Treatment with Interleukin-7 Restores Host Defense against Pneumocystis in CD4+ T-Lymphocyte-Depleted Mice

S. Ruan,1,2 D. R. Samuelslon,3 B. Assouline,4 M. Morre,3 J. E. Shellito1

Section of Pulmonary/Critical Care & Allergy/Immunology, Department of Medicine, Louisiana State University Health Sciences Center, New Orleans, Louisiana, USA1; Cytheris SA, Technopolis, Issy Les Moulineaux, France2

Pneumocystis pneumonia (PCP) is a major cause of morbidity and mortality in patients with HIV infection. CD4+ T lymphocytes are critical for host defense against this infection, but in the absence of CD4+ T lymphocytes, CD8+ T lymphocytes may provide limited host defense. The cytokine interleukin-7 (IL-7) functions to enhance lymphocyte proliferation, survival, and recruitment of immune cells to sites of infection. However, there is little known about the role of IL-7 in PCP or its potential use as an immunotherapeutic agent. We hypothesized that treatment with recombinant human IL-7 (rhIL-7) would augment host defense against Pneumocystis and accelerate pathogen clearance in CD4-depleted mice. Control and CD4-depleted mice were infected with Pneumocystis, and rhIL-7 was administered via intraperitoneal injection. Our studies indicate that endogenous murine IL-7 is part of the normal host response to Pneumocystis murina and that administration of rhIL-7 markedly enhanced clearance of Pneumocystis in CD4-depleted mice. Additionally, we observed increased recruitment of CD8+ T lymphocytes to the lungs and decreased apoptosis of pulmonary CD8+ T lymphocytes in rhIL-7-treated animals compared to those in untreated mice. The antiapoptotic effect of rhIL-7 was associated with increased levels of Bcl-2 protein in T lymphocytes. rhIL-7 immunotherapy in CD4-depleted mice also increased the number of gamma interferon (IFN-γ)-positive CD8+ central memory T lymphocytes in the lungs. We conclude that rhIL-7 has a potent therapeutic effect in the treatment of murine Pneumocystis pneumonia in CD4-depleted mice. This therapeutic effect is mediated through enhanced recruitment of CD8+ T cells and decreased apoptosis of lung T lymphocytes, with a preferential action on central memory CD8+ T lymphocytes.

Pneumocystis jirovecii was one of the first recognized complications of human immunodeficiency virus (HIV) infection (1). Despite a significant decline in the incidence of Pneumocystis pneumonia (PCP) following the introduction of 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 the loss of CD4+ T cells is the primary risk factor for developing PCP; HIV+ patients with CD4+ cell counts of <200/μL are highly susceptible to infection. Although a clear relationship between CD4+ T-cell counts and susceptibility to Pneumocystis jirovecii infection exists, the role of the other T-cell subsets is less clearly defined (2). It is well known that CD4+ T cells are critical for host defense against this infection, but in the absence of CD4+ T cells (as in HIV infection), CD8+ T cells may also be important. The depletion of CD8+ T cells in the CD4-depleted mouse model of Pneumocystis infection exacerbated Pneumocystis infection, suggesting a role for CD8+ T cells in host defense against Pneumocystis (3). In addition, it is known that gamma interferon (IFN-γ) is not essential for host defenses against Pneumocystis but is part of a cytokine response which is critical for optimal host defenses (4). Interleukin-7 (IL-7) is a 25-kDa glycoprotein produced by thymus and intestinal epithelial cells, bone marrow elements, and keratinocytes (5). Importantly, IL-7 is required for the normal development and survival of T cells and plays a critical role in modulating T-cell homeostasis (5–8). IL-7 can also induce proliferation of naive and memory T cells (9). IL-7 is also vital for the development of the immune system and profoundly enhances the function of mature T cells. Furthermore, IL-7 is a nonredundant cytokine in T-cell development and function, serving as a potent antiapoptotic cytokine that is essential for lymphocyte survival and expansion (5, 10–13).

In this study, we demonstrated that the continual administration of recombinant human IL-7 (rhIL-7) to CD4-depleted mice markedly increases T-lymphocyte cell number, especially CD8+ T cells and CD8+ T-cell subsets, and enhances T-cell functional potential, which is associated with enhanced clearance of P. murina infection in CD4-depleted mice.

MATERIALS AND METHODS

Mice. Specific-pathogen-free (SPF) female BALB/c mice were purchased at 6 to 7 weeks of age from Hilltop Laboratories (Scottsdale, PA). All animals were housed in filter-topped cages in an isolation room at the Louisiana State University Health Science Center (LSUHSC) animal care facility. All caging procedures and surgical manipulation were done under sterile conditions. Mice were fed autoclaved food, and sterile water was provided ad libitum. The animals were kept in the facility for at least 2 days prior to the beginning of treatment. All experiments were approved by the Institutional Animal Care and Use Committee of LSUHSC (protocol no. 2752).

