Regulatory T Cells Dampen Pulmonary Inflammation and Lung Injury in an Animal Model of Pneumocystis Pneumonia

Laura McKinley, Alison J. Logar, Florencia McAllister, Mingquan Zheng, Chad Steele and Jay K. Kolls

*J Immunol* 2006; 177:6215-6226; doi: 10.4049/jimmunol.177.9.6215
http://www.jimmunol.org/content/177/9/6215

References

This article cites 47 articles, 17 of which you can access for free at:
http://www.jimmunol.org/content/177/9/6215.full#ref-list-1

Why The *JI*? Submit online.

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Regulatory T Cells Dampen Pulmonary Inflammation and Lung Injury in an Animal Model of Pneumocystis Pneumonia

Laura McKinley, Alison J. Logar, Florencia McAllister, Mingquan Zheng, Chad Steele, and Jay K. Kolls

CD4+CD25+Fox3+ regulatory T cells are decreased in patients infected with HIV and have been shown to be critical in mediating Ag tolerance in the lung. Because a subset of Pneumocystis-infected individuals develop substantial lung injury, which can be modeled in immune reconstituted scid mice, we used mouse models of Pneumocystis carinii to investigate the role of regulatory T cells in opportunistic infection and immune reconstitution. In this study, we show that CD4+CD25+Fox3+ cells arepart of the host response to Pneumocystis in CD4+ T cell-intact mice. Moreover, lung injury and proinflammatory Th1 and Th2 cytokine levels in the bronchoalveolar lavage fluid and lung homogenate were increased following CD4+CD25− immune reconstitution in Pneumocystis-infected SCID mice but not in CD4+CD25+ T cell-reconstituted animals. The ability of CD4+CD25+ T cells to control inflammation and injury during the course of Pneumocystis was confirmed by treatment of wild-type C57BL/6 mice with anti-CD25 mAb. These data show that CD4+CD25+ T cells control pulmonary inflammation and lung injury associated with Pneumocystis infection both in the setting of immune reconstitution as well as new acquisition of infection. The Journal of Immunology, 2006, 177: 6215–6226.

Pneumocystis is a ubiquitous fungal pathogen that causes pneumonia in immunocompromised hosts. Despite a significant decline in the incidence of Pneumocystis pneumonia (PCP) following the introduction of anti-Pneumocystis prophylaxis and highly active antiretroviral therapy (HAART), PCP remains the leading opportunistic infection in HIV+ adults and children worldwide (1). Several studies have shown that loss of CD4+ T cells is the primary risk factor for developing PCP; HIV+ patients with CD4+ T cell counts <200 cells/µL are highly susceptible to infection (2, 3). Taking advantage of this observation has led to the development of mouse models of PCP where the selective depletion of CD4 cells by mAb injection followed by intratracheal inoculation with Pneumocystis results in persistent pulmonary infection (4, 5). SCID mice lacking functional B and T cells are also highly susceptible to PCP (6) and are often used to study the contribution of distinct cell subsets to clearance, as well as injury associated with PCP by adoptive transfer of purified wild-type (WT) cells. Reconstitution of CD4+ T cells in SCID mice infected with PCP results in organism clearance associated with hyperinflammation, lung injury, and death (7). Adoptive transfer of CD4+CD25+ regulatory T cells is tolerated by Pneumocystis-infected SCID mice and protects mice from lethality associated with the transfer of CD4+CD25− effector T cells (8).

Regulatory T cells are classified as natural, thymically derived, or inducible, which are induced in the periphery following encounters with Ag-loaded dendritic cells (9). CD4+CD25+ natural regulatory T cells specifically express the gene for forkhead transcription factor P3 (FoxP3), which is critical for their regulatory function and the only defining marker of this subset that is not also expressed on activated effector cells identified to date (10). These cells suppress proliferation, cytokine production, and cytotoxic activity of both CD4+ and CD8+ effector T cells, although the mechanisms of this suppression remains unclear. There are little data regarding the role these cells play during Pneumocystis infection. It was recently shown that the ratio of CD4+CD25−/CD4+CD25+ T cells in the bronchoalveolar lavage (BAL) fluid is altered in B cell-deficient mice (µMT) depleted of CD8+ T cells following Pneumocystis infection (11). Moreover, these CD8-depleted µMT mice exhibit an exacerbated Th2 phenotype and increased lung injury (11). These studies suggest regulatory T cells are important in controlling the inflammation associated with PCP.

In this study, we show CD4+CD25+Fox3+ regulatory T cells are recruited to the lung during the course of Pneumocystis infection in immunocompetent mice. Loss of this cell population due to treatment with anti-CD25 mAb results in enhanced lung injury associated with increased Th2 and inflammatory cytokine production. Furthermore, reconstitution of infected SCID mice with CD4+CD25− T cells leads to an even more prominent phenotype defined by exacerbated markers of lung injury and increased production of inflammatory cytokines and chemokines, as well as enhanced secretion of both Th1 and Th2 cytokines in the lung. Pneumocystis-infected SCID mice reconstituted with CD4+CD25− T cells are phenotypically similar to nonreconstituted (NR) control animals with regard to pulmonary inflammation and injury but show increased organism burden in the lungs. These data demonstrate that regulatory T cells, as defined by intracellular FoxP3 expression, are recruited to the lung as part of the immune response in a mouse model of PCP, and loss of this population results in increased morbidity. These findings are significant.

