Contribution of T cell subsets to the pathophysiology of Pneumocystis-related immunorestitution disease

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Bhagwat, Samir P., Francis Gigliotti, Haodong Xu, and Terry W. Wright. Contribution of T cell subsets to the pathophysiology of Pneumocystis-related immunorestitution disease. Am J Physiol Lung Cell Mol Physiol 291: L1256 –L1266, 2006. First published August 4, 2006; doi:10.1152/ajplung.00079.2006.—Immune-mediated lung injury is an important component of Pneumocystis pneumonia (PCp)-related immunorestitution disease (IRD). However, the individual contribution of CD4+ and CD8+ T cells to the pathophysiology of IRD remains undetermined. Therefore, IRD was modeled in severe combined immunodeficient mice, and specific T cell depletion was used to determine how T cell subsets interact to affect the nature and severity of disease. CD4+ cells were more abundant than CD8+ cells during the acute stage of IRD that coincided with impaired pulmonary physiology and organism clearance. Conversely, CD8+ cells were more abundant during the resolution phase following P. carinii clearance. Depletion of CD4+ T cells protected mice from the acute pathophysiology of IRD. However, these mice could not clear the infection and developed severe PCp at later time points when a pathological CD8+ T cell response was observed. In contrast, mice depleted of CD8+ T cells efficiently cleared the infection but developed more severe disease, an increased frequency of IFN-γ-producing CD4+ cells, and a prolonged CD4+ T cell response than mice with both CD4+ and CD8+ cells. These data suggest that CD4+ T cells mediate the acute respiratory disease associated with IRD. In contrast, CD8+ T cells contributed to neither lung injury nor organism clearance when CD4+ cells were present, but instead served to modulate CD4 function. In the absence of CD4+ cells, CD8+ T cells produced a nonprotective, pathological immune response. These data suggest that the interplay of CD4+ and CD8+ T cells affects the ultimate outcome of PCp-related IRD.

inflammation; pulmonary physiology; acquired immune deficiency syndrome

Pneumocystis carinii is an opportunistic fungus that causes pneumonia in immune-compromised patients (37), including patients with acquired immune deficiency syndrome (AIDS) (29), patients undergoing chemotherapy, and patients receiving treatment with immunosuppressive drugs during organ transplantation (34). Although exposure to P. carinii is nearly universal, as demonstrated by the appearance of anti-P. carinii antibody in 85% of children by the age of 20 mo (35), a period of immunosuppression is essential for the development of clinical disease (15, 34). Several studies have demonstrated a positive correlation between the degree of inflammation and the severity of disease (4, 23, 36), suggesting that the pathogenesis of Pneumocystis pneumonia (PCp) is primarily inflammatory in nature, in that infection with P. carinii is necessary to cause pneumonia, but certain aspects of the immune response against P. carinii are responsible for the associated pathophysiology (30, 39). For example, AIDS patients who demonstrate a rapid recovery of CD4+ T lymphocytes after institution of combined antiretroviral therapy may develop pulmonary decompensation in response to preexisting pulmonary infections, including P. carinii (26). This clinical syndrome has been termed “immunorestitution disease” (IRD) (9). At least one study that examined the number of possible cases of IRD based on the symptoms, and clinical profile suggests that IRD may be more prevalent than previously thought (9). At the onset of PCp-related IRD, patients no longer have heavy P. carinii infections, making it more likely that the severity of disease is directly related to the degree of immune recovery (9, 21). It is also possible that a dysregulated immune response may be a complicating factor affecting the intensity and duration of the disease. Importantly, patients who develop PCp-related IRD have a much greater mortality rate than patients with a classic AIDS-related presentation in which CD4+ T cell function is severely impaired (23). AIDS-related PCp is normally associated with higher P. carinii burdens, but disease severity correlates with levels of certain inflammatory markers (4) indicating that immune-mediated mechanisms of lung injury are also operative in this clinical presentation of PCp. Thus it is possible that variation in the degree of immunosuppression among AIDS patients affects their ability to mount an immune-mediated inflammatory response to P. carinii and may account for variability in the physiological presentation of PCp from one AIDS patient to the next.

