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T Cytotoxic-1 CD8⁺ T Cells Are Effector Cells against *Pneumocystis* in Mice

Florencia Mc Allister,* Chad Steele,* Mingquan Zheng,* Erana Young,† Judd E. Shellito,† Luis Marrero,† and Jay K. Kolls*2

Host defenses are profoundly compromised in HIV-infected hosts due to progressive depletion of CD4⁺ T lymphocytes. A hallmark of HIV infection is *Pneumocystis carinii* (PC) pneumonia. Recently, CD8⁺ T cells, which are recruited to the lung in large numbers in response to PC infection, have been associated with levels of host defenses as well as contributing to lung injury in BALB/c mice. In this study, we show that CD8⁺ T cells that have a T cytotoxic-1 response to PC in BALB/c mice, as determined by secretion of IFN-γ, have in vitro killing activity against PC and effect clearance of the organism in adoptive transfer studies. Moreover, non-T cytotoxic-1 CD8⁺ T cells lacked in vitro effector activity and contributed to lung injury upon adoptive transfer. This dichotomous response in CD8⁺ T cell response may in part explain the clinical heterogeneity in the severity of PC pneumonia. *The Journal of Immunology*, 2004, 172: 1132–1138.

**Materials and Methods**

**Ad vectors**

AdIFN is an E1-E3 replication-deficient rAd5-based vector containing and expressing the full-length murine IFN-γ cDNA driven by the CMV immediate early promoter (5, 9). The control AdEGFP vector is identical with this but encodes enhanced green fluorescent protein (EGFP) cDNA (Clontech, Palo Alto, CA). Viruses were propagated in 293 cells and purified as previously described (5, 10). Both viruses were propagated in 293 cells, purified by ultracentrifugation over a CsCl gradient, and titered by a plaque assay on 293 cells as described previously (5). Viral stocks contained <1 replication-competent Ad per 10⁶ PFU (as determined by a lack of cytotoxic effect on A549 cells, at a multiplicity of infection of 10). The particle/PFU ratio was <100:1, and virus stocks contained <0.01 ng/ml endotoxin as determined by the QCL-1000 Limulus lysate assay (BioWhittaker, Walkersville, MD).

**PC inoculum**

The PC inoculum was prepared as previously described (5, 11). Briefly, CB-17 *scid* mice with PC pneumonia were injected with a lethal dose of pentobarbital, and the lungs were aseptically removed and frozen for 30 min in 1 ml of PBS at −70°C. Frozen lungs were homogenized in 10 ml of PBS (model 80 stomacher; Tekmar Instruments, Cincinnati, OH), filtered through sterile gauze, and pelleted at 500 x g for 10 min at 4°C. The pellet was resuspended in PBS, and a 1/10 dilution was stained with modified Giemsa stain (Diff-Quik; Baxter, McGaw Park, IL). The number of PC cysts was quantified microscopically (11), and the inoculum concentration was adjusted to 2 x 10⁶ cysts/ml. Gram stains were performed on the inoculum preparation to exclude contamination with bacteria.

**Monoclonal Abs**

Anti-CD4 Ab was prepared as previously described (11). Briefly, the hybridoma GK1.5, which produces a rat IgG2b mAb against murine CD4 (12), was obtained from the American Type Culture Collection (Manassas, VA). Harvesting of the Ab as ascites from pristane-primed, uninfected athymic mice took place in the Monoclonal Ab Core Laboratory facility at...
Purification of lung CD8 T cells