Department of Pediatrics, and Division of Pulmonary Medicine, Allergy, and Immunology, Children’s Hospital of Pittsburgh, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213

Received for publication May 31, 2006. Accepted for publication July 28, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 J.K.K. acknowledges support from Public Health Service Grants 5R01HL062052 and 5R01HL061271.

2 Address correspondence and reprint requests to Dr. Jay K. Kolls, Children’s Hospital of Pittsburgh, Suite 3765, 3705 Fifth Avenue, Pittsburgh, PA 15213. E-mail address: jay.kolls@chp.edu

3 Abbreviations used in this paper: PCP, Pneumocystis pneumonia; HAART, highly active antiretroviral therapy; WT, wild type; BAL, bronchoalveolar lavage; LDH, lactate dehydrogenase; LN, lymph node; IRIS, immune reconstitution inflammatory syndrome; FoxP3, forkhead transcription factor P3; NR, nonreconstituted.
because they bring into question whether the subpopulation of HAART patients that suffer from a detrimental hyperinflammatory response following immune reconstitution are deficient in their ability to repopulate regulatory cells.

Materials and Methods

Mice

Six- to 8-wk-old, male C57BL/6 mice were obtained from National Cancer Institute (Frederick, MD). SCID mice were obtained from The Jackson Laboratory. Animals were housed in a pathogen-free environment and given food and water ad libitum. All experiments were approved by the Children’s Hospital of Pittsburgh Animal Research and Care Committee.

Monoclonal Abs

Anti-CD4 mAb was prepared as previously described (4). Briefly, the hybridoma GK1.5, which produces a rat IgG2b mAb against murine CD4 (12), was obtained from American Type Culture Collection. The Ab was harvested from the ascites of pristane-primed, uninfected athymic mice, precipitated with an equal volume of saturated ammonium sulfate and dialyzed against PBS overnight. The IgG content was quantified by cellulose acetate electrophoresis and densitometry. The Ab was stored as aliquots at −80°C until use. A hybridoma that produces a rat IgG1 against murine CD25 was obtained from American Type Culture Collection and harvested in accordance with the anti-CD4 mAb. The rat IgG1 content was quantified by ELISA kit (Bethyl Laboratories).

Pneumocystis inoculum

The isolation of Pneumocystis organisms from the lung tissue of C.B-17 SCID mice that had been inoculated with Pneumocystis has been previously described (4, 13). Briefly, C.B-17 SCID mice with PCP were sacrificed and the lungs were aseptically removed and frozen in 1 ml of sterile PBS at −80°C. Frozen lungs were homogenized through a 70-μm filter and pelleted at 500 g for 10 min at 4°C. The pellet was resuspended in 1 ml of PBS, and a 1/10 dilution was stained with modified Giemsa stain (Diff-Quik). The number of Pneumocystis cysts was quantified microscopically (13), and the inoculum concentration was adjusted to 2 × 10^6 cysts/ml.

Purification of T cell subsets and adoptive transfer

Spleens from WT mice were collected, teased apart, and filtered under sterile conditions. RBCs were lysed with ammonium chloride and CD4^+ T cells were purified by negative selection using a CD4 T cell isolation kit to deplete non-CD4 cells by magnetic bead separation (MACS, Miltenyi Biotec). CD4-negative cells were collected from the column and stained with

![Figure 1](http://www.jimmunol.org/)

**Figure 1.** Trafficking of CD4^+CD25^+FoxP3^+ T cells to the lung following Pneumocystis infection. Representative histograms from C57BL/6 mice infected with *Pneumocystis* for the indicated time. Day 0 animals were sacrificed immediately after inoculation. Surface staining of CD25 and intracellular FoxP3 expression are back-gated on the CD4^+ T cell population. The data indicate there is a rise in the percentage of CD4^+FoxP3^+ in BAL fluid peaking at day 14 postinfection as LN regulatory cells decline. All data are reported as mean ± SEM. Day 0, n = 3; day 7, n = 6; day 14, n = 9; days 21 and 28, n = 5. *p < 0.05 compared with LN day 0; #, p < 0.05 compared with BAL fluid day 0.
allophtocyanin-conjugated CD4 mAb (BD Pharmingen) to determine purity. The CD4-enriched cell population was then stained with allophtocyanin-conjugated CD4 mAb and PEcy7-conjugated CD25 mAb (BD Pharmingen) and sorted based on surface CD25 expression on a FACSAria (BD Biosciences). An aliquot of sorted cell populations was stained for intracellular Foxp3 expression with FITC-conjugated Foxp3 mAb (eBioscience) as per manufacturer’s protocol. A total of 3 × 10^5 CD4^+ , CD4^+ CD25^+ , and CD4^+ CD25^- cells was injected intraorbitally to SCID mice that had been infected with 2 × 10^5 Pneumocystis cysts by intratracheal inoculation 28 days before reconstitution. A control group of Pneumocystis-infected SCID mice received an intraorbital injection of PBS as a NR control.