P. murina inoculation. P. murina organisms for inoculation were isolated from lung homogenates from chronically infected scid/NCr (BALB/c background) mice as previously described (14). Briefly, mice with chronic P. murina infection were injected with a lethal dose of ket-
amine-xylazine, and their lungs were removed aseptically and frozen in 1 ml of phosphate-buffered saline (PBS) at –80°C. Frozen lungs were homogenized mechanically through a sterile 100-μm nylon strainer (BD Biosciences, Bedford, MA) in 10 ml of PBS and pelleted at 1,500 rpm for 10 min at 4°C. The pellet was then diluted 1:4 with PBS and fixed on a microscope slide for *P. murina* enumeration. The slide was stained with modified Giema stain (Diff-Quik; Dade Behring Inc., Newark, DE). The number of *P. murina* cysts was determined microscopically, and the inoculum concentration was adjusted to 2 × 10^5 cysts/ml. Recipient BALB/c mice were anesthetized with ketamine-xylazine and their lungs were removed aseptically and frozen in 1 ml of PBS at –80°C. The lungs were homogenized and the homogenate was assayed for the presence of *P. murina* cysts.

rhIL-7 preparation. The rhIL-7 used in this study was obtained from Cytheris (Issy Les Moulineaux, France). Human IL-7 can bind and signal via the murine IL-7 receptor (15). The rhIL-7 used throughout the study was fully glycosylated, has low immunogenicity, was manufactured using good manufacturing practices (GMP), and is currently being used by a Food and Drug Administration-sponsored clinic trial with patients with HIV-1 and cancer. Where indicated, mice were treated with 5 μg of rhIL-7 via intraperitoneal (i.p.) injection. This dose of rhIL-7 has been shown to have biological effects in a mouse model of sepsis (16). Specifically, CD4– intact animals were given rhIL-7 every 3 days, with the first dose coinciding with *P. murina* infection. CD4– depleted animals were infected with *P. murina*, and infection was allowed to establish for 2 weeks. CD4– depleted animals were then continuously treated with rhIL-7 every 3 days. rhIL-7 treatments were given continuously throughout the experiment.

BAL. Mice were sacrificed after infection with *P. murina* and treatment with rhIL-7. After a lethal dose of ketamine-xylazine, mice were exsanguinated by aortic transaction. The trachea was exposed through a midline incision and cannulated with a polyethylene catheter. The lungs were lavaged with 1-ml aliquots of sterile calcium- and magnesium-free PBS (Gibco/BRL, Gaithersburg, MD) containing 0.6 mM EDTA. A total of 10 ml of recovered bronchoalveolar lavage (BAL) fluid was centrifuged at 1,250 rpm for 10 min. Cells obtained from the BAL fluid were then used for cytometric analysis of cellular populations, as described below.

CD4 T-lymphocyte depletion. Mice were depleted of CD4+ T lymphocytes by i.p. injection of 0.1 mg of anti-CD4 monoclonal antibody purified from a mouse hybridoma cell line (SK1.5; ATCC TIB-207; ATCC, Manassas, VA) in 0.1 ml of PBS every 6 days, as described elsewhere (11). Depleted mice received a dose of anti-CD4 antibody 3 days prior to *Pneumocystis* challenge. Mice were treated with anti-CD4 antibody every 6 days to maintain CD4 depletion. This treatment produces a sustained and profound depletion of CD4+ cell lymphocytes from blood, spleen, and lungs, allowing progressive *P. murina* infection (17, 18).

Determination of absolute cell counts and apoptosis. Total cell counts per spleen and lung were determined as previously described (11). The percentages of individual cell subsets (e.g., CD8+ memory cells) were determined via flow cytometric analysis. The absolute cell counts for subpopulations of each spleen and lung were then calculated.

Cells were harvested from blood, spleen, and lungs by 1-month-old BALB/c mice infected with *P. murina*. The lung burden of *P. murina* was quantified via real-time PCR (RT-PCR) using a set of primers specific to the *P. murina* 18S ribosomal RNA. The RT-PCR reaction was performed in triplicate on each sample, and the results were normalized to the amount of 18S rRNA in each sample. The relative abundance of *P. murina* was then calculated using the 2^(-ΔΔCt) method.

rhIL-7 levels in mice after administration of rhIL-7 and endogenous IL-7 during infection with *P. murina*. To determine if the release of IL-7 is part of the normal host response to infection with *P. murina*, wild-type BALB/c mice were inoculated with *P. murina* and the endogenous levels of IL-7 were assessed in the plasma of infected mice. Infection of wild-type mice with *P. murina* results in a self-limiting infection with clearance of the pathogen from the lungs by 1 month (4). Additionally, we sought to determine the stability of rhIL-7 in both CD4-depleted and wild-type mice following i.p. rhIL-7 administration. rhIL-7 was only detected in the plasma of wild-type mice or CD4-depleted mice after the administration of rhIL-7 (Fig. 1A and B).
the plasma of both CD4-depleted and CD4-intact mice as early as 3 days after infection with *P. murina* and persisted for up to 6 weeks (Fig. 1C and D). In addition, treatment with rhIL-7 did not significantly alter the levels of endogenous IL-7 in CD4-intact or CD4-depleted mice (Fig. 1C and D). These results indicate that production of IL-7 is part of the normal host response to *P. murina*.