The immunopathogenesis of PCp is also supported by studies in animal models. In the absence of a host immune system, e.g., as in severe combined immunodeficient (SCID) mice, very little lung damage is induced by P. carinii infection until poorly characterized events take place during advanced stages of PCp (39). However, when the host immune system is restored by the adoptive transfer of congenic splenocytes (immunorestitution), an intense P. carinii-specific immune response brings about P. carinii clearance with the undesired consequence of severe lung damage (31, 39). Whereas the contribution of CD4+ T cells (17, 31, 33), TNF-α (7), and IL-1 (8) to P. carinii clearance has been established in this IRD model, the specific contributions of CD4+ and CD8+ T cells to the pathological response associated with IRD remains unknown. Work from several groups (17, 30) noted that the transfer of flow-sorted CD4+ cells from P. carinii-immunized donors to heavily infected SCID mice could induce a lethal
hyperinflammatory response, suggesting that CD4+ T cells could contribute to immune-mediated pathology if sensitized by prior exposure to \textit{P. carinii}. In addition, recent studies have demonstrated that the severity of IRD can be alleviated by the transfer of CD25+/CD4+ T regulatory cells (18) or the delivery of viral IL-10 to the lung (32). In addition to providing more support for the immunopathogenesis of PcP, these studies also suggest that specific immune modulation could decrease the severity of PcP-related IRD.

In the CD4-depleted model of AIDS-related PcP, mice develop progressive disease that is characterized by the accumulation of CD8+ T cells and polymorphonuclear leukocytes (PMNs) in the lung (3, 39). We have demonstrated that CD8+ T cells are required for maximal inflammation and lung injury, but have little effect on \textit{P. carinii} clearance (39). It has also been demonstrated that CD8+ T cells can suppress specific immune responses by helping to limit the intensity and duration of CD4+ T cell responses (19). Therefore, understanding the interplay of CD4+ and CD8+ T cells during IRD, and how each subset contributes to clearance, injury, and/or control of inflammation, is necessary to develop optimal treatment regimens for PcP-related IRD. We hypothesize that a balanced immune response consisting of both CD4+ and CD8+ T cells is more effective for efficiently resolving PcP-related IRD than a response dominated by either CD4+ or CD8+ T cells. The work described herein tests this hypothesis in vivo.

**MATERIALS AND METHODS**

**Animal model.** B6.CB17-Prkdcsidc/SzJ (B6 SCID) female mice were purchased from the Jackson Laboratory (Bar Harbor, ME). SCID mice were infected with \textit{P. carinii} formae speciales muris by either direct inoculation or cohabbing and then immune-reconstituted with an intraperitoneal injection of 5 × 10⁷ splenocytes from naive C57BL/6J donor females. For direct inoculation experiments, mice were given 1 × 10⁷ \textit{P. carinii} cysts by the intranasal route and then immune-reconstituted 17 days later. For cohabbing experiments, mice were housed with heavily infected SCID mice for 6 wk before immune reconstitution. We have determined that both routes of exposure result in similar \textit{P. carinii} lung burdens at the time of reconstitution. Our laboratory now routinely uses the direct inoculation method because it accelerates the infection process and therefore requires less time to complete individual experiments. Depletion of specific lymphocyte subsets was achieved by intraperitoneal injection of specific CD4+ T cell- and CD8+ T cell-depleting monoclonal antibodies (MAb). Antibody injections were given 1 day before and 1 day after immune reconstitution. Thereafter, antibodies were administered every 4 days for the duration of the experiment. Reconstituted animals received either 300 µg of control rat IgG (Sigma), 300 µg of CD4+ T cell-depleting MAb [clone GK1.5, American Type Culture Collection (ATCC) clone TIB207], or 250 µg of CD8+ T cell-depleting MAb (clone 24.3, ATCC clone TIB210). Our immune reconstitution model is described in detail elsewhere (39). On specific days mice from each group were anesthetized with pentobarbital for lung resistance and compliance measurements (see below) and further tissue analysis. All animal protocols were preapproved by University Committee for Animal Research at the University of Rochester Medical Center.

**Preparation of mouse \textit{P. carinii} organisms for inoculation.** \textit{P. carinii}-infected CB.17 scid/scid mice were treated with dexamethasone (4 mg/l) and tetracycline (500 mg/l) in the drinking water 3–7 days before death to increase the \textit{P. carinii} burden in the lung. The lungs were removed, and \textit{P. carinii} were isolated from the lung tissue as previously described (38, 41). The final prep was then stained with ammoniacal silver to enumerate cysts and Diff-Quick (Dade, Dudingen, Switzerland) to ensure no bacterial contamination was present. In addition, the preps were routinely plated on commercially available chocolate blood agar plates to test for the presence of other microorganisms.

**Physiological assessment of pulmonary compliance and resistance in live, ventilated mice.** Dynamic lung compliance and resistance was measured in live mice using a previously described method (5, 39, 41) with modifications. Mice were anesthetized by intraperitoneal injection of 0.13 mg of pentobarbital sodium per gram body wt. A tracheostomy was performed and a 20-gauge cannula was inserted 3 mm into an anterior nick in the exposed trachea. The thorax was then opened to equalize airway and transpulmonary pressure. To assure that the mice tolerated the procedure, they were examined for spontaneous respirations before proceeding further. Mice were immediately placed into a plethysmograph designed for anesthetized mice (Buxco Electronics, Wilmington, NC) and connected to a Harvard rodent ventilator (Harvard Apparatus, Southnack, MA). Mice were ventilated with a tidal volume of 0.01 ml per gram body wt at a rate of 150 breaths/min. Respiratory flow and pressure were measured using transducers attached to the plethysmograph chamber. Data were collected and analyzed using the Biosystems XA software package (Buxco Electronics). Dynamic lung compliance was calculated in ml·cm⁻¹·H₂O⁻¹ from the flow and pressure signals using the method by Amur and Mead (1) and then normalized for peak body weight. Lung resistance values were calculated in cmH₂O·ml⁻¹·s⁻¹ from the same input signals.