**BAL fluid and lung tissue collection**

At time of sacrifice, mice were anesthetized with ketamine/xylazine and tracheas were cannulated. Five 1-ml volumes of PBS were instilled through the cannula and aspirated back; the first 1-ml aliquot was kept separate whereas the subsequent 4 ml were combined. BAL fluid cells were pelleted by centrifugation at 1000 × g for 5 min. The supernatant from the first 1-ml aliquot was stored at −80°C for later analysis and the remaining supernatant was discarded. Cell pellets from both aliquots were combined by resuspending in 200 μl of PBS. Total white blood cells in BAL fluid were counted using a particle counter (Z1; Beckman Coulter).

Lungs were harvested following the collection of lavage fluid. The right lungs were harvested and homogenized in 1 ml of PBS containing 0.05% Triton X and Complete protease inhibitor (Roche). Homogenate was centrifuged at 12,000 × g for 15 min, and supernatant was stored at −80°C for later cytokine analysis. Left lungs were excised and homogenized in 1 ml of TRIzol (Invitrogen Life Technologies) for RNA extraction.

**RNA isolation and TaqMan probes and primers for Pneumocystis rRNA**

The assay for determination of Pneumocystis copy number per whole lung has been previously described (14). Briefly, real-time PCR was conducted using one-step TaqMan RT-PCR reagents (Applied Biosystems). The PCR amplification was run in triplicate using the ABI Prism 7700 SDS (Applied Biosystems). The threshold cycle values were averaged from the values obtained from each reaction, and data were converted to rRNA copy number by using a standard curve of known copy number of Pneumocystis rRNA.

**Parameters of lung injury**

Granzyme B is a serine protease that enters target cells in a perforin-dependent manner to mediate apoptosis. Others report epithelial cell damage following bleomycin-induced lung injury was associated with increased expression of lung tissue granzyme B; perforin and granzyme B are also increased in the lung tissue of idiopathic pulmonary fibrosis patients and acute respiratory distress syndrome patients (15, 16). Therefore, granzyme B levels in BAL fluid and lung homogenate were determined by ELISA as a measure of lung injury; all Abs and recombinant protein were obtained from R&D Systems. Goat anti-mouse granzyme B coating Ab was used at a concentration of 0.4 μg/ml in PBS, and a standard curve was generated from recombinant mouse granzyme B. Biotinylated anti-mouse granzyme B detection Ab was used at a concentration of 0.25 μg/ml. The lower limit of detection of the assay was 30 pg/ml.

Total protein in the BAL fluid was assayed with a BCA protein assay kit (Pierce) as per manufacturer’s instructions. Lactate dehydrogenase (LDH) levels in the BAL fluid were analyzed by in vitro toxicology kit (Sigma-Aldrich) as per manufacturer’s instructions.

**Cytokine analysis**

BAL fluid and lung homogenate samples were analyzed for protein levels of G-CSF, IL-1β, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, KC, MCP-1, and TNF-α using a Bio-Plex multiplex suspension cytokine array (Bio-Rad Laboratories) according to the manufacturer’s instructions. The data were analyzed using Bio-Plex Manager software (Bio-Rad Laboratories). Active TGF-β was measured by ELISA using a TGF-β1 DuoSet (R&D Systems).

**Statistical analysis**

Data are reported as mean ± SEM. GraphPad Prism, version 4.0 (GraphPad Software), was used to calculate p values using one-way ANOVA with a Newman-Keuls multiple comparison posttest. A value of p < 0.05 was considered statistically significant.
Results
Recruitment of regulatory T cells to the lung following Pneumocystis infection

To determine whether CD4^+CD25^+FoxP3^+ T cells are recruited to the lung during the course of Pneumocystis infection, WT C57BL/6 mice were infected with 2 × 10^5 cysts intratracheally and sacrificed at various time points thereafter. The cell surface expression of CD4 and CD25, as well as intracellular FoxP3 expression in draining mediastinal lymph nodes and BAL fluid cell populations were analyzed by flow cytometry. Day 0 indicates animals sacrificed immediately following inoculation; at time of initial infection, 25% of lymph node (LN) cells were CD4^+ (Fig. 1). Of these CD4^+ cells, there is a distinct regulatory population as defined by surface expression of CD25 and intracellular FoxP3 protein expression, with nearly all FoxP3^+ cells coexpressing CD25. As expected, there were very few CD4^+ cells present in the BAL fluid at the time of initial infection. By day 7 postinfection, there was a significant decrease in FoxP3-expressing CD4^+ T cells in the LN, which remained low through 28 days postinfection. Concurrent with this drop in the percentage of CD4^+CD25^+FoxP3^+ T cells in LN, there was an increase in the percentage of BAL fluid CD4^+CD25^+FoxP3^+ T cells peaking at 14 days postinfection (Fig. 1). The regulatory population was still evident in the BAL fluid 28 days postinfection, although the percentage approached that of early infection. These results indicate CD4^+CD25^+FoxP3^+ regulatory T cells are recruited to the lung during the course of Pneumocystis infection.