Left lungs suspended in RPMI 1640 (Invitrogen) supplemented with 1% BSA (Sigma-Aldrich) were digested with collagenase type IV (Sigma-Aldrich) at a concentration of 1–2 mg/ml for 60–90 min at 37°C using a rotating shaker. Thereafter, the cell mixture was passed successively through a 70-μm filter and a 40-μm filter (BD Falcon, Franklin Lakes, NJ). After centrifugation at 1800 rpm for 10 min, the pellet was resuspended in ice-cold RBC lysis buffer (8.02 g of NH4Cl, 0.84 g of NaHCO3, 0.37 g of NaH2PO4, 0.37 g of EDTA, 100 ml of H2O at pH 7.4 and 4°C) for 3 min. After centrifugation and double washing, cells were resuspended in medium and enumerated in a hemocytometer (1/100 dilution). Trypan blue staining was used for viability determination. After lung digestion and cell count, CD8+ T cells were isolated via MACS (Miltenyi Biotech, Auburn, CA) using negative and positive selection, following the manufacturer’s protocol. The cells were first incubated with a biotin-Ab mixture of biotin-conjugated mAbs against CD4 (L3T4, rat IgG2b), CD11b (Mac-1, rat IgG2b), CD45R (B220, rat IgG2a), DX5 (rat IgM), and Ter-119 (rat IgG2b) (Sigma-Aldrich) and at 3 μg/ml biotinylated anti-CD8α (clone 53-6.7) or anti-CD8β (clone 53-5.8) (BD PharMingen). The cells were incubated on the Ab-coated plates at 37°C in 5% CO2 for 2 h. Duplicate wells were washed with (5 μg/ml) and without added Con A (Sigma-Aldrich). The cells were incubated for 3 h with and then for 3 h with PBS/0.05% Tween to remove bound cells. Biotinylated detecting Ab then was added to appropriate wells at 4 μl/ml biotinylated anti-IFN-γ (XM1G1.2, BD PharMingen) and at 3 μl/ml biotinylated anti-IL-4 (24G2; Endogen). Plates were incubated at 4°C for 18 h. Wells were washed four times with PBS-Tween, and 100 μl of streptavidin-conjugated peroxidase (1/400 dilution of 1 ml in 1% PBS-Tween; Sigma-Aldrich) was added to each well. The plates were incubated with peroxidase for 2 h at room temperature in the dark and then were washed four times with PBS-Tween. Substrate (3-aminon-9-ethylcarbazole; Sigma-Aldrich) then was prepared according to the manufacturer’s directions and was added at 200 μl/well. The plates were incubated for 15–30 min to develop spots and then were washed with water. Plates were shaken free of water and were air dried before the spots were counted under a dissecting microscope. Spots were counted in duplicate wells, and data were expressed as the number of IFN or IL-4 spots per 105 CD8+ T lymphocytes.

IL-10 secretion assay

Spontaneous or stimulated (with Con A or Ag) cell culture supernatant of IFN-γ was analyzed in lung digest CD8+ T cells with an IFN-γ secretion assay kit (Miltenyi Biotec) per the manufacturer’s instructions. Digested lung cells (described above) were cultured for 16 h at 37°C, 5% CO2 in medium alone (spontaneous production), with Con A (0.5 μg/ml; nonspecific stimulated production), or with PC Ag-pulsed bone marrow dendritic cells as previously described (16). At the conclusion of the assay, the cells were incubated with optimal dilutions of FITC-conjugated anti-CD8α (clone 53-6.7) or PE-conjugated anti-IFN-γ (from BD PharMingen and Miltenyi Biotec, respectively) for 20 min at room temperature. Thereafter, 10,000 cells were analyzed with a FACSComp flow cytometer (BD Biosciences, San Jose, CA). Gates were set for lymphocytes in the analyzed cell population using forward and right-angle light scatter, and the percentage of dual-positive cells was calculated. Controls included isotype-matched control Ab conjugated to identical fluorochromes. To ensure accurate enumeration of activated lymphocytes that may have altered forward- and side-scatter profiles, sample standards were back-gated against the forward- and side-scatter profiles. Data are expressed as the percentage of dual-positive cells (IFN-γ/CD8+).
In vitro effector activity of lung CD8\(^+\) T cells

Thioglycollate-elicited peritoneal macrophages were obtained from 6- to 8-wk-old BALB/c mice and resuspended at a concentration of 10\(^6\) per milliliter. Cells (100 \(\mu\)l) were cocultured with PC (10\(^3\) cysts) with or without lung CD8\(^+\) T cells for 16 h at 37\(^\circ\)C and 5% CO\(_2\). Controls included PC incubated with medium alone. The contents of each well were collected and pelleted at 800 \(\times\) g for 5 min. The supernatants were discarded, and total RNA was isolated from the cell pellets using TRIzol reagent (Invitrogen). Viability of PC was analyzed through real-time PCR measurement of rRNA copy number and quantified by using a standard curve of known copy number of PC rRNA as previously described (16, 18). This methodology detects viable PC organisms as confirmed by the absence of detectable PC RNA in samples subjected to heat inactivation or exposure to trimethoprim/sulfamethoxazole. Percent killing was defined as follows: 1 – (rRNA copy number from wells containing macrophages + PC/rRNA copy number from wells containing PC alone) \(\times\) 100.