**Bronchoalveolar lavage and lung tissue preparation.** Bronchoalveolar lavage (BAL) and lung tissue samples were obtained following dynamic compliance measurements. The chest cavity was surgically opened to expose the lungs and trachea, and the left lung lobe was tied off securely at the bronchus with surgical silk and removed with sterile scissors. The isolated lung tissue was immediately snap-frozen in liquid nitrogen and stored at −80ºC for RNA isolation. The remaining lung lobes were lavaged with four 1-ml aliquots of 1× Hanks’ balanced salt solution (HBSS) via the tracheal cannula. Recovered lavage fluid (~3.5 ml per mouse) was centrifuged at 250 g for 5 min, and the cell pellet was stored at −80ºC. BAL cell numbers were determined by fluorescence-activated cell sorting (FACS) analysis and was used to calculate numbers of CD4+ or CD8+ cells from total cell counts. Closed circles represent CD4+ T cells, and open circles represent CD8+ T cells. Values in brackets above each CD4+ time point shows \textit{P. carinii} burden expressed as log₂(kexin DNA copies) at that particular data point as assessed by quantitative real-time PCR and was measured as described in MATERIALS AND METHODS. Each data point is expressed as the arithmetic mean ± SE (n = 4–7 mice at each time point; *P ≤ 0.05).
5 min to obtain the cellular fraction, and the supernatant was removed and frozen at −80°C for subsequent ELISA analyses of cytokine/chemokine content. The cells were resuspended in fresh HBSS, enumerated, centrifuged onto glass slides, and stained with Diff-Quick for differential counting. In addition, multiparameter flow cytometric analysis was performed on BAL cells following staining with fluorochrome-conjugated antibodies. Anti-CD4-fluorescein (clone RM4-4), and anti-CD8a-peridinin chlorophyll-a protein (clone 53–6.7) were purchased from BD Biosciences (San Jose, CA). These MAb were distinct from the MAb used to deplete CD4+ and CD8+ T cells in vivo. At least 5,000 events per BAL sample were routinely analyzed on a FACSCalibur cell sorter (BD Biosciences, San Jose, CA).

For lung tissue fixation, the lungs were inflated with 15 cm gravity flow pressure of 10% formalin (Sigma, St. Louis, MO). The lungs were fixed for 10 min under gravity flow pressure and then carefully removed from the animal and placed in fixative for 16 h at 4°C. The lungs were rinsed and stored at 4°C in 70% ethanol. Before embedding, the lower right lung lobe of each animal was removed and placed in a tissue cassette. The lobe was embedded in paraffin, and 4-μm sections were cut from the tissue blocks. Slides were stained with hematoxylin and eosin to visualize lung architecture and inflammatory infiltrates. Hematoxylin-eosin-stained tissue sections were visualized under high-power magnification (×600), and inflammatory cells were blindly counted by a pathologist. Ten random fields from each slide were counted with three slides per each lung sample. Results are expressed as number of inflammatory cells per field in Figs. 3 and 8.

Measurement of *P. carinii* burden by quantitative real-time PCR. *P. carinii* burden in the lungs of experimental mice was determined by real-time PCR as previously described (41). Briefly, the right lung lobes were homogenized with PBS (1 ml of PBS per 150 mg of lung tissue) in a mechanical homogenizer. Homogenates were boiled for 15 min, vigorously vortexed for 2–3 min, and then centrifuged for 5 min at 12,000 g. The supernatant was carefully removed and stored at −80°C for real-time PCR analysis. Boiled samples were assayed by quantitative PCR using TaqMan primer/fluorogenic probe chemistry and a Prism 7000 Sequence Detection System (Foster City, CA). A primer/probe set specific for a 96-nucleotide region of the mouse-derived *P. carinii* kexin gene (22) was designed using the Primer Express software (Applied Biosystems). The sequences of the primers and probe used were as follows: forward primer, 5′-GCACGCATT-TATACTACCGATGTT-3′; reverse primer, 5′-GAGCTATAAACGCTGTGCAA-3′; and fluorogenic probe, 5′-CAGCAGCTCATCTCAGATTTCTGCTTCC-3′. Quantitation was determined by extrapolation against standard curves constructed from serial dilutions of known copy numbers of plasmid DNA containing the target kexin sequence. Data were analyzed using the ABI Prism 7000 SDS v1.0