Depletion of total CD4^+ T cells, but not CD25^+ T cells dampens Pneumocystis-associated lung injury and inflammatory cytokine production

Regulatory T cells express cell surface CD4, IL-2R (CD25), CTLA-4, and glucocorticoid-inducible TNFR; however, these markers are also present on subsets of activated effector cells and no defining cell surface marker for regulatory T cells has been identified to date (17). Nearly all FoxP3-expressing cells were CD25^+ (Fig. 1); therefore, we used a single 400-μg dose of anti-CD25 mAb (PC61) i.p. to deplete regulatory T cells, knowing a portion of effector cells would also be depleted under these conditions. Animals were sacrificed 14 days postinfection, the peak of regulatory T cell recruitment to the lung. Treatment with anti-CD4 mAb, but not anti-CD25 before infection resulted in decreased cell recruitment to the lung following Pneumocystis infection compared with control Ab-treated animals (Fig. 2A). Pneumocystis burden per whole lung was assessed by real-time PCR. Organism burden was increased in the anti-CD4 treatment group compared with control Ab-treated animals (Fig. 2A). Pneumocystis burden per whole lung was assessed by real-time PCR. Organism burden was increased in the anti-CD4 treatment group compared with control Ab-treated animals (Fig. 2A). Anti-CD25 treatment resulted in a drastic increase in the level of granzyme B in the lung compared...
with both control Ab-treated mice and anti-CD4-treated mice infected with Pneumocystis (Fig. 2E). Substantial increases were also seen in the levels of inflammatory cytokines and chemokines in the lungs of anti-CD25-treated animals. The mouse homolog of human CXCL8, KC, was 4-fold higher in the anti-CD25 treatment group compared with control Ab, whereas anti-CD4 treatment led to slightly decreased KC levels in the lung (Fig. 3A). TNF-α levels were variable across all treatment groups 14 days postinfection, although the anti-CD25 treatment group tended to have decreased TNF-α in the lungs at this time (Fig. 3B). There was a marked increase in the protein levels of proinflammatory cytokines IL-1β and IL-6 in the lungs of infected animals treated with anti-CD25 but not anti-CD4 compared with control Ab-treated animals (Fig. 3, C and D). MCP-1 (MCP-1, CCL2 in human) is a potent chemoattractant of monocytes and has been implicated in contributing to the pathogenesis of various pulmonary disorders including

**FIGURE 4.** Treatment with anti-CD25 results in an exacerbated Th2 phenotype in the lungs of Pneumocystis-infected mice. A, BAL IL-4. B, Lung homogenate IL-4. C, BAL IL-5. D, Lung homogenate IL-5. E, BAL IL-13. F, Lung homogenate IL-13. G, BAL IFN-γ. H, Lung homogenate IFN-γ. I, Lung homogenate IL-17. BAL IL-17 was below detection. All data are reported as mean ± SEM for n = 7–9. *, p < 0.05 compared with WT. #, p < 0.05 compared with anti-CD4-treated animals.
asthma, acute respiratory distress syndrome, and idiopathic pulmonary fibrosis (18). There is substantial MCP-1 in the lungs of infected animals and treatment with anti-CD25 exacerbated this response (Fig. 3E). In addition to exacerbated proinflammatory responses following anti-CD25 treatment, this treatment also resulted in higher levels of G-CSF. It has been suggested that G-CSF is involved in the differentiation and trafficking of neutrophils, as well as CD4\(^+\)CD25\(^+\) FoxP3\(^+\) regulatory T cells (19). Although the levels of G-CSF were relatively low, anti-CD25 treatment resulted in a 3-fold increase in this cytokine (Fig. 3F).

**Treatment with anti-CD25 results in an exacerbated Th2 response in the lung**

The Th2-type cytokines IL-4, IL-5, and IL-13, as well as Th1-type IFN-γ and Th\(_{IL-17}\)-type IL-17, were measurable in BAL fluid and lung homogenate 14 days postinfection. Treatment with anti-CD25 mAb led to significantly increased secretion of IL-4 in both BAL fluid and lung homogenate (Fig. 4, A and B), and lung homogenate IL-5 (Fig. 4D) compared with both control Ab- and anti-CD4-treated animals. BAL fluid IL-5 and IL-13 (Fig. 4, C and E) secretion were also enhanced by treatment with anti-CD25. The level of IL-13 in lung homogenate was high and comparable between all treatment groups. Interestingly, IFN-γ production in BAL fluid was also enhanced by anti-CD25 treatment (Fig. 4G) with similar levels to that of BAL fluid IL-4. However, this enhanced Th1 response was not seen in lung homogenate (Fig. 4H). Lung homogenate IFN-γ levels were relatively low, and treatment with anti-CD25 resulted in an attenuated response. A recently described subset of Th cells distinct from Th1 and Th2 that secrete IL-17 have been termed Th\(_{IL-17}\) cells (20, 21). IL-17 is a proinflammatory cytokine involved in neutrophil migration and granulopoiesis (22). IL-17 was not detectable in the BAL fluid; lung homogenate levels were low compared with Th1 and Th2 cytokine production but were similar between groups (Fig. 4I). These data indicate that loss of regulatory T cell function results in an exacerbated Th2 phenotype in the lung.

