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

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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.

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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-I10°(m1Cm)m) and C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or bred in our animal facilities. Colonies of CB-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 × 10⁷ 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 RBCs were lysed by treatment with a hypotonic buffer. Lung cells were resuspended in HBSS for enumeration. TBLN cells 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 prepared 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: log₁₀ 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 log₁₀ 3.6.

Preparation of lung and TBLN cells for flow cytometric analysis

Cells derived from digested lung and TBLN tissue were enumerated and ~5 × 10⁶ to ~1 × 10⁷ 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 10⁷ 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 >log₁₀ 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.
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⁺ (CD44^{high}CD62^{low}) cells and 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. 2A). Similarly, infiltration of activated CD8⁺ T cells into the interstitium and alveolar spaces of IL-10 KO mice was significantly higher than in the WT mice on days 15 and 23 postinfection (Fig. 2B). 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...
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 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. 5A 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. 5A 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. 5B). 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 log10 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
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 and the same time points.](http://www.jimmunol.org/)

![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 CD62L^- 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.](http://www.jimmunol.org/)
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-depleted 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 pro-

References