Effect of immunotherapeutic administration of rhIL-7 on clearance of *P. murina* in CD4-depleted mice. We then sought to determine if rhIL-7 immunotherapy could enhance the clearance of *P. murina* in CD4-intact and CD4-depleted mice. Mice were depleted of CD4+ T cells (starting 3 days prior to infection) and infected with *P. murina* for 2 weeks prior to initiation of rhIL-7 treatment. CD4-depleted mice were then treated with rhIL-7 every 3 days for the remainder of the experiment. Additionally, CD4-depleted mice continued to receive injections of GK1.5 every 3 days for the duration of the experiment. Control mice were treated with PBS and isotype control by following the same dosing protocol. In CD4-intact mice, treatment with rhIL-7 coincided with infection with *P. murina*. CD4-intact mice were continuously treated with rhIL-7 every 3 days throughout the duration of the experiment. CD4-intact mice were sacrificed 3, 7, and 14 days postinfection (dpi), and *P. murina* lung burden was determined by RT-PCR (Fig. 2A). Compared to that in control mice, *P. murina* burden in mice treated with rhIL-7 was significantly decreased at both 28 and 42 dpi (Fig. 2B). These results support our hypothesis that rhIL-7 can enhance host defenses against *P. murina* in immunosuppressed mice.

rhIL-7 increases T lymphocyte recruitment to sites of infection. T lymphocytes play a vital role in host defense against fungal infections (17, 19). We investigated the effect of rhIL-7 immunotherapy on the recruitment of lymphocytes to the site of *Pneumocystis* infection in both normal and CD4-depleted mice. Control and rhIL-7 treated mice were generated and treated as described above.

Normal (CD4 intact) mice were sacrificed at 3, 7, and 14 dpi, and lung lymphocyte populations of each mouse were assessed via flow cytometry. The absolute cell count for each cell population was then calculated for each animal. We also evaluated the absolute number of T-lymphocyte subset populations. Wild-type mice treated with rhIL-7 exhibited an increase in lung CD4+ T lymphocyte absolute cell counts at 3, 7, and 14 dpi (Fig. 3A). We also evaluated the absolute numbers of CD4+ effector memory and CD4+ central memory T cells in the lung following rhIL-7 treatment and *P. murina* infection. Similar to the results obtained with CD4+ T cells, mice treated with rhIL-7 exhibited an increase (although not significant) in lung CD4+ effector memory cells (Fig. 3B). However, mice treated with rhIL-7 exhibited a significant increase in the number of CD4+ central memory T cells in the lungs 7 dpi (Fig. 3C). We then sought to

FIG 1 IL-7 secretion is part of the normal host defense against *P. murina* infection. Wild-type BALB/c mice and CD4-depleted BALB/c mice were administered 5 μg of rhIL-7 via i.p. injection. CD4-intact animals were given rhIL-7 every 3 days with the first dose, coinciding with *P. murina* infection. CD4-depleted animals were infected with *P. murina*, and infection was allowed to establish for 2 weeks. CD4-depleted animals were then continuously treated with rhIL-7 every 3 days. rhIL-7 treatments were given continuously throughout the experiment. CD4-intact and CD4-depleted mice were sacrificed, and rhIL-7 and endogenous IL-7 levels in the serum were assayed. (A) CD4-intact mice exhibit a significant increase in the levels of rhIL-7 at 3, 7, and 14 days postinfection (dpi) as determined by ELISA. (B) CD4-depleted animals had significant increases in the levels of rhIL-7 at 28 and 42 dpi compared to the values for untreated mice. (C) CD4-intact mice exhibit significant increases in the levels of endogenous IL-7 at 3, 7, and 14 dpi. (D) CD4-depleted animals had significant increases in the levels of endogenous IL-7 at 28 and 42 dpi compared to the values for untreated mice. Data are reported as means ± SEM; n = 6, *, P < 0.01 compared to the value for mice treated with PBS, as determined by repeated-measures two-way ANOVA with post hoc comparison of the means using Bonferroni’s multiple-comparison test. N.D., not detected.
evaluate the levels of CD8⁺ T cells in the lungs following rhIL-7 treatment and *P. murina* infection. Wild-type mice treated with rhIL-7 exhibited an increase (not significant) in lung CD8⁺ T lymphocyte absolute cell counts at 3, 7, and 14 dpi (Fig. 4A). We likewise evaluated the absolute numbers of CD8⁺ effector memory and CD8⁺ central memory T cells in the lungs following rhIL-7 treatment and *P. murina* infection. Similar to the results observed with CD8⁺ T cells, mice treated with rhIL-7 exhibited an increase in lung CD8⁺ effector memory cells (Fig. 4B). However, mice treated with rhIL-7 exhibited a significant increase in the number of lung CD8⁺ central memory T cells 7 dpi (Fig. 4C).

