# T Cytotoxic-1 CD8$^+$ T Cells Are Effector Cells against *Pneumocystis* in Mice[1]

**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 some level of host defense 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-$\gamma$, 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.**

Despite current strategies to treat HIV infection and its complications, *Pneumocystis carinii* (PC)[3] pneumonia remains a common clinical problem. Although there is a clear relationship between CD4$^+$ lymphocyte count and the risk of PC infection (1, 2), the roles of mononuclear phagocytes, CD8$^+$ cells, NK cells, and their secreted cytokines in host defense against this infection are far less clear. Because it remains unclear whether highly active antiretroviral therapy will result in long-term immune reconstitution of patients with AIDS (3, 4), CD4$^+$ T cell-independent host defense mechanisms operative in opportunistic infections may be critical. Our laboratory and others have previously shown that delivery of IFN-$\gamma$ as an aerosol or by overexpression using adenoviral-mediated gene transfer using AdIFN (5) results in clearance of PC in the absence of CD4$^+$ T cells. The putative mechanism for clearance of PC with AdIFN involved CD8$^+$ T cells, because depletion of this T cell subset abrogated the effect of AdIFN on PC infection (5). Despite this protective role of CD8$^+$ T cells, CD8$^+$ T cells have also been associated with lung injury in severe PC pneumonia (6, 7). Based on these data, we hypothesized that AdIFN resulted in the pulmonary recruitment of T cytotoxic (Tc)1-like CD8$^+$ T cells, defined by high levels of endogenous IFN-$\gamma$ production (8), that are effector cells against PC, whereas non-Tc1 cells may contribute to lung injury in PC pneumonia. In this study, we show that AdIFN, as opposed to a control adenoviral vector, results in the recruitment of PC-specific Tc1 CD8$^+$ T cells, which have in vitro effector activity against PC in a macrophage coculture assay, and effect clearance upon adoptive transfer into PC-infected *scid* mice. Moreover, CD8$^+$ T cells without a Tc1 phenotype, from mice treated with a control adenovirus (Ad) vector, lacked any in vitro effector activity against PC and, upon adoptive transfer into PC-infected *scid* mice, resulted in significantly worse lung injury. Taken together, these data suggest that Tc1-like CD8$^+$ T cells are capable of effector activity, whereas non-Tc1 CD8$^+$ T cells may exacerbate lung injury in PC pneumonia.

## Materials and Methods

### *Ad vectors*

AdIFN is an E1-E3 replication-deficient rAd5-based vector containing and expressing the full-length murine IFN 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 $10^7$ PFU (as determined by a lack of cytopathic 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, C.B-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 $500 \times g$ for 10 min at 4°C. The pellet was resuspended in PBS, and a 1/4 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 $2 \times 10^6$ 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

*Department of Pediatrics, Children's Hospital of Pittsburgh, University of Pittsburgh, Pittsburgh, PA 15213; and †Gene Therapy Program, Louisiana State University Health Sciences Center, School of Medicine, New Orleans, LA 70112

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

[3] Abbreviations used in this paper: PC, *Pneumocystis carinii*; Tc, T cytotoxic; Ad, adenovirus; EGFP, enhanced green fluorescent protein; BAL, bronchoalveolar lavage; Mig, monokine induced by IFN-$\gamma$; IP-10, IFN-$\gamma$-inducible protein-10; LDH, lactate dehydrogenase; GMS, Gomori methenamine silver.



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Louisiana State University Health Sciences Center. The Ab was precipitated with an equal volume of saturated ammonium sulfate and dialyzed against PBS overnight, and the IgG content was quantified by cellulose acetate electrophoresis and densitometry. The Ab was stored at −80°C until use. All lots of Ab contained <0.01 ng/ml endotoxin as determined by the QCL-1000 *Limulus* lysate assay. Heat denaturation of the Ab ablates its CD4-depleting capacity as well as its ability to modify lung host defenses (13).