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**Fig. 2.** Effect of lymphocyte subsets on lung function during *Pneumocystis* pneumonia (PcP) in IRD mice. Pulmonary function testing was carried out on Pc-infected IRD mice at 9, 14, 21, and 28 days post-immune reconstitution. Lung compliance (A) and resistance (B) measurements were taken on nondepleted (closed circles), CD4-depleted (open circles), and CD8-depleted (closed triangles) IRD mice. C: polymorphonuclear leukocytes (PMN) numbers in the BAL fluid of experimental mice. D: *P. carinii* burden expressed as Log_{10}(kexin DNA copies) as measured by quantitative real-time PCR. The results were pooled data from 3 independent experiments, and each data point is the arithmetic mean ± SE [n = 8–16 mice for all groups at all time points except CD4-depleted mice at day 21 (n = 3)]. *P < 0.05 compared with nondepleted IRD mice.
Cytokine and chemokine ELISAs. ELISAs were performed on the cell-free lavage fluid collected from the experimental mice. Quantikine ELISA kits for the quantitation of TNF-α, IFN-γ, regulated on activation, normal T cell expressed, and presumably secreted (RANTES), and monocyte chemoattractant protein-1 (MCP-1) were purchased from R&D Systems (Minneapolis, MN). Assays were performed according to the manufacturer’s instructions.

Intracellular staining of IFN-γ. Cells were isolated from the lungs of experimental mice using a previously published protocol (25). Briefly, the lungs were excised, minced, and dissociated by pushing through a 60-gauge stainless steel mesh screen in a total volume of 10 ml of saline. Aliquots measuring 0.5 ml were stored at −80°C for DNA isolation and kexin copy number estimation using quantitative real-time PCR. Aliquots of 1 ml were centrifuged (13,000 rpm for 10 min in a microcentrifuge), and the supernatants were stored at −80°C for ELISA. The remaining 8 ml of lung homogenates were processed to isolate cells for fluorescence-activated cell sorting (FACS) analysis as follows. Cells were first centrifuged at 300 g for 10 min. The pellets were resuspended in 5-ml complete RPMI + 3% FBS + 1 mg/ml type IV collagenase (Invitrogen) + 50 U/ml−1 of DNase I (Sigma).

Fig. 3. Histopathology of PcP in IRD mice. At 14 days postreconstitution, lungs from nondepleted (A), CD4-depleted (B), CD8-depleted (C), or CD4- and CD8-depleted (D) IRD mice were inflation-fixed with 10% buffered formalin. Four-micrometer sections were stained with hematoxylin and eosin and photographed at ×100 magnification. The number of inflammatory cells per field were determined as described in MATERIALS AND METHODS and are represented as a bar graph in E. Bars represent arithmetic mean ± SE (*P < 0.05 compared with nondepleted group).
Cells were incubated by rocking at 37°C for 1 h in 5% CO₂ incubator. They were first filtered through 70- and 40-μm filters and centrifuged at 300 g for 10 min. The pellet was resuspended in 5 ml of ice-cold red blood cell lysis buffer (25) and incubated on ice for 3 min. Cells were washed with 20 ml of complete RPMI + 3% FBS centrifuged, and the pellet was suspended in 2 ml of complete RPMI + 3% FBS. Aliquots were removed to perform cell counts, and remaining cells were stimulated with phorbol myristate acetate (PMA; 50 ng/ml final concentration) and ionomycin (0.5 μg/ml final concentration) at 37°C for 1.5 h before intracellular staining of IFN-γ and FACS analysis using intracellular cytokine staining kit (BD Biosciences).

Statistical analyses. All values reported for each experimental group are means ± SE. For each individual experiment, P values were determined by performing a one-way analysis of variance (ANOVA) with the SigmaStat software package (Jandel Scientific, San Rafael, CA). The Student-Newman-Keuls method was used for all pairwise multiple comparisons of experimental groups.