**IL-10 and TGF-β production are not altered by treatment with anti-CD25**

Studies have shown membrane-bound TGF-β on FoxP3-expressing regulatory T cells is an important mechanism of cell contact-dependent suppression (23, 24). Two weeks postinfection with *Pneumocystis*, >80% of BAL fluid CD4\(^+\)FoxP3\(^+\) T cells expressed TGF-β on the cell surface in control animals (Fig. 5A). Treatment with anti-CD25 resulted in near-complete loss of TGF-β-expressing cells. Active TGF-β was high in lung homogenate following *Pneumocystis* infection, and there was no effect by Ab treatment (Fig. 5B). Anti-CD4 treatment resulted in a slight but significant decrease in BAL fluid TGF-β compared with control and anti-CD25 treatment groups. Despite the loss of cell surface TGF-β, treatment with anti-CD25 did not significantly alter BAL fluid or lung homogenate TGF-β levels compared with control Ab treatment, suggesting the exacerbated responses seen in these animals is not due to dampened secretion of regulatory cytokines. IL-10 was below detection in the BAL fluid of all treatment groups and the levels of IL-10 in lung homogenate were not altered by treatment with either anti-CD4 or anti-CD25 (Fig. 5D).

**Reconstitution of SCID mice with CD4\(^+\)CD25\(^-\) effector T cells but not CD4\(^+\)CD25\(^+\) regulatory T cells results in lung injury**

To further distinguish the effects of CD4\(^+\)CD25\(^-\) and CD4\(^+\)CD25\(^+\) T cells on lung injury following *Pneumocystis* infection, a SCID reconstitution model was used. Immune reconstitution of SCID mice infected with *Pneumocystis* results in clearance of organism associated with a hyperinflammatory response.

---

**FIGURE 5.** Secretion of IL-10 and TGF-β in the lung are not altered by treatment with anti-CD25. A. Representative histogram of BAL fluid cells stained for cell surface TGF-β and intracellular FoxP3 from control Ab-treated animals (left panel) or anti-CD25 mAb-treated animals (right panel). B. Active lung homogenate TGF-β. C. Active BAL fluid TGF-β. D. Lung homogenate IL-10. BAL fluid IL-10 was below detection. All data are reported as mean ± SEM for *n* = 7–9. *, *p < 0.05 compared with WT. #, *p < 0.05 compared with anti-CD4-treated animals.
that leads to lung injury and death (7). To determine the T cell subsets involved in this detrimental response, splenocytes from WT C57BL/6 mice were enriched for CD4 and sorted based on expression of CD25. Groups of mice were given intraorbital PBS as a NR control or reconstituted with $3 	imes 10^5$ CD4$^+$, CD4$^+$CD25$^-$, or CD4$^+$CD25$^+$ cells. As seen in Fig. 6, negative selection of CD4 T cells using magnetic bead isolation resulted in at least 90% enrichment of CD4$^+$T cells (Fig. 6B); the CD4$^+$ cell population had <1% of cells expressing CD4 (Fig. 6C). The majority of CD4$^+$ cells in the starting population did not coexpress CD25 (Fig. 6B). Sorting of the enriched CD4$^+$ T cell population based on CD25 expression resulted in a CD4$^+$CD25$^+$ pure population and a CD4$^+$CD25$^-$ cell population that consistently exhibited >90% purity. Analysis of intracellular FoxP3 protein expression showed ~75% of CD4$^+$ CD25$^+$ cells were FoxP3-expressing regulatory cells (Fig. 6E).

SCID mice infected for 28 days with *Pneumocystis* were reconstituted with the various cell populations or PBS and sacrificed on day 11 postreconstitution. Cell recruitment to the lung was significantly increased following reconstitution with CD4$^+$CD25$^-$ cells, whereas reconstitution with CD4$^+$CD25$^+$ cells had a similar response to NR control animals (Fig. 7A). Reconstitution with CD4$^-$ cells also led to enhanced BAL fluid cell recruitment compared with NR, although this response was not significant (data not shown). Organism burden in the lung was higher in animals reconstituted with CD4$^+$ or CD4$^+$CD25$^+$ cells and slightly reduced in CD4$^+$CD25$^-$ cell reconstituted animals (Fig. 7B). Reconstitution of *Pneumocystis*-infected SCID mice with effector CD4$^+$CD25$^-$ T cells caused lung injury as seen by increased LDH, total protein, and granzyme B in the BAL fluid of these animals compared with NR mice (Fig. 7, C–E). Interestingly, reconstitution with CD4$^-$ cells had no effect on parameters of lung injury. KC and IL-1$\beta$ levels in the lung were substantial in NR mice; reconstitution with the various cell populations had minimal effect on these cytokines (Fig. 8, A and C). TNF-$\alpha$ in the lungs of SCID infected animals was 10-fold lower compared with C57BL/6 WT strain (Figs. 8B and 3B, respectively). Reconstitution of the SCID mice with CD4$^+$CD25$^-$, but not CD4$^-$ cells, led to exacerbated TNF-$\alpha$, IL-6, and MCP-1 responses (Fig. 8, B, D, and E). MCP-1 levels were also significantly enhanced by reconstitution with CD4$^+$CD25$^-$ cells compared with NR control, although the level of MCP-1 in the lungs of these animals was still 2-fold lower than that of CD4$^+$CD25$^-$ reconstituted animals (Fig. 8E).