### *Adenoviral gene transfer and PC inoculation*

Male BALB/c mice (6–8 wk) were obtained from Charles River Laboratory (Wilmington, MA). The mice were housed in the Louisiana State University Health Sciences Center Animal Care facility and were cared for according to institutional standards. BALB/c mice received 0.3 mg of depleting anti-CD4 Ab (GK1.5; American Type Culture Collection) by i.p. injection and 24 h later were randomized to receive $10^9$ PFU of AdIFN or AdEGFP intratracheally. Three days after the vector administration, animals were challenged with $2 \times 10^5$ PC cysts intratracheally. Mice continued to receive rat IgG or GK1.5 weekly until sacrifice. We have previously shown that, in BALB/c mice, weekly GK1.5 maintains a continued state of >97% CD4 depletion in blood and lymphoid tissue for up to 14 wk (11). The vector and PC inoculations were done on mice that were anesthetized with ketamine/xylazine. Based on our prior studies of inflammatory cell influx into the lung in response to PC (5), mice were sacrificed at day 3, 14, or 28 after PC inoculation.

### *Bronchoalveolar lavage (BAL) and lung tissue RNA*

Cells from the lower respiratory tract were obtained by BAL of mice anesthetized with i.p. pentobarbital as previously described (5). Briefly, lungs were lavaged through an intratracheal catheter with prewarmed (37°C) calcium- and magnesium-free PBS supplemented with 0.6 mM EDTA. The first milliliter was processed at $500 \times g$, and the supernatant was stored at −80°C until use. The remaining cell pellet and the other 10 ml of lavage fluid were pooled and centrifuged at $800 \times g$ for 10 min, and the cells were collected for flow cytometry and $CD8^+$ T cell isolation. In another subgroup of animals, both lungs were tied off at the bronchial airway and then removed with sterile scissors. The right lungs were homogenized in 1 ml of TRIzol for total lung RNA isolation and stored at −80°C, and the left lungs were kept in RPMI 1640 medium (Invitrogen, Grand Island, NY) supplemented with 1% BSA (Sigma-Aldrich, St. Louis, MO) for subsequent enzyme digestion.

### *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 $NH_4Cl$, 0.84 g of $NaHCO_3$, 0.37 g of EDTA, 100 ml of $H_2O$ at pH 7.4 and 4°C) for 3 min. After centrifugation and double washing, cells were resuspended in medium and enumerated in hematocytometer (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 Biotec, 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). After 10 min of incubation at 4–8°C, anti-biotin microbeads (colloidal magnetic microbeads conjugated to a monoclonal anti-biotin Ab, mouse IgG1) and buffer (PBS supplemented with 0.5% BSA and 2 mM EDTA) were added. After an additional 15 min of incubation at the same temperature, cells were washed, resuspended in an adequate amount of buffer, applied onto columns, and allowed to pass through, and effluent was collected (unlabeled cells), representing the enriched $CD8^+$ T cell fraction. Cells were then exposed to CD8a (Ly-2) microbeads and incubated for 15 min at 6–12°C. After washing and resuspension in buffer, cells were applied onto columns, and negative cells were allowed to pass through. Columns were removed from the separator; an appropriate amount of buffer was added, and the positive fraction was flushed out. To determine purity of the separation, cells were stained with PE-conjugated anti-CD8a (BD PharMingen, San Diego, CA). The purity of double-selected cells was >95% by flow cytometry.

### *ELISPOT assay for IFN-γ-secreting $CD8^+$ T cells*

The ELISPOT assay for frequency of cells that secrete IFN-γ and IL-4 was applied to purified $CD8^+$ T lymphocytes from lymph nodes and lung tissue in a modification of the method described by Czerkinsky et al. (14) and as previously described by our group (15). In brief, selected wells in a 96-well cellulose membrane plate (Millipore, Bedford, MA) were coated with anti-IFN Ab (6 g/ml R4-6A2; BD PharMingen) or anti-IL-4 Ab (10 g/ml 1D11; Endogen, Woburn, MA) for 24 h at 4°C. The plates then were blocked by incubation with 5% BSA for 30 min at 37°C. The $CD8^+$ T lymphocytes were added to washed wells in 100 μl of culture medium (RPMI supplemented with 10% FBS, 50 M 2-ME, 0.1 mg/ml gentamicin, 100 U/ml penicillin, 100 g/ml streptomycin, and 2 mM L-glutamine). For lymph node $CD8^+$ cells, we added $10^5$ cells for the detection of IFN-γ and $0.5 \times 10^5$ cells for the detection of IL-4. For lung tissue $CD8^+$ cells, we added $0.5 \times 10^5$ cells for the detection of IFN-γ and $0.25 \times 10^5$ cells for the detection of IL-4. The cells were incubated on the Ab-coated plates at 37°C in 5% $CO_2$ in air for 22 h. Duplicate wells were cultured with (5 mg/L) and without added Con A (Sigma-Aldrich). The plates were washed three times with PBS and then three times with PBS/0.05% Tween to remove bound cells. Biotinylated detecting Ab then was added to appropriate wells at 4 g/ml biotinylated anti-IFN-γ (XMG1.2; BD PharMingen) and at 3 g/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 mg 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 three times with PBS-Tween. Substrate (3-amino-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 $10^5$ $CD8^+$ T lymphocytes.