We further sought to explore the recruitment of CD8⁺ T cells to the lungs following rhIL-7 treatment by examining CD8⁺ T and CD8⁺ T-cell subsets in CD4-depleted mice. CD4-depleted mice infected with *P. murina* were continuously treated with rhIL-7, and CD8⁺ T-cell populations were assessed at 28 and 42 dpi. CD4-depleted mice treated with rhIL-7 showed a significant increase in the amounts of CD8⁺ T cells in the lungs at both 28 and 42 dpi compared to the values for control mice (Fig. 5A). We then evaluated the absolute numbers of CD8⁺ effector memory and CD8⁺ central memory T cells in the lungs. Similar to the results obtained with CD8⁺ T cells, mice treated with rhIL-7 exhibited a significant increase in lung CD8⁺ effector memory cells 28 dpi (Fig. 5B). Additionally, mice treated with rhIL-7 exhibited a significant increase in the numbers of lung CD8⁺ central memory T cells 28 and 42 dpi (Fig. 5C). These results indicate that treatment of mice with rhIL-7 results in the recruitment of CD4⁺ and CD8⁺ central memory T lymphocytes to the site of infection in normal (CD4-intact) and CD4-depleted mice.

**rhIL-7 increases precursor memory cell survival and promotes expansion of long-lived memory cell populations.** We then sought to further characterize the CD8⁺ T-cell and CD8⁺
T-cell subset phenotypes in CD4-depleted mice following treatment with rhIL-7. A majority (90 to 95%) of antigen-specific CD8^+ T cells generated by clonal expansion die after antigen clearance; however, the antigen-experienced cells that survive after antigen clearance become long-lived memory cells. IL-7R^hi T lymphocytes are often considered to be memory T-lymphocyte precursors (20). CD4-depleted mice infected with P. murina received continuous treatment with rhIL-7 every 3 days (beginning

FIG 4 Treatment with rhIL-7 significantly increases the number of CD8 central memory cells. CD4-intact mice were treated with rIL-7, and the numbers of CD8^+ T cells and CD8^+ T-cell subsets in the BAL fluid at 3, 7, and 14 dpi were determined via flow cytometry. Treatment of CD4-intact mice with rhIL-7 increased the absolute numbers of CD8^+ T cells (A) and effector memory CD8^+ T cells (B); rhIL-7 treatment significantly increased the number of central memory CD8^+ T cells (C) 7 dpi compared to the value for mice that did not receive rhIL-7. Data are reported as means ± SEM; n = 6. *, P < 0.01 compared to the value for mice treated with PBS, determined by repeated-measures two-way ANOVA with post hoc comparison of the means using Bonferroni’s multiple-comparison test.

FIG 5 Treatment of CD4-depleted mice with rIL-7 significantly increases the numbers of CD8^+ T cells and CD8^+ T-cell subsets. CD4-depleted mice were treated with rIL-7, and the numbers of CD8^+ T cells and CD8^+ T-cell subsets in the BAL fluid at 28 and 42 dpi were determined via flow cytometry. Treatment of CD4-depleted mice with rhIL-7 significantly increased the absolute numbers of CD8^+ T cells (A), effector memory CD8^+ T cells (B), and central memory CD8^+ T cells (C) at both 28 and 42 dpi compared to the values for mice that did not receive rhIL-7. Data are reported as means ± SEM; n = 6. *, P < 0.01 compared to the value for mice treated with PBS, determined by repeated-measures two-way ANOVA with post hoc comparison of the means using Bonferroni’s multiple-comparison test.
Treatment of CD4-depleted mice with rIL-7 increases the numbers IL-7R<sup>hi</sup> (CD127<sup>+</sup>) CD8<sup>+</sup> T cells and CD8<sup>+</sup> T-cell subsets. Mice were administered rIL-7 as described in Materials and Methods. CD4-depleted mice were then sacrificed 6 weeks after infection with <i>P. murina</i>. The absolute numbers of BAL fluid (A to D) and spleen (E to H) CD8<sup>+</sup> T cells and CD8<sup>+</sup> T-cell subsets were assessed via flow cytometry. CD8<sup>+</sup> T cells and CD8<sup>+</sup> T-cell subsets from the BAL fluid of CD4-depleted mice treated with rIL-7 exhibited 7.9-, 6.13-, 9.69-, and 11.57-fold increases in IL-7R expression on CD8<sup>+</sup> T cells (A), naive CD8<sup>+</sup> T cells (B), effector memory CD8<sup>+</sup> T cells (C), and central memory CD8<sup>+</sup> T cells (D), respectively. CD8<sup>+</sup> T cells and CD8<sup>+</sup> T-cell subsets from the spleens of CD4-depleted mice treated with rIL-7 exhibited 6.12-, 6.7-, 3.35-, and 4.77-fold increases in IL-7R expression in CD8<sup>+</sup> T cells (E), naive CD8<sup>+</sup> T cells (F), effector memory CD8<sup>+</sup> T cells (G), and central memory CD8<sup>+</sup> T cells (H), respectively. Data are reported as means ± SEM; n = 6. *, P < 0.01 compared to the value for mice treated with PBS, as determined by unpaired Student’s t test analysis.