RESULTS

Kinetics of T cell recruitment during PcP-related IRD. Normal naïve splenocytes were adoptively transferred into P. carinii-infected SCID mice, and groups of mice were killed 9, 14, 21, and 28 days later. The average P. carinii burden of each group was determined by real-time PCR (Fig. 1), and the absolute numbers of CD4⁺ and CD8⁺ T cells in the BAL were enumerated by FACS analysis (Fig. 1). It was evident that of the lymphocytes infiltrating the lungs of infected mice with IRD, there were more CD4⁺ T cells than CD8⁺ T cells at earlier time points (Fig. 1; days 9 and 14; P < 0.05). However, CD4⁺ T cell numbers subsequently decreased with time, coinciding with organism clearance from the lungs by day 28 postreconstitution. This observation was consistent with previous studies (17, 39) showing that CD4⁺ T cells form the main line of defense that is responsible for P. carinii clearance. In contrast, there were fewer CD8⁺ than CD4⁺ T cells during the early stages of IRD (Fig. 1; days 9 and 14), but their relative proportion and total numbers increased dramatically with time postreconstitution (there was an average of 1.3 × 10⁵ CD8⁺ cells on day 14, 4.2 × 10⁵ on day 21, and 3.9 × 10⁵ on day 28 postreconstitution). Increased CD8⁺ T cell numbers coincided with the resolution phase of IRD in this model. This influx of immune cells is specific to Pc infection and immune reconstitution, since noninfected but immune-reconstituted mice did not show a significant influx of CD4⁺ or CD8⁺ T cells (data not shown).

Fig. 4. Cytokine/chemokine levels in the BAL of P. carinii-infected IRD mice. TNF-α (A), IFN-γ (B), monocyte chemoattractant protein-1 (MCP-1) (C), and regulated on activation, normal T cell expressed, and presumably secreted (RANTES) (D) protein levels were measured in the BAL of nondepleted (closed circles), CD4-depleted (open circles), and CD8-depleted (closed triangles) IRD mice. Each data point represents the arithmetic mean of the combined data of 3 independent experiments ± SE [n ≥ 7 mice for each data point except CD4-depleted mice at day 21 (n = 3)]. *P < 0.05 compared with nondepleted IRD mice.
Contribution of CD4+ T cells to acute lung injury during PcP-related IRD. To determine whether CD4+ T cells contributed to the acute lung injury associated with PcP-related IRD, normal splenocytes were adoptively transferred into P. carinii-infected SCID mice. Experimental groups of mice were treated with control IgG or anti-CD4 MAb. Disease severity was evaluated by measuring dynamic lung compliance, lung resistance, and weight loss. Pulmonary inflammation was assessed by examining cellular infiltrates, cytokine levels, and histology. Uninfected control SCID mice did not show any signs of pulmonary disease following immune reconstitution, supporting the requirement of P. carinii for the generation of the pathological IRD immune response (data not shown). As expected, Pneumocystis-infected immune-reconstituted mice (IRD mice) mounted an intense inflammatory response against the preexisting P. carinii infection and exhibited severe abnormalities in pulmonary physiology at 14 and 21 days postreconstitution (Fig. 2, A and B). In addition, the lungs of IRD mice contained significantly elevated numbers of PMNs, which is indicative of PCP-related lung injury (Fig. 2C). However, by day 28, the IRD mice had nearly cleared the infection (Fig. 2D) and had improved lung function (Fig. 2, A and B). In contrast, the CD4-depleted IRD mice were protected from the acute stage of disease occurring at 14 and 21 days postreconstitution, but, as a consequence of impaired CD4+ T cell function, were unable to clear the P. carinii infection (Fig. 2D) and subsequently deteriorated at the later time points as a result of progressive PcP (Fig. 2, A and B). CD4-depleted IRD mice exhibited considerably better lung compliance and resistance than nondepleted IRD mice (Fig. 2, A and B; P < 0.05), had fewer lung PMNs (Fig. 2C), and suffered little body weight loss (data not shown). Whereas histological evidence of severe pulmonary inflammation was obvious in the nondepleted IRD mice (Fig. 3A), the CD4-depleted IRD mice displayed little evidence of lung inflammation or injury at day 14 (Fig. 3, B and D). Pathological scoring of lung sections confirmed that nondepleted IRD mice had much more severe pulmonary pathology than the CD4-depleted mice (Fig. 3E). Analysis of BAL revealed that increased pulmonary inflammation and injury in IRD mice were associated with elevated cytokine in the lungs (Fig. 4). In contrast, CD4 depletion resulted in severely impaired lung TNF, IFN-γ, MCP-1, and RANTES responses at day 14 postreconstitution (Fig. 4; P < 0.05). These data demonstrate that depletion of CD4+ T cells prevents the onset of the acute stage of immune-mediated lung injury associated with IRD.