Adoptive transfer

Male C.B-17 BALB/c scid mice were obtained from Charles River Laboratory and housed at the Louisiana State University Health Sciences Center Animal Care facility. scid mice were anesthetized with ketamine/xylazine, and challenged with 2 \(\times\) 10\(^3\) PC cysts intratracheally. Mice were adoptively transferred 24 h later with 10\(^3\) CD8\(^+\) T cells obtained from the digested lungs (isolated by double selection as previously described) of AdIFN- or AdEGFP-treated CD4-depleted mice 14 days after inoculation with PC (the time of peak CD8\(^+\) T cell influx in the AdIFN group). CD8\(^+\) T cells were administered in a volume of 1 ml of PBS by i.p. injection. A third group of scid mice group was injected with PBS but not immunologically reconstituted. All of the mice were sacrificed 28 days after the adoptive transfer. Lung injury was assessed by assaying lactate dehydrogenase (LDH; Sigma-Aldrich) and total protein (Pierce, Rockford, IL) in BAL fluid. The right lung was harvested for PC organism burden as measured by TaqMan RT-PCR and silver staining of histological lung sections as previously described (16, 19).

Histopathology

Paraffin-embedded sections were stained with H&E or Gomori methenamine silver (GMS) and scored blindly for alveolar and perivascular inflammation. This method has been shown to correlate in a quantitative scale ranging from 0 (no visible infection) to 4 (cysts throughout most alveolar regions). This method was shown to correlate in a double-blind fashion with organism counts from homogenized lung tissue.

Real-time PCR analysis of PC infection

Total RNA was isolated from the right lung of infected mice by a single-step method using TRIzol reagent (Life Technologies, Gaithersburg, MD) as per the manufacturer’s instructions. Thereafter, RNA was transcribed to cDNA and real-time PCR was performed as previously described (16, 17). This assay has a correlation coefficient of 0.98 with PC/rRNA copy number (16). Results were expressed as PC copy number normalized to 18s rRNA content, also determined by real-time RT-PCR.

Statistical analysis

Data were analyzed using StatView statistical software (Brainpower, Calabasas, CA). Comparisons between groups where data were normally distributed were made with Student’s t test, and comparisons among multiple groups or nonparametric data were made with ANOVAs and appropriate follow-up testing. The Mann-Whitney test or the Wilcoxon paired-sample test was used to make ordinal comparisons. Significance was accepted at a value of p < 0.05.

Results

Pharmacokinetics of AdIFN in CD4-deficient mice and clearance of PC

As previously reported by our laboratory (5), administration of AdIFN intratracheally into CD4-deficient BALB/c mice 3 days before PC inoculation resulted in significantly higher levels of IFN in BAL fluid at all time points compared with AdEGFP-treated control mice. This IFN response was compartmentalized to the lung, because serum IFN levels were <10 pg/ml at each time point. The overexpression of IFN was associated with comparable levels of PC burden for the first 14 days followed by significant organism clearance in the AdIFN-treated group by day 28, which was sustained to the end of the analysis at day 42 (5). Analysis of T cells in prior studies in this model showed that day 14 was associated with the greatest CD8\(^+\) T cell influx in AdIFN-treated mice (Fig. 1). Based on the kinetics of this CD8\(^+\) T cell response, the fact that CD8\(^+\) T cell depletion abrogates the effect of AdIFN, and that PC burdens were similar at this maximal CD8\(^+\) T cell response, we focused on phenotyping the CD8\(^+\) T cell response at this 14-day time point.

Induction of CXCL9 and CXCL10 and lung recruitment of CXCR3\(^+\) CD8\(^+\) T cells by AdIFN

As it has been reported that chemokines induced by strong Th1/ Tc1 polarizing cytokines such as IFN can lead to the selective recruitment of CXCR3\(^+\) T cells (21), we assayed the induction of CXCL9 (Mig) and CXCL10 (IP-10) in this model. AdIFN-treated mice had similar levels of Mig and IP-10 compared with AdEGFP controls at day 3 of PC infection; however, by day 14, at the time of maximal CD8\(^+\) T cell influx in this model, there were significantly higher concentrations of these CXCR3 ligands in AdIFN-treated animals (Fig. 2, A and B). The induction of these ligands was associated with a significantly higher number of CXCR3\(^+\) CD8\(^+\) T cells as measured by absolute number and percentage of CD8\(^+\) T cells in the BAL fluid of AdIFN-treated mice at day 14 (Fig. 2, C and D). These data
suggested that there was a selective recruitment of Tc1 CD8+ T cells in AdIFN-treated mice. To test this hypothesis, we analyzed the phenotype of recruited CD8+ T cells.