FIG 6 14 days after infection) for 48 days after infection with <i>P. murina</i>. CD4-depleted mice were then sacrificed, and CD8<sup>+</sup> T cells and CD8<sup>+</sup> T-cell subsets expressing IL-7 receptor (IL-7R) in both the BAL fluid and spleen were determined (Fig. 6). BAL T lymphocytes isolated from mice treated with rIL-7 compared to mice treated with PBS exhibited significant increases—7.9-, 6.13-, 9.69-, and 11.57-fold—in IL-7R levels on CD8<sup>+</sup> cells (Fig. 6A), naive CD8<sup>+</sup> cells (Fig. 6B), effector memory CD8<sup>+</sup> cells (Fig. 6C), and central memory CD8<sup>+</sup> cells (Fig. 6D), respectively. Furthermore, splenic T lymphocytes isolated from mice treated with rIL-7 compared to mice treated with PBS resulted in significant increases—6.12-, 6.7-, 3.35-, and 4.77-fold—in IL-7R levels on CD8<sup>+</sup> (Fig. 6E), naive CD8<sup>+</sup> (Fig. 6F), effector memory CD8<sup>+</sup> (Fig. 6G), and central memory CD8<sup>+</sup> (Fig. 6H) T lymphocytes, respectively.

**rIL-7 increases lymphocyte proliferation in CD4-depleted mice challenged with <i>P. murina</i>**. IL-7 promotes the survival and proliferation of CD8<sup>+</sup> T cells (20–22). To determine the effect of rIL-7 on CD8<sup>+</sup> T-cell proliferation, BAL fluid and spleen cells were harvested from CD4-depleted mice challenged with <i>P. murina</i> that were continuously treated with rIL-7. BAL fluid and spleen cells were stained with Ki-67, an indicator of cell proliferation. After challenge with <i>P. murina</i>, no significant increase in CD8<sup>+</sup> T-cell proliferation in spleen cells was observed in mice treated with rIL-7 compared to the value for CD4-depleted mice treated with PBS (data not shown). However, compared to control animals, BAL fluid cells isolated from rIL-7-treated CD4-depleted mice challenged with <i>P. murina</i> exhibited a significant (>3.5-fold) increase in proliferation of CD8<sup>+</sup> (Fig. 7A), naive CD8<sup>+</sup> (Fig. 7B), effector memory CD8<sup>+</sup> (Fig. 7C), and central memory CD8<sup>+</sup> (Fig. 7D) T lymphocytes.

**rIL-7 decreases apoptosis of lung CD8<sup>+</sup> T cells in CD4-depleted mice challenged with <i>P. murina</i>**. We also sought to determine if rIL-7 immunotherapy enhances or stimulates antiapoptosis pathways, thus mitigating apoptosis of CD8<sup>+</sup> T cells and CD8<sup>+</sup> memory T-cell subsets in CD4-depleted mice. To assess if rIL-7 immunotherapy enhances antiapoptosis signaling, CD8<sup>+</sup> T cells and CD8<sup>+</sup> memory T-cell subsets were harvested from the BAL fluid and incubated for 4 h in <i>in vitro</i> culture with camptothecin (an apoptosis-inducing agent). Protection from camptothecin-induced apoptosis in CD8<sup>+</sup> T cells and CD8<sup>+</sup> T-cell subsets was then determined by the cellular PARP apoptosis assay coupled with flow cytometry (23, 24). The levels of apoptosis in CD8<sup>+</sup> T cells and CD8<sup>+</sup> memory T-cell subsets following a 4-h <i>in vitro</i> culture with camptothecin were significantly different in mice treated with rIL-7 than in control mice (Fig. 8). Specifically, apoptosis (the percentage of PARP<sup>+</sup> CD8<sup>+</sup> T cells) was 2.43-fold higher in mice treated with PBS than in mice treated with rIL-7 (Fig. 8A). We also observed 2.0-, 2.5-, and 2.7-fold increases in naive CD8<sup>+</sup> (Fig. 8B), effector memory CD8<sup>+</sup> (Fig. 8C), and central memory CD8<sup>+</sup> (Fig. 8D) T lymphocytes in mice treated with PBS compared to the values for mice treated with rIL-7, respectively. The results indicate that rIL-7 reduces camptothecin-induced apoptosis and also increases the antiapoptosis potential of CD8<sup>+</sup> T cells and CD8<sup>+</sup> T-cell subsets in CD4-depleted mice responding to a pulmonary challenge.