### *IFN-γ secretion assay*

Spontaneous or stimulated (with Con A or Ag) cellular secretion 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% $CO_2$ 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, cells were incubated with optimal dilutions of FITC-conjugated anti-CD8a (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 FACSCalibur 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 Abs conjugated to identical fluorochromes. To ensure accurate enumeration of activated lymphocytes that may have altered forward- and side-scatter profiles, stained samples were back-gated against the forward- and side-scatter profiles. Data are expressed as the percentage of dual-positive cells ($IFN\text{-}\gamma^+CD8^+$).

### *IL-10 secretion assay*

Spontaneous or stimulated cellular secretion of IL-10 was analyzed with an IL-10 secretion assay kit (Miltenyi Biotec) per the manufacturer's instructions. Cells (isolated from digested lungs as described for the IFN-γ secretion assay) were cultured for 16 h at 37°C and 5% $CO_2$ in medium alone (spontaneous production), with Con A (0.5 μg/ml; stimulated production), or with DC pulsed with Ag (specific stimulated production). At the end of the assay, cells were incubated with optimal dilutions of FITC-conjugated anti-CD8 (clone 53-6.7; BD PharMingen) and PE-conjugated anti-IL-10 (Miltenyi Biotec) for 20 min at 4–8°C. Thereafter, 10,000 cells were analyzed with a FACSCalibur flow cytometer (BD Biosciences).

### *ELISA*

BAL fluid was assayed for IFN-γ, monokine induced by IFN-γ (Mig or CXCL9) and IFN-γ-inducible protein-10 (IP-10 or CXCL10), by ELISA using commercially available Abs (BD PharMingen; and R&D Systems, Minneapolis, MN). Standard curves were generated using the respective recombinant murine proteins. The assays were performed in high-protein-binding enzyme immunoassay/A2 96-well tissue culture plates (Costar; Corning, Corning, NY) as previously described (17). The absorbance values and concentrations of each cytokine were determined using a μQuant automated microplate reader (Bio-Tek, Winooski, VT) and Kineticalc software (Bio-Tek). Data were expressed as picograms of cytokine per milliliter.

### *In vitro effector activity of lung CD8$^+$ T cells*

Thioglycolate-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°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 rRNA 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 $\times$ $10^5$ PC cysts intratracheally. Mice were adoptively transferred 24 h later with $10^6$ 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 inmunologically 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 and PC infection score, respectively, as previously described (20). Briefly, tissue blocks were sectioned at 5-$\mu$m thickness and stained with GMS. The extent of infection with PC was scored using a semiquantitative scale ranging from 0 (no visible infection) to 4 (cysts throughout most alveolar regions). This method has been 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.

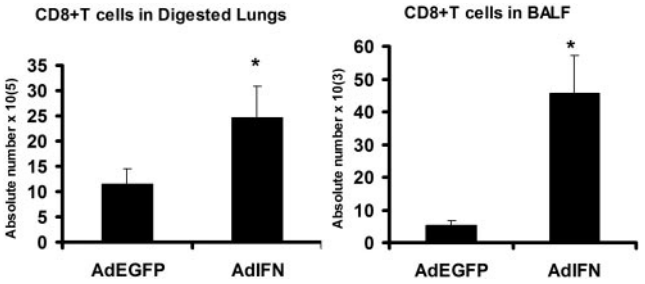


**FIGURE 1.** AdIFN augments the absolute numbers of CD8$^+$ T cells per animal in lung digest and BAL fluid ($n$ = 5–7; ∗, $p < 0.05$, ANOVA).