CD8+ T cells modulate CD4+ T cell-dependent acute lung injury during PcP-related IRD. To determine whether CD8+ T cells contribute to the acute injury following IRD, normal splenocytes were adoptively transferred into P. carinii-infected SCID mice. Experimental groups of mice were treated with either control IgG or anti-CD8 MAb. Unexpectedly, the absence of CD8+ T cells actually enhanced the PcP-related IRD observed at day 9 and 14 postreconstitution. Mice depleted of CD8+ T cells had significantly decreased lung compliance, increased lung resistance, and more weight loss compared with nondepleted and CD4-depleted mice IRD mice (Fig. 2, A and B; P < 0.05). At day 21 postreconstitution, lung function measurements were similar in nondepleted and CD8+-depleted mice, but by this time both groups suffered from severe disease. Surviving mice in both groups effectively cleared the P. carinii infection (Fig. 2D) and began to show improved lung function and weight gain by day 28 postreconstitution. CD8-depleted mice demonstrated histological patterns of pulmonary inflammation that were generally similar to nondepleted IRD mice (Fig. 3, C vs. A). Importantly, CD8-depleted IRD mice had significantly greater amounts of TNF-α, MCP-1, and RANTES (Fig. 4; P < 0.05 for each cytokine) in the BAL fluid at day 9 postreconstitution than nondepleted IRD mice. However, the most striking finding was the >5-fold increase in IFN-γ production (Fig. 4B; P < 0.05). Although the peak number of CD4+ T cells in lungs of CD8-depleted and nondepleted IRD mice were similar (Fig. 5; day 14), the CD8-depleted IRD mice exhibited an earlier increase in CD4+ T cell recruitment and a prolonged CD4+ T cell response that persisted out to 28 days postreconstitution (Fig. 5). These data demonstrate that in the absence of CD8+ T cells, the early proinflammatory CD4+ T cell response is enhanced, leading to more severe IRD-related pulmonary disease.

Enhanced IFN-γ production in CD8-depleted mice is associated with increased numbers of CD4+/IFN-γ+ T cells. To determine the source of elevated IFN-γ in the CD8-depleted mice, lung cells were isolated from experimental mice, stimulated with PMA and ionomycin, and stained for intracellular IFN-γ as well as surface CD4. A representative FACS histogram confirms that specific staining for surface CD4 and intracellular IFN-γ, which was blocked with nonlabeled anti-IFN antibody, was achieved (Fig. 6, A and B). Importantly, the CD8-depleted IRD mice had significantly more CD4+/IFN-γ+ cells in the lung than the nondepleted IRD group (1.5 × 105 ± 4.8 × 104 in CD8-depleted group vs. 1.4 × 104 ± 8 × 103 in nondepleted group; P < 0.05; Fig. 6C). In addition, we also

*Fig. 5. CD4+ T cells in the BAL of IRD mice. Cells were recovered from nondepleted (closed circles), CD4-depleted (open circles), and CD8-depleted (closed triangles) IRD mice by BAL and then stained with fluorescence-conjugated anti-CD4 antibodies. FACS analysis was used to determine total number of CD4+ T cells. The data represents the arithmetic mean of samples pooled from 3 independent experiments ± SE [n = 5–14 mice for all groups at all time points, except for CD4-depleted mice at day 21 (n = 3)]. *P < 0.05 compared with nondepleted IRD mice.
compared IFN-γ levels in the lung homogenates of these mice. Nondepleted mice had $330 \pm 81 \text{ pg/ml}$ of IFN-γ in their lung homogenate compared with $1,773 \pm 814 \text{ pg/ml}$ of IFN-γ in CD8-depleted mice ($P < 0.05; n = 3$ for each group). These results were consistent with our prior results (Fig. 4B). Very few CD8$^+$ or NK1.1$^+$ cells stained positive for IFN, suggesting that CD4$^+$ cells are the major source of IFN-γ at this time point (data not shown). Together, these data suggest that CD8$^+$ T cells modulate the nature and intensity of the CD4$^+$ T cell response to *P. carinii*.

CD8$^+$ T cells mediate damage but not *P. carinii* clearance in the absence of CD4$^+$ T cells. To determine the effect CD8$^+$ T cells have on IRD in the absence of CD4$^+$ T cells, normal splenocytes were adoptively transferred into *P. carinii*-infected SCID mice. Experimental groups of mice were treated with anti-CD4 antibody or both anti-CD4 and anti-CD8 antibodies. A control group was *P. carinii*-infected, but did not receive splenocytes. Because each group lacked CD4$^+$ T cells, none of the mice cleared the *P. carinii* infection over the 4-wk study, and each group had similar *P. carinii* lung burdens (Fig. 2C). Thus there was no protective anti-*P. carinii* benefit associated with the presence of CD8$^+$ T cells in this model. As expected, the nonreconstituted *P. carinii*-infected mice exhibited nearly normal pulmonary function (Fig. 7, A and B) and no body weight loss (data not shown). By wk 4 post reconstitution, the CD4-depleted group showed a significant accumulation of CD8$^+$ T cells ($2.8 \times 10^5 \pm 6.9 \times 10^4$ CD8$^+$ T cells as measured by FACS analysis) in the lung, which was associated with a decrease in lung function (Fig. 7, A and B), and a $>10\%$ loss of body weight (data not shown). In contrast, CD4/CD8-depleted IRD mice demonstrated less impairment of pulmonary function (Fig. 7, A and B; $P < 0.05$), and continued to gain body weight. Enhanced disease in the CD4-depleted IRD mice that retained CD8$^+$ T cell function was associated with obvious histological signs of inflammation and lung injury, which was quantified by a blinded pathologist (Fig. 8, A–C).