**AdIFN results in the selective recruitment of Tc1 CD8+ T cells during PC infection**

To phenotype recruited CD8+ T cells, these cells were purified from hilar lymph nodes or BAL fluid at day 7 and day 14 of infection and assayed by ELISPOT for IFN or IL-4 production in response to Con A. There were significant numbers of IFN-producing CD8+ T cell cells in AdIFN-treated mice compared with AdEGFP mice, by day 7 in hilar lymph nodes followed by BAL fluid at day 14 (Fig. 3A). We observed very little spontaneous secretion of IFN by CD8+ T cells from AdIFN- or AdEGFP-treated mice, because cells not stimulated with Con A had <5 spots/10^5 cells. Moreover, we observed a similarly low frequency of IL-4-producing cells even after Con A stimulation (data not shown).

To assess the Ag specificity of these cells, we harvested cells from enzyme-digested lung at day 14. Cells were stained with a bispecific Ab for CD45 and anti-IFN and then stimulated with Con A or PC Ag-pulsed bone marrow-derived dendritic cells as previously described (16). After stimulation, cells were stained for CD8 and IFN and analyzed with two-color FACS. Similar to the ELISPOT data, CD8+ T cells from the lungs of AdEGFP control mice routinely showed <1% positive IFN-producing CD8+ T cells, whereas cells from AdIFN-treated mice showed between 8 and 15% IFN+CD8+ T cells (Fig. 3B). Thus, the Tc1-phenotype is PC Ag specific. Using this whole-lung stimulation procedure, we demonstrated that CD8+ T cells from AdEGFP mice are capable of producing IFN in response to Con A (Fig. 3B, bottom left panel), but not to PC Ag (upper right panel). Using the same technology, we assayed IL-10 secretion in response to PC Ag and observed <2% of CD8+ T cells stained positive for IL-10 in either AdIFN or AdEGFP groups (data not shown).

**In vitro effector activity of Tc1 CD8+ T cells against PC**

To test in vitro effector activity, we used a recently developed macrophage:PC coculture assay where thioglycolate-elicited peritoneal macrophages are cocultured with PC organisms at a 100:1 macrophage:PC cyst ratio (or ~1:1 macrophage:trophozoite ratio). Optimization of this assay revealed that this ratio results in a 50% reduction in viability of PC as assessed by assaying the integrity of the PC rRNA subunit integrity by TaqMan PCR (16). The loss of viability of PC in this assay is dependent on phagocytosis and the release of reactive-oxygen species (18). CD8+ T cells purified from lung digest on day 14 were mixed in either a 90:10% or 50:50% ratio with macrophages. Assaying either of these conditions revealed no greater PC killing than that ascribed to the equivalent number of macrophages alone (Fig. 4). However, the addition of Tc1 CD8+ T cells from AdIFN-treated mice significantly enhanced killing at the 90:10% ratio (Fig. 4) and was associated with slightly greater killing at the 50:50% ratio, but this was not statistically significant. There was no augmentation of in vitro killing with CD8+ T cells from AdEGFP control mice (Fig. 4). We could not rigorously test ratios below 90:10 to assess any direct effect of CD8+ T cells on PC, because CD8+ T cells purified from lung digest using magnetic beads always had 2–5% macrophages that coeluted.

**In vivo effector activity of Tc1 CD8+ T cells against PC**

To test whether these Tc1 cells from day 14 after AdIFN treatment had in vivo effector activity, we adoptively transferred purified CD8+ T cells (>95% pure) that were extensively washed to remove any potential contaminating anti-PC Ab, into PC-infected scid mice. Twenty-eight days later, mice were sacrificed, and PC infection was assessed by histological scoring and by TaqMan PCR for PC rRNA (16). scid mice that were mock adoptively transferred (given PBS i.p.) had significant PC infection by day 28 (Fig. 5A), whereas those given Tc1 CD8+ T cells had significantly lower organism burdens (Figs. 5A and 6). Interestingly, scid mice...
given non-Tc1 CD8⁺ T cells from AdEGFP showed a trend to slightly higher PC organism load (Fig. 5A) but significantly more lung injury as assayed by LDH and total protein in BAL fluid (B and C). These data were corroborated by lung histology that showed significantly more proteinaceous material in the alveolar space of scid mice given non-Tc1 CD8⁺ T cells (Fig. 6).

**Discussion**

Host defense against infection is critically dependent upon recruitment into infected tissue of immune effector cells. The resultant influx of inflammatory cells into a site of infection may result in either clearance of infection or, in some cases, tissue damage. Tissue damage during the inflammatory phase of the host response to infection may be the result of an overexuberant recruitment of immune effector cells. Alternatively, there may be specific immune effector cells with the recruited cells that mediate clearance or tissue damage. The results of these studies would support the latter possibility for infection with *Pneumocystis*.