### *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

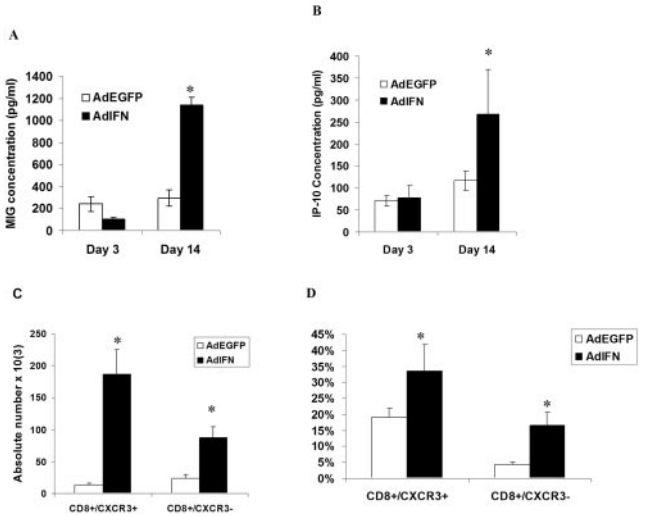


**FIGURE 2.** CXCR3 ligands and CXCR3 status of recruited CD8$^+$ T cells. *A* and *B*, Levels of Mig and IP-10, respectively, were measured in BAL fluid ($n$ = 4–6; ∗, $p < 0.05$ ANOVA). *C* and *D*, The absolute number of CXCR3$^+$CD8$^+$ T cells and the percentage (of the cells in the lymphocyte gate) were determined by dual-color FACS on lung BAL cells, respectively ($n$ = 4–6; ∗, $p < 0.05$, ANOVA).

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. 3*A*). 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 with $<5$ spots/$10^5$ cells in both AdIFN and AdEGFP treatment groups (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. 3*B*). 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. 3*B*, *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 thiogycolate-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. 5*A*), whereas those given Tc1 $CD8^+$ T cells had significantly lower organism burdens (Figs. 5*A* and 6). Interestingly, *scid* mice

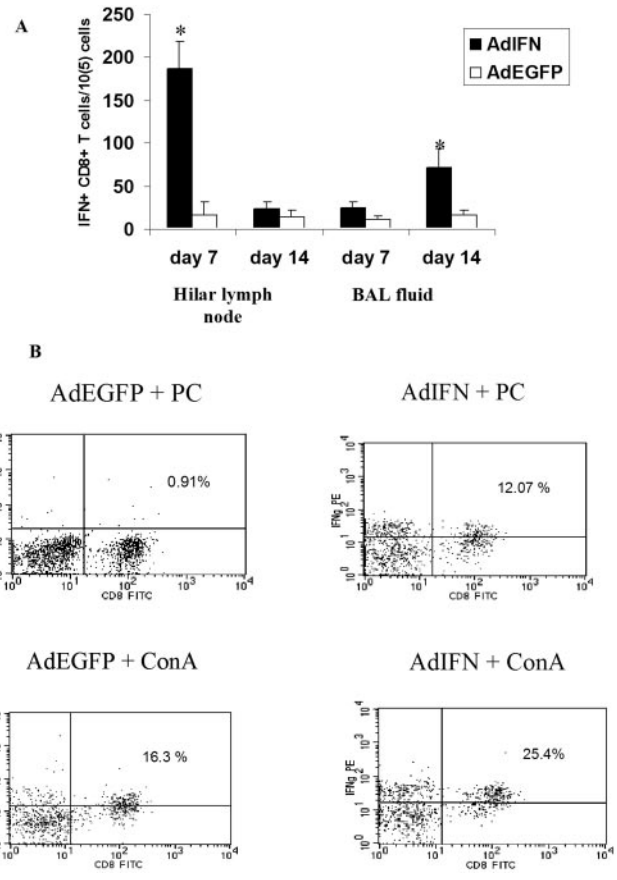


**FIGURE 3.** Recruitment of Tc1 $CD8^+$ T cells in PC by AdIFN. *A*, IFN-producing $CD8^+$ T cell precursor frequency in response to Con A stimulation. *B*, Representative FACS profile of Ag-specific Tc1 phenotyping of lung $CD8^+$ T cells day 14 after PC infection.