Fig. 6. CD4$^+$ IFN-γ$^+$ T cells are readily detected in the lung tissue of experimental mice. Lung cells were isolated from experimental mice killed on the 7th day after immune reconstitution, stimulated with PMA and ionomycin, and stained with fluorescent monoclonal antibodies (MAb) against the following surface markers: CD4 (APC), CD8, and intracellular IFN-γ (FITC). Results of pooled cells from CD8-depleted IRD mice are shown in A. The same cells pretreated with $10 \mu g$ of unlabeled anti-IFN-γ shows nearly complete blocking of IFN staining. The percentage of CD4$^+$ IFN-γ$^+$ cells (cells in the gate R2) from a similar plot for each sample were used to calculate total number of IFN-γ-producing CD4$^+$ T cells in that sample, and the results are shown in C. Bars represent arithmetic means of 3 samples and error bars represent SE; $*P < 0.05$. 

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and increased proinflammatory cytokine production in the lung (Fig. 9; \( P < 0.05 \)). These data demonstrated that in the absence of CD4\(^+\) T cells, CD8\(^+\) T cells caused significant inflammatory injury in this IRD model. However, the injurious response was delayed compared with the acute CD4\(^+\) T cell-dependent injury, and, importantly, CD8\(^+\) T cell-mediated injury proceeded without the beneficial effect of organism clearance.
in patients who present with PcP as their initial manifestation of AIDS, and our animal model data suggesting that a major contributor to IRD is the kinetics of return of CD4+ and CD8+ T cells, we speculate that delaying institution of antiretroviral therapy for several weeks until the treatment of PcP has been completed may reduce the incidence of PcP-associated IRD. A study which will test this hypothesis is underway through the National Institutes of Health AIDS Clinical Trials Group (ACTG protocol A5164).

Our experiments highlight the contribution of T lymphocytes to immune-mediated lung damage during PcP-related IRD. Consistent with previously published studies (16, 17, 30, 33, 39), our studies show that CD4+ T cells are absolutely essential to clear the P. carinii infection. It is possible that features of CD4 function that are essential for P. carinii clearance are also responsible for inflammatory lung injury. Alternatively, certain aspects of CD4 function may mediate clearance, whereas other aspects drive lung injury. In either case, further understanding of CD4 function and the mechanisms controlling the intensity and duration of their responses are needed.

Our murine IRD model has allowed us to study the effect of CD8+ T cells have on P. carinii clearance and pathophysiology in the presence or absence of CD4+ T cells, something that has not been done before. Based on our prior work demonstrating that CD8+ T cells mediate inflammation and injury in a CD4-depleted mouse model of PcP (39, 41), we hypothesized that IRD-related injury was the result of the combined CD4+ and CD8-mediated injurious response, and that the depletion of CD8+ T cells would alleviate some of this injury. However, depleting CD8+ T cells appeared to enhance the CD4-mediated lung injury that is characteristic of IRD. CD8+ T cell-depleted mice had reduced lung function compared with non-depleted mice, and more often appeared moribund. Interestingly, the more severe IRD observed when CD8+ T cells were depleted was associated with higher IFN-γ levels in the lungs at seven days postreconstitution. Intracellular staining for IFN-γ revealed that depletion of CD8+ T cells resulted in more CD4+/IFN-γ+ cells in the lungs, suggesting that CD8+ T cells can modulate the CD4+ T cell response during PcP-related IRD. Since IFN-γ is one of the hallmarks of T helper type 1-mediated immune responses (6, 27), CD8+ T cells are likely regulating the intensity or polarity of the CD4+ T cell response. The number of CD4+/IFN-γ+ T cells in the CD8-depleted group was greater at day 6 postreconstitution (see Fig. 6C), suggesting that differences in IFN-γ could be due to either differences in the level of IFN production by individual CD4+ T cells or differences in the number of IFN-γ producing CD4+ T cells. CD8+ T cells have been reported to act as negative regulators of inflammation in certain models of disease. For example, a regulatory role for CD8+ T cells in controlling CD4+ T cell-mediated inflammatory response has been described in a mouse model of Mycoplasma pulmonis respiratory disease (19). It is apparent that a similar phenomenon is occurring in our studies that merits further study. One possible explanation for the observed regulatory effect of CD8+ T cells is they might be somehow affecting CD4+ helper T cell function. It is also possible that a specific subset of CD8+ T cells regulates the immune response in an antigen specific manner. These possibilities are subjects of further study.

