreated WT mice. Note that there were no differences in cellular response between the IL-10 KO and WT mice at any time examined. Data are expressed as the mean ± SD of five to six mice per group. +, $p < 0.05$ compared with the *P. carinii*-infected groups at the same time points.

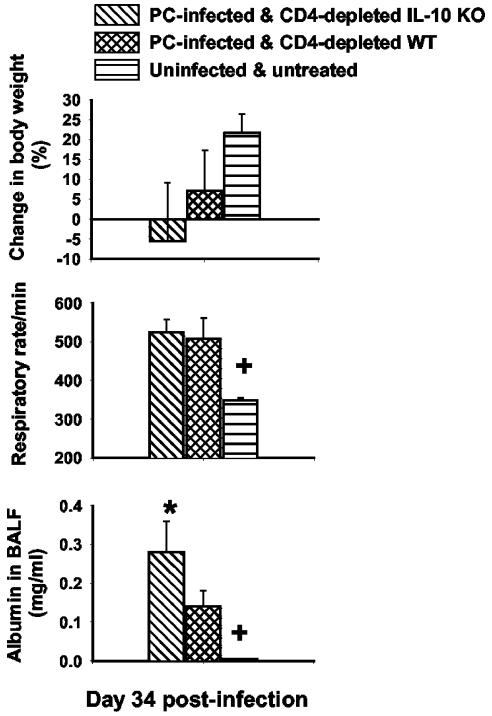

FIGURE 8. Effects of IL-10 and $CD4^+$ T cells on respiratory rates, BALF albumin concentrations, and body weights during PCP in mice. IL-10 KO and WT mice were depleted of $CD4^+$ T cells and infected as described in Fig. 6. Body weights, respiratory rates, and albumin content in BALF were measured at indicated time points. Controls were uninfected and untreated WT mice. Data represent the mean ± SD of five to six mice per group. *, $p < 0.05$ compared with CD4-depleted WT mice at the same time points. +, $p < 0.05$ compared with the *P. carinii*-infected groups at the same time points.

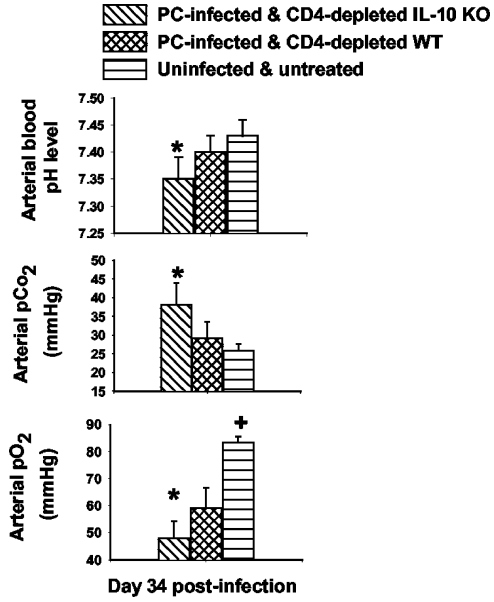

FIGURE 9. Respiratory impairment during PCP was exacerbated in IL-10 KO mice compared with that of the WT mice after $CD4^+$ T cell depletion. Mice were depleted of $CD4^+$ T cells and infected as per Fig. 6. Measurements of P_aO_2, P_aCO_2, and arterial blood pH were performed on day 34 postinfection. Uninfected and untreated WT mice were used as control. Data represent the mean ± SD of five to six mice per group. *, $p < 0.05$ compared with CD4-depleted WT mice at the same time points. +, $p < 0.05$ compared with the *P. carinii*-infected mice at the same time points.

the CD4-depleted WT mice gained weight, although not nearly as much as untreated and uninfected WT mice. Respiratory rates were ~2-fold higher in the CD4-depleted mice compared with those of untreated controls (Fig. 8), although there was no difference in the respiratory rates of the CD4-depleted IL-10 KO mice compared with those of the CD4-depleted WT mice. However, albumin content of BALF, an indicator of lung permeability, was significantly elevated in both *P. carinii*-infected, CD4-depleted WT and IL-10 KO mice compared with the uninfected, untreated WT mice (Fig. 8). Interestingly, albumin content was significantly higher in the BALF of CD4-depleted IL-10 KO mice compared with that of CD4-depeleted WT mice (Fig. 8), indicating that lung injury in the IL-10 KO mice was more intense than in WT mice.