**rIL-7 increases intracellular Bcl-2 expression in CD8<sup>+</sup> T lymphocytes**. Given that rIL-7 induces protection from apopto-
sis, we sought to evaluate the antiapoptotic cell signaling pathways of CD8⁺ T cells and CD8⁻ T-cell subsets following rhIL-7 treatment. IL-7 is a potent antiapoptotic cytokine that enhances immune effector cell function and is essential for lymphocyte survival (16, 25, 26). Although IL-7 inhibits apoptosis via several mechanisms, one of its most important antiapoptotic actions is due to increased expression of Bcl-2 in immune effector cells (16, 25). We then sought to determine if rhIL-7 alters the levels of Bcl-2 in CD8⁺ T cells and CD8⁻ T-cell subsets following infection with P. murina in CD4-depleted mice. We administered rhIL-7 to CD4-depleted mice every 3 days starting 2 weeks after infection with P. murina challenge. BAL fluid cells were harvested 6 weeks after infection with P. murina, and CD8⁺ T cells and CD8⁻ subset T cells was determined via flow cytometry. Treatment of CD4-depleted mice with rhIL-7 resulted in marked increases in the levels of intracellular Bcl-2 in CD8⁺ T cells and CD8⁻ subset T cells after challenge with P. murina compared to those in PBS-treated mice (Fig. 9). Total CD8⁺ Bcl-2+ absolute cell numbers were significantly increased (20.06 × 10⁵ in rhIL-7-treated mice versus 5.14 × 10⁵ in PBS-treated mice) 6 weeks after infection with P. murina (Fig. 9A). We also observed a significant increase in the absolute numbers of CD8⁺ naïve CD8⁺ (Fig. 9B), effector memory CD8⁺ (Fig. 9C), and central memory CD8⁺ (Fig. 9D) T lymphocytes in mice treated with PBS compared to the values for mice treated rhIL-7. Our results indicate that rhIL-7 treatment leads to a significant increase in the levels of Bcl-2 in CD8⁺ T lymphocytes and CD8⁻ T-lymphocyte subsets in CD4-depleted mice following P. murina challenge, suggesting that rhIL-7 promotes CD8⁺ T-lymphocyte survival by upregulating the levels of the antiapoptotic protein Bcl-2.

rhIL-7 treatment increases the levels of IFN-γ in CD8⁺ T cells and CD8⁻ memory T-cell subsets in CD4-depleted mice. Finally, we sought to determine if treatment of CD4-depleted mice with rhIL-7 results in increased numbers of IFN-γ⁺ CD8⁺ T cells and IFN-γ⁺ CD8⁻ T-cell subsets. CD4-depleted mice were treated with rhIL-7 via i.p. injection every 3 days beginning 2 weeks after infection with P. murina. Mice were then sacrificed 6 weeks postinfection, and BAL fluid was collected. CD4-depleted mice treated with rhIL-7 showed a significant increase in the numbers of IFN-γ⁺ CD8⁺ T cells and IFN-γ⁺ CD8⁻ T-cell subsets compared to the values for mice treated with PBS (Fig. 10). More precisely, we found a significant increase in the numbers of IFN-γ⁺ CD8⁺ (Fig. 9A), naïve CD8⁺ (Fig. 9B), effector memory CD8⁺ (Fig. 9C), and central memory CD8⁺ (Fig. 9D) T cells in CD4-depleted mice treated with rhIL-7 compared to the values for control mice. These results indicate that rhIL-7 treatment results in a significant increase in the levels of IFN-γ in both CD8⁺ T cells and CD8⁻ T-cell subsets compared with the values for mice that did not receive rhIL-7.

**DISCUSSION**

IL-7 is a nonredundant pluripotent cytokine produced by bone marrow and thymic stromal cells that is required for T-cell survival (11–13, 25, 27–29). The human IL-7 locus consists of 6 exons and 9 introns with extensive 3' and 5' untranslated regions and is located on chromosome 8q12-13. There is 81% homology between the human and the murine IL-7 sequences in the coding regions (murine IL-7 lacks the 54-bp exon 5); however, the homology in the 3' and 5' noncoding regions is only 60 to 70%. Interestingly, human IL-7 has activity in murine cells, but murine cells...
IL-7 fails to stimulate human pre-B cells (30). Additionally, IL-7 mediates early lymphocyte development by regulating V(D)J re-arrangement (31) and promoting thymocyte proliferation and survival (32), which helps to regulate the immune response following antigen exposure. Similarly, IL-7 has been shown to regulate the generation of memory CD4+ and CD8+ T cells following antigen exposure (33). As a result, IL-7 is currently being evaluated in multiple clinical trials (e.g., for hepatitis and HIV) as a potent immunotherapeutic agent (21, 34, 35). The ability of IL-7 to enhance host immunity and promote disease resolution has also been demonstrated using various bacterial and viral infection models (16, 36). Importantly, CD8+ T cells play a dominant role in resistance to fungal infection (37, 38), and recently, IL-7 has been shown to promote survival in an animal model of fungal (Candida albicans) sepsis (29).