**FIGURE 6.** SCID reconstitution model of *Pneumocystis*. A, SCID mice were inoculated on day 0 with $2 \times 10^5$ *Pneumocystis* cysts/mouse intratracheally. On day 28, mice were reconstituted with $3 \times 10^5$ WT cells as indicated; animals were sacrificed 11 days postreconstitution (day 39). Representative histograms of cell populations used for adoptive transfer. B, WT splenocytes were enriched for CD4 by negative selection. The starting cell population was consistently >90% CD4$^+$; 5–10% of these cells coexpressed CD25. C, CD4$^+$ cells are those left after negative selection of CD4; <1% of this population expressed CD4. D, Purity of CD4$^+$CD25$^-$ was >99%, and almost none of these cells expressed FoxP3. E, The majority of CD4$^+$CD25$^+$ cells express intracellular FoxP3.
CD4\(^+\)CD25\(^+\) cell transfer causes a mixed Th1/Th2 phenotype in Pneumocystis-infected SCID mice independent of IL-10 and TGF-\(\beta\) secretion

Th1, Th2, and Th\(_{IL-17}\) cytokine levels in BAL fluid were below detection for all treatment groups, as was lung homogenate IL-4. An exacerbated Th2 response in the lung homogenate was seen as a result of effector cell transfer; lung homogenate IL-5 and IL-13 protein levels were increased following CD4\(^+\)CD25\(^-\) cell transfer (Fig. 9, A and B). Surprisingly, lung homogenate IFN-\(\gamma\) was increased >40-fold by adoptive transfer of CD4\(^+\)CD25\(^-\) cells (16 ± 1.5 in NR; 931 ± 275 in CD4\(^+\)CD25\(^-\); Fig. 9C). There was minimal lung homogenate IL-17 present at this time point (Fig. 9D), suggesting this cytokine is not involved in the inflammatory responses associated with CD4\(^+\)CD25\(^-\) effector T cell transfer. As with WT animals (Fig. 5), lung injury and inflammatory responses exhibited by reconstituted SCID mice seem to be independent of IL-10 and TGF-\(\beta\). Levels of both regulatory cytokines were neither decreased in effector cell reconstituted mice nor enhanced by adoptive transfer of regulatory cells (Fig. 10). In fact, lung homogenate IL-10 was increased in mice reconstituted with CD4\(^+\)CD25\(^-\) cells, suggesting that IL-10 alone is not enough to dampen the hyperinflammatory response initiated by WT effector cell transfer.

Discussion

Thymically derived CD4\(^+\)CD25\(^+\) regulatory T cells exhibit immunosuppressive properties in various disease states and acute injury models (25, 26). In addition to the IL-2R \(\alpha\)-chain, these cells express cell surface CTLA-4 and glucocorticoid-inducible TNFR; however, these markers are also present on activated effector cells, making it difficult to isolate a pure regulatory cell population (9). Intracellular expression of the forkhead winged-helix family transcription factor FoxP3 is the most specific marker of CD4\(^+\)CD25\(^+\) regulatory cells. FoxP3 expression is sufficient to induce suppressive properties and vital to regulatory cell function (10). We show, for the first time to our knowledge, that CD4\(^+\)CD25\(^+\)FoxP3\(^+\) T cells are recruited to the lung during the course of Pneumocystis infection, peaking in the BAL fluid 14 days after inoculation in WT C57BL/6 mice.

Although CD4\(^+\) T cells are essential for the proper clearance of Pneumocystis from the lung, neither the mechanism of control and clearance nor the subset(s) of CD4\(^+\) T cells involved is known at this time. Studies characterizing the lymphocyte subsets involved in murine host defense against Pneumocystis found the precursor frequency of both Th1 and Th2 cells were increased in the LN within 1 wk of infection followed by a delayed increase in IL-4- and IFN-\(\gamma\)-producing cells in the lung tissue (27). Neither a pure Th1 nor pure Th2 response is required for clearance of infection, because both IFN-\(\gamma\) and IL-4 are able to clear Pneumocystis from the lungs; although IFN-\(\gamma\) and IL-4 are to same extent as CD4\(^+\)CD25\(^+\) regulatory T cells are able to suppress both Th1 and Th2 cell differentiation and cytokine production in mouse models of Leishmania major and colitis (30).
determine whether CD4+CD25+FoxP3+ cells mediate the immune response to Pneumocystis, two models were used. First, WT C57BL/6 mice were treated with either anti-CD4 mAb or anti-CD25 mAb and subsequently inoculated with Pneumocystis. To focus on the roles of distinct cell populations, the SCID reconstitution model was used where mice were infected with Pneumocystis and then reconstituted with purified cell populations, 4 wk postinoculation when organism burden in the lungs was substantial. IL-17-producing cells are a newly defined Th cell subset (21), and their involvement in PCP has yet to be defined. We show IL-17 in the lung tissue is relatively low in both models compared with that of type 1 and type 2 cytokine levels. Treatment with anti-CD25 results in exacerbated Th1 and Th2 cytokine production in the lungs of infected animals compared with anti-CD4 treatment. The increase in Th2 cytokine levels in both BAL fluid and lung homogenate was more pronounced following anti-CD25 treatment than that of Th1 cytokine levels. Treatment with anti-CD25 mAb has been shown to promote Th2 cytokine secretion in models of allergic asthma, a classic Th2 disorder, as well as Helicobacter pylori, which is characterized by a Th1 inflammatory phenotype (31, 32). Reconstitution of infected SCID mice with CD4+CD25− but not CD4+CD25+ T cells leads to increased levels of IL-5, IL-13, and IFN-γ in the lungs. Interestingly, WT C57BL/6 mice had relatively higher levels of Th2 cytokines compared with CD4+CD25− reconstituted SCID mice of the same background that had more substantial IFN-γ production, suggesting differences in the ratio of Th1:Th2 cells between the two models. Our data concur with previous reports showing the immune response to PCP is not dichotomous (27, 28) and suggest CD4+CD25+ regulatory T cells are able to suppress both Th1- and Th2-mediated immune responses during PCP.