Respiratory impairment is exacerbated in the absence of IL-10

To further characterize the respiratory impairment in WT and IL-10 KO mice, after $CD4^+$ cell depletion, we measured arterial oxygen tension (P_aO_2), arterial carbon dioxide tension (P_aCO_2), and arterial blood pH. As shown in Fig. 9, both WT and IL-10 KO mice lacking $CD4^+$ cells had lower P_aO_2 compared with the uninfected mice by day 34 postinfection. However, *P. carinii*-infected CD4-depleted IL-10 KO mice averaged nearly 15 mmHg lower P_aO_2 compared with the CD4-depleted WT mice (Fig. 9). Consistent with this observation, P_aCO_2 was significantly increased and pH levels were significantly decreased in the CD4-depleted IL-10 KO mice compared with the CD4-depleted WT mice in response to *P. carinii* infection (Fig. 9). These data indicate that the absence of IL-10 exacerbated the respiratory impairment normally caused by *P. carinii* infection.

Discussion

IL-10 is known to be important in controlling inflammatory responses to infection at the expense of efficient clearance of the organisms (15–18, 27, 28). As has been reported for other infectious agents (10), we have demonstrated that IL-10 KO mice clear *P. carinii* infections more rapidly than do WT mice. This enhanced clearance corresponded with elevated levels of IFN-γ, IL-12, and IL-18 in BALF, suggesting that alveolar macrophages were more active in the IL-10 KO than in WT mice. In addition to increased expression of proinflammatory cytokines and chemokines, the cellular responses in the lungs were also significantly greater in the IL-10 KO mice than in WT mice which is consistent with a lack of control of inflammatory mediators. Interestingly, when $CD4^+$ T cells were depleted from IL-10 KO mice, rather than observing enhanced protection from PCP, these mice were unable to control infection and had significantly worsened lung injury compared with $CD4^+$ T cell-depleted WT mice. Together, these data confirm that IL-10 is an important anti-inflammatory mediator in the lungs and is also important in modulating lung injury from PCP.

P. carinii continues to be a significant problem for immunocompromised individuals including AIDS patients. Most patients with <200 $CD4^+$ T cells/μl of blood are placed on prophylaxis for PCP. Although effective, the drug of choice for prophylaxis or PCP treatment, trimethoprim/sulfamethoxazole, is not well tolerated by all individuals, has bone marrow toxicity associated with it, and can fail (29–33). It would be of significant benefit if alternative strategies, particularly those that use innate host defense mechanisms, were used to treat or prevent PCP. Because IL-10 exerts many of its functions by inhibiting cytokine production of macrophages (10, 34, 35), it was thought that releasing IL-10 pressure may contribute to enhanced phagocytosis and killing activity by alveolar macrophages. Indeed, unlike WT mice, IL-10 KO mice were able to control lung *P. carinii* burden and largely clear the

infection within 15 days. This expedited clearance of PCP corresponded to elevated concentrations of IL-12, IL-18, and IFN-γ in BALF and increased chemokine mRNA expression in the lungs.

It is generally thought that alveolar macrophages are the cells responsible for killing of *P. carinii* (36, 37). It has been reported that depletion of macrophages in rat lungs resulted in the inability to control *P. carinii* infection (38). However, macrophages alone are not sufficient for clearance of infection because mice deficient in T cells but containing functional macrophages are susceptible to PCP (39). $CD4^+$ T cells are required for clearance of *P. carinii*; however, their function is not to merely provide cytokines such as IFN-γ, because adoptive transfer of T cells from IFN-γ-deficient donors into infected SCID mice does not affect clearance of PCP (11). In the experiments presented in this study, it is likely that the release of IL-10 pressure on alveolar macrophages resulted in increased production of IL-12 and IL-18 that then stimulated production of IFN-γ by T cells. The net effect was that phagocytosis and killing of *P. carinii* were more efficient in the absence of IL-10. Notably, there was no difference in the lung *P. carinii* burdens of WT compared with IL-10 KO mice at day 5 postinfection, before T cell infiltration into the lungs. This was unexpected because it has been shown with other fungal infections that, in the absence of IL-10, innate antifungal activity is enhanced within 24–48 h of challenge (18, 28). Moreover, neutrophil responses were elevated in the lungs of IL-10 KO mice in response to *P. carinii* at these early time points; however, they appeared ineffective at controlling infection. This is consistent with our unpublished observations that depletion of neutrophils in mice using a mAb specific for GR-1 has no effect on clearance of *P. carinii* infection. These data suggest that $CD4^+$ T cells must be present at the site of infection for clearance to take place.