It has been previously reported that CD8⁺ T cells are recruited in significant numbers into the lungs of experimental animals and patients with PC pneumonia (19, 22). However their role in host defense and lung injury remains unclear. Beck et al. (23) have previously shown that depletion of CD8⁺ T cells in the CD4⁻ depleted mouse model of PC infection exacerbated PC infection, suggesting a role for CD8⁺ T cells in host defense against PC. Moreover, γδ TCR knockout mice, which clear PC more rapidly, demonstrate that augmented recruitment of IFN-producing CD8⁺ T cells and depletion of CD8⁺ T cells in this model reverses the augmented clearance seen in this mouse strain (17). These data suggested that Tc1 cells might be beneficial in host defense against PC. To test this further, we enriched for Tc1, PC-specific CD8⁺ T cells in the lung using gene transfer of the IFN-γ gene into CD4⁻ depleted mice infected with PC.

Using this model, we confirmed that overexpression of IFN resulted in the induction of the IFN-regulated chemokines Mig (24) and IP-10 (25). Moreover, overexpression of IFN was associated with augmented CXCR3⁺/H11001 CD8⁺ T cells, a marker consistent with a Tc1 phenotype (26). The Tc1 phenotype was further demonstrated by ELISPOT and Ag-induced IFN secretion. CD8⁺ T cells harvested from the lungs of mice 14 days after PC infection (the time of peak CD8⁺ T cell influx) demonstrated in vitro and in vivo effector activity. One caveat of these experiments is that the Tc1

**FIGURE 5.** Adoptive transfer of CD8⁺ T cells. A, Twenty-eight-day PC infection burden in 6- to 8-wk-old scid mice infected with PC and left unreconstituted or reconstituted with 10⁶ CD8⁺ T cells from day 14 AdIFN or AdEGFP mice (n = 3–5; *, p < 0.05, ANOVA). B, LDH activity in BAL fluid in 28 day after CD8⁺ T cell reconstitution in PC-infected scid mice (n = 3–5; *, p < 0.05, ANOVA). C, Total protein in BAL fluid 28 days after CD8⁺ T cell reconstitution in PC-infected scid mice (n = 3–5; *, p < 0.05, ANOVA).

**FIGURE 6.** Representative histology in adoptively transferred scid mice at 28 days. Upper panel, Lung sections stained with GMS revealed less infection in scid mice adoptively transferred with Tc1 CD8⁺ T cells. Lower panel, Lung sections stained with H&E revealed more alveolar exudates in mice adoptively transferred with non-Tc1 cells.
phenotyping was done at the 14-day time point, and that, upon adoptive transfer, the phenotype could have changed. However, we feel that this is unlikely, because it has been shown in other systems that Th1/Tc1 and Th2/Tc2 differentiation occurs within three to four cell divisions of T cell proliferation (27), and this appears to be a fixed response associated with DNA silencing of Th1 loci in the case of Th2/Tc2 cells and Th2 loci in the case of Th1/Tc1 cells (28). Taken together, these data clearly demonstrate that Tc1 CD8\(^+\) T cells are capable of host defense against PC. What remains unclear from these studies is the mechanism by which these cells are effective. The in vitro data suggest that they augment macrophage-mediated clearance of PC. This may be through an apoptotic process or through soluble factors such as TNF-\(\alpha\) or TNF-\(\beta\) that may augment macrophage killing of PC (29, 30).

We thank Dr. Imtiaz Khan for helpful discussions during the writing of this manuscript.

**References**

CORRECTIONS


The word “apoptosis” was misspelled in the title, in the fourth sentence of the Abstract, in the first sentence of the third paragraph of the Introduction, and in the abbreviations list in Footnotes. The error has been corrected in the online version, which now differs from the print version as originally published.


In Results, in Fig. 7, the arrows indicating IL-4 and IFN-γ are inverted. The corrected figure is shown below.

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![Corrected Figure](image-url)

In **Results and Discussion**, after the first sentence in the last paragraph, the authors wish to add the sentences shown below. In **References**, the authors also wish to add the citation shown below.

Recently, Bielekova et al. described their findings concerning the induction of CD56bright NK cells by anti-IL2R therapy in multiple sclerosis patients (31). This is consistent with our observations in uveitis patients.


In **Disclosures**, a potential financial conflict of interest was inadvertently omitted. The disclosure shown below should have been declared.

R. A. B. Ezekowitz is cofounder and serves on the Board of Directors of NatImmune, a privately held biotechnology company.


The first author’s name is listed incorrectly. The correct name is Florencia McAllister.


The fifth author’s name is listed incorrectly. The correct name is John F. Elliott.