CD4+ T cells are not the only source of these cytokines; there are also distinct subsets of IL-4- and IFN-γ-secreting CD8+ T cells (33, 34). Depletion of CD8+ T cells does not render animals susceptible to PCP as is the case when CD4+ T cells are depleted (35). Furthermore, unlike CD4-deplete animals, animals depleted of CD8+ T cells are able to clear infection (5, 35). The role of CD8+ T cells in the absence of CD4+ cells remains uncertain due to contradicting results with regard to CD8-mediated organism clearance and morbidity associated with PCP. Although Ab depletion of CD8+ T cells in CD4-deplete animals results in more significant infection than depletion of CD4+ cells alone (35), Pneumocystis-infected SCID mice reconstituted with CD8+ T cells were unable to clear infection (7). However, adoptive transfer of CD8+ cells isolated from AdIFN-treated animals to generate Tc1 cells resulted in decreased organism burden in the lungs of Pneumocystis-infected SCID mice (13, 36). Furthermore, γδ-TCR+ T cell-deficient mice have augmented clearance of Pneumocystis associated with increased IFN-γ producing CD8+ T cells (37), suggesting the Tc1 subset of CD8+ T cells are involved in host defense against Pneumocystis. We show Pneumocystis-infected SCID mice reconstituted with CD8+ T cells exhibit a moderately increased organism burden compared with NR animals. The frequency of IFN-γ-producing CD8+ cells in our population of

**FIGURE 8.** Hyperinflammatory profile in the lungs of Pneumocystis-infected mice reconstituted with CD4+CD25+ T cells but not CD4− cells. Lung homogenate levels of KC (A), TNF-α (B), IL-1β (C), IL-6 (D), MCP-1 (E), and G-CSF (F). All data are reported as mean ± SEM for n = 9–11. *, p < 0.05 compared with NR.
CD4^+ cells may not be sufficient to mount an effective host response. Previous studies from our laboratory have shown that reconstitution of *Pneumocystis*-infected SCID mice with nonpolarized CD8^+ cells is not sufficient to promote organism clearance (36). Although IFN-γ levels in the lungs of animals reconstituted with CD4^+ cells was increased compared with NR mice, the levels were 4-fold lower compared with those following adoptive transfer of CD4^+CD25^+ T cells. Organism burden in the lungs of SCID mice reconstituted with CD4^+CD25^+ T cells was slightly reduced compared with NR animals 11 days postreconstitution. In contrast, adoptive transfer of CD4^+CD25^+ regulatory cells led to a substantial increase in organism burden. One explanation for the inability of any cell population to clear *Pneumocystis* from the lungs is that 11 days was not sufficient; to further investigate, clearance studies were conducted to 28 days postreconstitution (data not shown). Immune reconstitution of *Pneumocystis*-infected SCID mice with CD4^+ cells or CD4^+CD25^+ cells resulted in 100% day 28 all-cause mortality, whereas NR mice and those reconstituted with CD4^+CD25^+ T cells all survived (n = 4; data not shown). The *Pneumocystis* copy number in the lungs of mice reconstituted with regulatory cells was >10^11/lung but was not significantly different from NR mice. These data suggest CD4^+CD25^+ T cells are not effector cells against *Pneumocystis*.

The 28-day all-cause mortality associated with reconstitution of CD4^+CD25^+ T cells is not surprising in that inflammatory mediators and markers of lung injury were elevated even 11 days postreconstitution. Others have shown adoptive transfer of CD4^+ cells into *Pneumocystis*-infected SCID mice results in hyperresponsive- ness and pulmonary injury (7); in this study, transfer of 5×10^5–5×10^6 CD4^+ cells from WT donors did not result in mortality but led to significant morbidity on day 28. It is likely the prolonged survival of these animals compared with our study is due to the presence of CD4^+ regulatory cells in the transferred population. This would be consistent with a recent report by Hori et al. (8), which found transfer of CD4^+CD25^+ cells to *Pneumocystis*-infected SCID mice was fatal but cotransfer of CD25^+ cells at a 1:1 ratio significantly decreased mortality.

We show adoptive transfer of CD4^+ cells does not increase parameters of lung injury 11 days postreconstitution compared with NR control animals, and only reconstitution with CD4^+CD25^+ effector T cells led to significant pulmonary injury at this time. However, CD4^+ cell transfer did result in 100% mortality by day 28 postreconstitution, suggesting there is delayed pulmonary impairment. It has been suggested that CD8^+ cells contribute to pulmonary injury during immune response to PCP. Wright et al. (38) report Ab depletion of CD4^+ cells before *Pneumocystis* inoculation results in a significant decline in lung function, whereas depletion of both CD4^+ and CD8^+ cells results in minimal injury associated with infection. The authors suggest these data demonstrate CD8^+ T cells are responsible for exacerbated lung injury in the absence of CD4^+ cells. The decline in lung

**FIGURE 9.** Reconstitution with CD25^+ cells results in a mixed Th cytokine profile. A, Lung homogenate IL-5. B, Lung homogenate IL-10. C, Lung homogenate IFN-γ. D, Lung homogenate IL-17. BAL cytokine levels were below detection, as was lung homogenate IL-4. All data are reported as mean ± SEM for n = 9–11. *, p < 0.05 compared with NR.