![Graph showing effect of CD8+ T cells on cytokine/chemokine levels in the BAL during PcP in CD4-depleted IRD mice.](http://ajplung.physiology.org/Downloaded from 10.220.33.3 on August 14, 2017)
Our studies suggest an important role for IFN-γ in initiating and regulating inflammatory response to P. carinii in this mouse model. In the two groups that show severe CD4+ T cell mediated inflammation and lung injury (nondepleted and CD8− T cell-depleted IRD groups), elevated IFN-γ levels were detected at 9 days postreconstitution. The role of IFN-γ in regulating the immune response to P. carinii has been described before. It has been shown to play a role in TNF-α- and IL-1-activated-killing of P. carinii by rat alveolar macrophages (10). However, even though it collaborates with TNF-α in anti-P. carinii defenses, it is not absolutely necessary and sufficient for P. carinii clearance (12). Kolls et al. (20) have shown that expression of IFN-γ through an intracellular delivery system can prime CD8+ T cells to protect CD4-depleted mice against subsequent challenge with P. carinii. In contrast, IFN-γ has also been shown to play a more regulatory role in controlling the inflammation associated with P. carinii infection (12). In a bone marrow transplantation mouse model, treatment with anti-IFN-γ antibodies worsened P. carinii-induced pneumonitis (13), and in a model of Pcp-related IRD the lack of IFN appeared to delay the resolution of pulmonary inflammation (12). Our results suggest that IFN-γ may play a role in enhancing the CD4-mediated inflammatory response important for the generation of Pcp-related IRD. Since both anti- and proinflammatory roles for IFN-γ have been demonstrated, the timing and magnitude of the IFN-γ response may dictate the specific role it plays during Pcp-related IRD. We are currently investigating this role further.

It must be noted that whereas the studies presented herein were designed to focus specifically on the role of T cell subsets during Pcp-related IRD, other variables also affect the ultimate outcome of disease. For example, the intensity of the P. carinii infection at the time of immune reconstitution is critical. In some experiments we have performed, a higher P. carinii burden led to a very severe IRD in both CD8-depleted and nondepleted mice, resulting in an altered time frame of disease and/or lethal IRD, and obscuring the immunomodulatory effects of CD8+ T cells. In other experiments, a very light P. carinii burden at the time of reconstitution has resulted in little lung injury in either group. Furthermore, we have noted an alteration in the time course of disease onset in some experiments, possibly also related to the P. carinii burden, or, alternatively, to the quality of the donor lymphocytes. Thus we have found that the time course of Pcp-related IRD is very dependent upon the intensity of infection and the number and composition of the donor lymphocytes. Future experiments will need to examine a more detailed time course of disease progression.

Our studies also suggest that CD8+ T cells contribute to a delayed injurious immune response in the absence of CD4+ T cells. All the parameters of lung injury and inflammation are better in CD8+/CD4+ T cell double-depleted mice compared with mice depleted of only CD4+ T cells, suggesting an inflammatory role for the CD8+ T cells. It will be interesting to study whether CD8+ T cell-mediated inflammation observed in Pcp lungs is major histocompatibility complex class I restricted, which antigen of P. carinii is recognized by CD8+ T cells, and how P. carinii antigens are presented to the CD8+ T cells since P. carinii is not an intracellular pathogen.

We have demonstrated dual roles for both CD4+ and CD8+ T cells during Pcp-related IRD. CD4+ T cells were required for P. carinii clearance, but also induced the severe pulmonary inflammatory syndrome associated with IRD. In the absence of CD4+ T cells, CD8+ T cells were responsible for a delayed inflammatory response that was not capable of clearing the P. carinii infection but did cause significant lung injury. However, when CD4+ T cells were present, CD8+ T cells played an immunomodulatory function by limiting the intensity and/or altering the polarity of the injurious CD4 response.

Based on this study and our previously published experiments (2, 14, 39, 40), we propose the following conceptual framework to think about how T cells affect the outcome of Pcp. We suggest that the overall inflammatory response and outcome of infection with P. carinii is dependent on the balance between CD4+ and CD8+ T cells. With a balanced or physiologic inflammatory response, as would be seen in the normal host, infection is easily cleared with minimal inflammation and little apparent “disease” (2, 14). With a CD4+ T cell dominant response in the absence of adequate CD8+ T cell suppression, e.g., as might be seen in IRD, infection is rapidly cleared but at the expense of excessive inflammation and bystander injury to the lung (as shown in this study). Finally, with a CD8+ T cell dominant response in the absence of adequate CD4+ T cell help (39, 40), e.g., as might be seen in patients receiving chemotheraphy or in AIDS patients with high CD8+ to CD4+ T cell ratios, there is futile inflammation with bystander injury to the lung and failure to clear infection. A better understanding of these T cell effects should allow for improved therapy of Pcp.

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