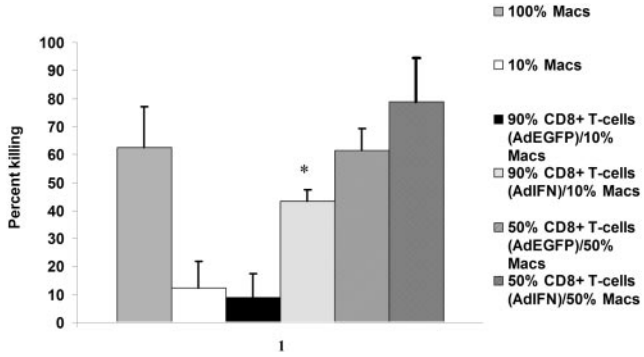


**FIGURE 4.** In vitro effector activity of lung $CD8^+$ T cells. $CD8^+$ T cells were purified from lung digest and cocultured with macrophages described in *Materials and Methods*. Percent killing was assayed by quantifying the integrity of the PC rRNA subunit by TaqMan compared with PC organisms cultured without macrophages ($n = 4–6$; $*$, $p < 0.05$, ANOVA).

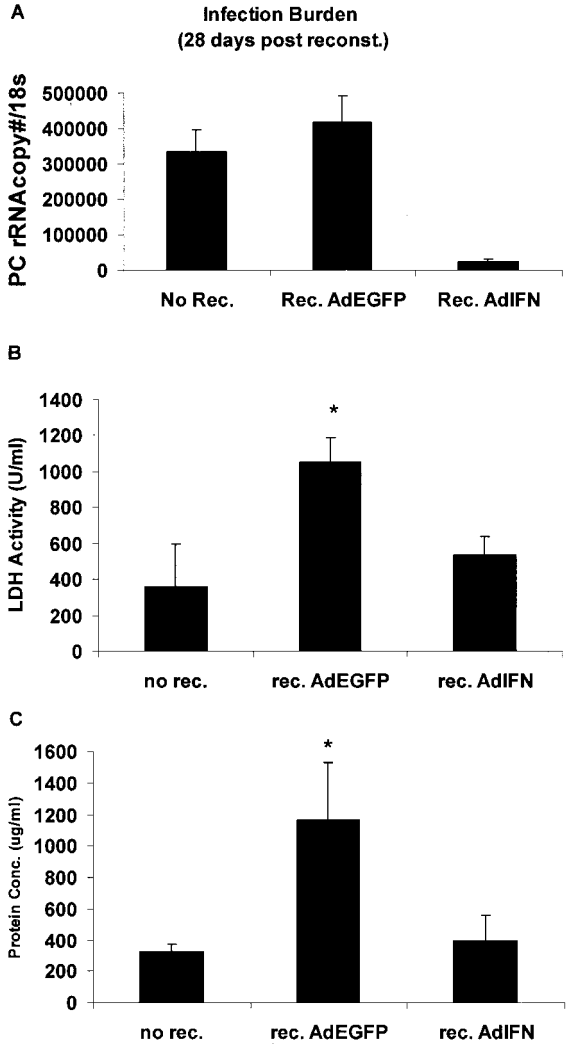


**FIGURE 5.** Adoptive transfer of $CD8^+$ T cells. A, Twenty-eight-day PC infection burden in 6- to 8-wk-old *scid* mice infected with PC and left unreconstituted or reconstituted with $10^6$ $CD8^+$ T cells from day 14 AdIFN or AdEGFP mice ($n = 3–5$; $*$, $p < 0.05$, ANOVA). *B*, LDH activity in BAL 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).

given non-Tc1 $CD8^+$ T cells from AdEGFP showed a trend to slightly higher PC organism load (Fig. 5*A*) 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, $\gamma\delta$ 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-*$\gamma$ 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 $CXCR3^+CD8^+$ 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