However, the role of IL-7 in host defense against infection with P. murina remains unclear. It is noteworthy that immunocompetent mice inoculated with P. murina experience a self-limiting infection, with clearance of the organisms from the lungs by 4 weeks (14). We first sought to determine whether IL-7 participates in the normal host response to infection with P. murina, and develop progressive pneumonia after inoculation of organisms into the lungs (17). Specifically, we determined that systemic administration of recombinant human IL-7 (rhIL-7) in the absence of CD4+ cells promotes the influx of both CD8+ T cells and CD8+ memory T cells to the site of infection (i.e., the lungs), which also correlates with clearance of P. murina. Additionally, our data suggest that rhIL-7 immunotherapy may be effective for the prevention or establishment of infection (as seen in CD4-intact animals), as well as the clearance of infection (as seen in CD4-depleted animals).

While several studies have demonstrated that CD4+ T cells are crucial for resistance to fungal infection (39–41), less is known about CD8+ T cells. We have previously demonstrated that both CD4 and CD8 T cells are important for host defense against Pneumocystis (4). Specifically, we found that the administration of both anti-CD4 and anti-CD8 neutralizing monoclonal antibody leads to a more severe form of Pneumocystis infection than does depletion of CD4+ cells alone. This suggests that CD8+ T cells also play a critical role in host defense against Pneumocystis infection (4). Beck et al. also demonstrated that depletion of CD8+ T cells in CD4-depleted mice exacerbated P. murina infection (3). We did not observe any increased cellular pathology associated with increased levels of CD8+ T cells following treatment with rhIL-7. However, others have reported that CD8+ T cells have little importance in the control of P. murina (42). In support of a protoc-
tive role for CD8⁺ T cells, we found in this study that systemic administration of rhIL-7 induces the proliferation and activation of CD8⁺ T cells independently of CD4⁺ T cells and that this treatment provided enhanced resistance against *P. murina* infection.

Current immunological dogma states that the generation and maintenance of long-lived memory CD8⁺ T cells require CD4⁺ T-cell help (43). In addition, it is known that chronic IL-7-mediated signaling induces antiapoptotic and costimulatory responses, but when T cells become activated, the expression of IL-7Rα (CD127) is downregulated (6). However, in contrast to this, we see an increase in the levels of CD127 on CD8⁺ T cells following treatment with rhIL-7. This may be due to the fact that there is an exception to this rule which occurs during primary immune responses, when IL-7Rα is selectively expressed on a small minority of effector T cells that are destined to enter the central memory T-cell pool, thus implicating IL-7 as a modulator of the effector-to-memory cell transition (6). This is supportive of our findings indicating that rhIL-7 treatment increases the numbers of central memory CD4⁺ T cells and CD8⁺ T cells. This may also suggest that during *P. murina* infection treatment, rhIL-7 promotes T-cell development to central memory T-cell subsets. Given that IL-7Rα promotes the expansion and activation of CD8⁺ T cells, we chose to investigate the maintenance of memory CD8⁺ T cells during *P. murina* infection following administration of IL-7 in the absence of CD8⁺ T cells. Specifically, we sought to dissect CD8 T cell proliferation, Bcl-2 expression, apoptosis, and production of intracellular IFN-γ. Administration of rhIL-7 significantly increased the recruitment and activation of CD8⁺ T cells to the lungs of *P. murina*-infected animals, even in the absence of CD4⁺ T cells. A number of investigative groups, as in our study, have demonstrated that administration of IL-7 prevents the loss of CD8⁺ T cells in the absence of CD4⁺ T cells (19, 56). Our results demonstrate an increase in the absolute numbers of CD8⁺ cells and CD8⁺ central memory T cells and a significant decrease in the percentage of cells undergoing apoptosis in mice treated with rhIL-7.

We then sought to investigate how CD8⁺ memory T cells are generated, maintained, and activated independently of CD4⁺ T-cell help. IL-7 is integral to the generation and maintenance of CD8⁺ memory T cells. Importantly, it has been demonstrated that insufficient production of IL-7 limits the survival and persistence of CD8⁺ memory T cells (44). IL-7 has also been shown to have antiapoptotic effects which promote the survival, proliferation, and activation of CD4⁺ T cells, B cells, and gamma/delta T cells (5, 13, 45). In addition, IL-7 inhibits apoptosis via several mechanisms; however, one of its most important antiapoptotic actions is due to its ability to increase Bcl-2 expression (46, 47). Other groups have demonstrated that administration of rhIL-7 rapidly induces a significant increase in the expression of intracellular Bcl-2 in both CD4 and CD8 T cells (27, 29, 48). Our data indicate that CD4-depleted mice infected with *P. murina* exhibit a marked increase in the levels of intracellular Bcl-2 in lung CD8⁺ T cells and CD8⁺ T-cell subsets following the administration of rhIL-7. Increased levels of Bcl-2 were also correlated with decreased camptothecin-induced apoptosis of lung CD8⁺ T cells and CD8⁺ T-cell subsets. Importantly, we have previously demonstrated that mice depleted of CD4⁺ T cells and then infected with *P. murina* have significantly increased levels of apoptotic CD8⁺ T