In the absence of IFN-γ, mice are capable of clearing *P. carinii* infections (11, 12). This suggests that other proinflammatory cytokines compensate for the absence of IFN-γ. Surprisingly, it was found that IFN-γ is also important for controlling inflammatory responses in the lungs, because in the absence of IFN-γ, mice developed severe interstitial pneumonia that persisted after clearance of *P. carinii* infections (11, 40). IFN-γ is generally considered a proinflammatory cytokine; however, it has been shown in other systems to be important for controlling inflammation. In the absence of IL-10, the levels of IFN-γ were increased in the lung BALF. This was likely an important reason for the early increased expression of chemokine mRNA in the lungs of the IL-10 KO mice, resulting in a more intense T cell and neutrophil response (26, 41).

Because we observed enhanced clearance of *P. carinii* in IL-10 KO mice, we hypothesized that neutralization of IL-10 in immunodeficient individuals may afford some protection against development of PCP. In this regard, enhancement of early innate responses to *Listeria*, *T. gondii*, *T. cruzi*, *C. albicans*, *A. fumigatus*, and *C. neoformans* have been previously described in IL-10 KO mice (10, 18, 27, 28). Surprisingly, there appeared to be no protection against PCP in CD4-depleted IL-10 KO mice compared with similarly treated WT mice. This may have been due to the failure of IFN-γ, IL-12, and IL-18 to be produced in increased quantities in CD4-depeleted IL-10 KO mice compared with the similarly treated WT mice. This was an unexpected result, because it has been demonstrated that IL-10 acts directly on macrophages to inhibit production of IL-12 and IFN-γ (10, 42). Consistent with our results, Neyer et al. (43) reported that, in the absence of lymphocytes to drive a lethal inflammatory response, SCID/IL-10 KO mice survived *T. gondii* infection significantly longer than either IL-10 KO mice or SCID mice. These studies indicate that a significant portion of the proinflammatory responses to some infectious agents are driven by T cells.

Finally, *P. carinii*-infected, CD4-depleted IL-10 KO mice suffered more severe lung injury than did CD4-depleted WT mice. This increase in severity of lung injury manifested itself as decreased body weight, increased albumin concentrations in the BALF, increased P_aCO_2, and decreased arterial blood pH and P_aO_2 in the CD4-depleted IL-10 KO mice compared with similarly treated WT mice. It has recently been reported that lung injury in *P. carinii*-infected mice is driven by immune-mediated inflammation because depletion of both $CD4^+$ and $CD8^+$ resulted in the inability to clear the infection, whereas markers of lung injury including vascular leak, dynamic lung compliance, and respiratory rate were normal (9). However, *P. carinii*-infected mice depleted of only $CD4^+$ cells had significant vascular leak, decreased dynamic lung compliance, and decreased P_aO_2, suggesting that $CD8^+$ cells contributed to the lung injury (9). Interestingly, in our studies, we found that, in the absence of IL-10, the markers of lung injury were more severe in *P. carinii*-infected and CD4-depleted mice than in WT mice. However, this was not due to increased numbers of $CD8^+$ cells or neutrophils in lungs because they were not different between the IL-10 KO and WT mice. Moreover, there was no difference in the concentrations of IFN-γ, IL-12, or IL-18 in the CD4-depleted IL-10 KO compared with those in WT mice, which may suggest that macrophage activation is not driving the lung injury.

It has been reported in immune complex models of lung injury that IL-10 protects against vascular albumin leak and hemorrhage and that this corresponded to reduced production of TNF-α in the lungs (44, 45). Additionally, Wright et al. (9) found that there was a temporal relationship between decreased dynamic lung compliance and increased lung TNF-α mRNA expression after immune reconstitution of *P. carinii*-infected SCID mice. Together, these studies suggest a model in which IL-10 is integral in limiting inflammation and lung injury in response to *P. carinii* infection by controlling the expression of TNF-α. However, our preliminary studies indicate that the concentrations of TNF-α in the BALF of *P. carinii*-infected CD4-depleted mice were not different between WT and IL-10 KO mice (data not shown). Further studies are underway to confirm these findings and to determine whether other proinflammatory cytokines, such as IL-6, are differentially controlled in WT and IL-10 KO mice. It is clear from these studies that attempts at bolstering the innate response to *P. carinii* in immunosuppressed hosts by depleting IL-10 will result in significant lung injury. Finding the mechanism involved in this exacerbated injury is important for devising new therapies for PCP.

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