**FIGURE 10.** Reconstitution of infected SCID mice with regulatory T cells does not alter IL-10 or TGF-β secretion. A, BAL fluid IL-10 was below detection. A, Lung homogenate IL-10. B, Active BAL fluid TGF-β. All data are reported as mean ± SEM for n = 9–11. *, p < 0.05 compared with NR.
function following depletion of CD4+ cells, but not both CD4+ and CD8+ cells may also be worsened by the loss of CD4+ regulatory cells capable of suppressing the CD8 immune response.

The mechanism of CD4+CD25+Fox3+ T cell suppression is unclear; numerous mechanisms of suppression have been suggested including competitive consumption of IL-2, CTLA-4-mediated suppression through the metabolism of tryptophan, and the secretion of IL-10 and/or TGF-β (9, 39). We measured the levels of IL-10 and soluble TGF-β in the BAL fluid and lung homogenate in the Ab depletion model and the SCID reconstitution model and demonstrate these cytokines do not seem essential to regulatory function. Treatment with anti-CD25 did not alter IL-10 or TGF-β in the lungs at the time point studied. Moreover, reconstitution of SCID mice with CD4+CD25− T cells did not alter TGF-β and significantly increased IL-10, whereas CD4+CD25+ T cell reconstitution did not increase levels of IL-10 or TGF-β compared with NR. The source of IL-10 in CD4+CD25− reconstituted SCID mice was not determined but various cell types including mast cells, monocytes, and dendritic cells, in addition to T and B cells produce IL-10 (40). It has been suggested membrane-bound TGF-β, as opposed to the soluble form, is important in the contact-dependent suppression by CD4+CD25+Fox3+ T cells (23). In a recent study, CD4+CD25−Fox3+ T cells expressing cell surface TGF-β were shown to mediate immunosuppression in a model of Ag-induced tolerance through the activation of Notch in target cells (24). We show the majority of Fox3+ expressing cells express TGF-β on the cell surface. Furthermore, treatment with anti-CD25 results in near-complete loss of membrane-bound TGF-β. Future studies in our laboratory will concentrate on elucidating the mechanism(s) of suppression during PCP.

The involvement of CD4+CD25+Fox3+ T cells in PCP in humans has yet to be demonstrated. Although CD4+ T cells decline in HIV-infected patients, HAART generally results in reconstitution of immune cells as well as decreased incidence of opportunistic infections and death. However, a subpopulation of HIV+ patients receiving HAART develop a hyperinflammatory disorder referred to as immune restoration disease or immune reconstitution inflammatory syndrome (IRIS) despite decreased viral load (41). This detrimental immune response is believed to be directed against previously treated or subclinical opportunistic infections such as Mycobacterium tuberculosis, Mycobacterium avium intracellulare, or Pneumocystis (42, 43). Previous studies have shown antiretroviral therapy results in an initial reconstitution of CD4+CD45RO+ memory T cells (44) but the reconstitution of CD4+ regulatory populations has not been investigated to date. Autran et al. (44) showed CD4+CD45RA+CD25− naïve T cells did not begin to significantly increase until 6 mo after the initiation of antiretroviral therapy, but whether this population is regulatory is not known because Fox3+ had not been identified as a marker of regulatory function at the time of this study. It has been shown that CD4+CD25+Fox3+ regulatory T cells express the HIV coreceptor CCR5 and are susceptible to HIV infection (45). There is a decrease in CD4+CD25− T cells and Fox3+ mRNA levels in the peripheral blood of HIV+ patients, whereas lymphoid tissues have increased regulatory cells, suggesting a redistribution of CD4+ T cell subsets following infection (46, 47). However, it remains unclear whether the ratio of effector T cells to regulatory T cells is altered as a result of HIV infection and HAART, nor is it known whether patients with IRIS have a lag in CD4+CD25+Fox3+ T cell reconstitution that is permissive of chronic inflammation. Further study of the distinct subsets of CD4+ and CD8+ T cells in the clinical setting is essential to defining the patient population at risk for opportunistic infections and IRIS.

In summary, our studies demonstrate CD4+CD25+Fox3+ regulatory T cells are recruited to the lung during the course of Pneumocystis infection. Loss of this population, by Ab treatment, results in exacerbated pulmonary inflammation, Th1 and Th2 cytokine production, and lung injury. It is also apparent that CD4+CD25+Fox3+ T cells are not effector cells against Pneumocystis. The role of CD8 T cells in organism clearance and lung injury associated with PCP is a complex issue with contradicting reports (13, 35, 36, 38). Although further study is necessary to clearly define CD8+ T cell responses, our data suggest there is a delayed injury response mediated by these cells as opposed to CD4+CD25− T cells. These studies demonstrate the necessity to define CD4 and CD8 lymphocyte subsets in disease states as opposed to the total cell population that consists of both regulatory and effector cells.

Disclosures

The authors have no financial conflict of interest.

References