![Graphs showing the effects of rhIL-7 on T cells](https://iai.asm.org/)

**FIG 9** Mice treated with rhIL-7 exhibit increased levels of Bcl-2 in CD8⁺ T cells and CD8⁺ T-cell subsets. CD4-depleted mice were inoculated with *P. murina*. Two weeks postinoculation, mice were treated with 5 μg of rhIL-7. BAL fluid cells were harvested 6 weeks later, and intracellular levels of Bcl-2 were determined for CD8⁺ T cells and CD8⁺ T-cell subsets by flow cytometry. Mice treated with rhIL-7 exhibited significantly increased absolute numbers of cells expressing Bcl-2 in CD8⁺ T cells (A), naive CD8⁺ T cells (B), effector memory CD8⁺ T cells (C), and central memory CD8⁺ T cells (D). Data are reported as means ± SEM; n = 5. *, P < 0.01 compared to the value for mice treated with PBS, as determined by unpaired Student’s *t* test analysis.
The production of IFN-γ/H9253 CD8 +/H9253 mechanism is that rhIL-7 increases the number of IFN-γ-secreting cells (49). These data suggest that IL-7, in part, mitigates the induction of apoptosis in CD8 + T cells seen in CD4-depleted mice infected with P. murina. Our results indicate that the 2-fold increase in CD8 + T cells and CD8 + central memory T cells after IL-7 treatment is, in part, due to an rhIL-7-mediated increase in Bcl-2 levels and decrease in PARP levels. We cannot rule out an effect of IL-7 treatment on chemotaxis and migration of CD8 + T cells into lung tissue, as has been demonstrated in other model systems (50).

A key goal of our study was to determine the therapeutic potential of rhIL-7 in host defense against P. murina infection in the context of CD4 T-cell depletion. The mechanism(s) for the therapeutic effect described here is not clear. One potential mechanism is that rhIL-7 increases the number of IFN-γ-secreting CD8 + T lymphocytes present at the site of infection and these cells are cytotoxic to Pneumocystis. It is noteworthy that IL-7 stimulates the production of IFN-γ by T lymphocytes (29). In addition, IL-7 has been shown to regulate CD8 + T-cell pore-forming protein gene expression, gamma interferon production, and cytotoxicity of human peripheral blood lymphocyte subsets (51). This mechanism is supported by the fact that gene transfer of murine IFN-γ into the lungs of mice challenged with P. murina results in the clearance of the pathogen independent of CD4 + T cells (4), suggesting that the recruitment of IFN-γ-secreting CD8 + T lymphocytes to the site of infection may be sufficient for P. murina host defense. In support of this, we found that systemic administration of rhIL-7 to CD4-depleted mice infected with P. murina led to a significant increase in the recruitment of IFN-γ-positive CD8 + T cells and CD8 + T-cell subsets in lungs and enhanced clearance of P. murina. Our results indicate that memory CD8 + T cells are induced and maintained in the absence of CD4 + T cells by administration of rhIL-7. These data are supported by several papers that demonstrate that activated CD8 + T cells are critical for host-mediated defense against other fungal pathogens (52, 53). Additionally, these data suggest that an immunocompromised host may be treated for fungal infections by the direct targeting of IFN-γ-secreting CD8 + T cells (4, 26, 39, 54). However, we cannot exclude the possibility that IL-7 treatment promotes macrophage-mediated killing and/or enhances the P. murina-specific antibody response. We are currently evaluating all of the potential mechanisms involved in the IL-7 response to Pneumocystis.

In summary, we demonstrate that rhIL-7 is a potent immune stimulatory agent in P. murina-infected mice. Specifically, we show that IL-7 significantly improved lymphocyte functions, including proliferation, T-cell activation, IFN-γ expression, and decreased apoptosis at the site of infection. Treatment with IL-7 enhanced host defense against Pneumocystis independently of CD4 + T lymphocytes through the recruitment and activation of CD8 + T cells and CD8 + T-cell subsets. Additionally, both lung CD8 + T cells and CD8 + T-cell subsets exhibited decreased apoptosis and enhanced expression of IFN-γ. Our data provide strong evidence that IL-7 has potential as an immune therapy for Pneumocystis infection and could potentially serve as a novel adjuvant.
for a Pneumocystis vaccine by increasing the size and breadth of the host T-cell response.

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