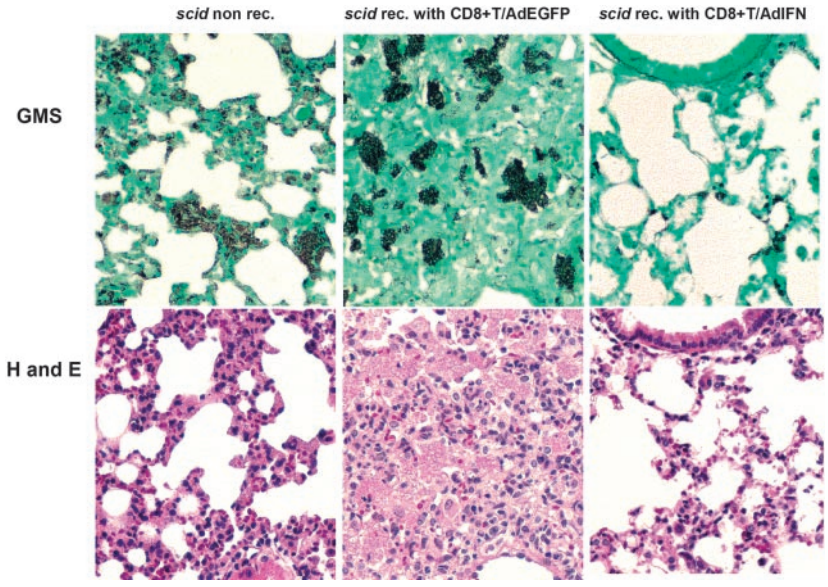


**FIGURE 6.** Representative histology in adoptively transferred *scid* mice at 28 days. *Upper panel*, Lung sections stained with GMS revealed less infection in *scid* mice adoptively transferred with Tc1 $CD8^+$ T cells. *Lower panel*, Lung sections stained with H&E revealed more alveolar exudates 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 do not believe that this is due to IFN secretion in and of itself, because rIFN at doses up to 100 U/ml in the macrophage coculture assay does not augment killing (our unpublished observations) and IFN knockout mice are capable of eradicating a pulmonary challenge with PC (31).

Interestingly, $CD8^+$ T cells from AdEGFP control mice exacerbated PC-mediated lung injury in *scid* mice. These data are consistent with Harmsen and colleagues (7, 32) that showed that $CD8^+$ T cell depletion can also attenuate lung injury in the CD4-depleted PC model. Moreover, a high level of rapidly proliferating $CD8^+$ T cells has been observed in BAL fluid of patients who developed PC-related pneumonitis after starting on highly active retroviral therapy (33). In our study, these cells had an anergic response to PC Ag in terms of IFN-$\gamma$, IL-4, or IL-10 production. We did not examine TGF-$\beta$ production in this study. Another possibility is that, in AdEGFP-treated mice, the PC-specific $CD8^+$ T cell response was below the limit of our detection. Also, the $CD8^+$ T cells used in this study were derived from mice transduced with adenoviral vectors, and this could influence the effector function observed in these studies. However, we have previously shown that CD4 depletion significantly attenuates the generation of adenoviral-specific $CD8^+$ T cells (34, 35), and thus, it is unlikely that the adenoviral transduction is influencing the $CD8^+$ T cell phenotype, and that the phenotype is more specific to the transgene encoded by the Ad. Despite this, it will be critical to further sort PC Ag-specific cells to further define the $CD8^+$ T cell effector activity.

Mechanisms by which $CD8^+$ T cells contribute to lung injury include disruption of surfactant homeostasis (6) and Fas ligand-mediated apoptosis (36) that can occur in an Ag-independent manner. Our data suggest that resolution of PC infection or the development of lung injury may be due to the phenotype of the $CD8^+$ T cells that are recruited to the lung. This is reminiscent of the data in respiratory syncytial virus vaccination where the recruitment of Fas ligand-expressing $CD8^+$ T cells (37–39) is associated with worse lung injury. Thus, in future studies, it will be critical to phenotype these cells further, in response to both Ag-specific as well as nonspecific stimuli, to gain insight into the mechanisms of $CD8^+$ T cell-mediated host defense vs lung injury.

Our data support the idea that Ag-specific Tc1 $CD8^+$ T lymphocytes are critical to clearance of infection in hosts with deficient numbers of $CD4^+$ T lymphocytes. In contrast, $CD8^+$ T lymphocytes without the Tc1 phenotype (Tc0 or Tc2) appear to mediate tissue damage. Because both Tc1 and non-Tc1 $CD8^+$ T lymphocytes respond to many of the same chemotactic signals to enter the lung, we suggest that the end result (pathogen clearance vs lung injury) of inflammatory influx into the alveolar spaces during *Pneumocystis* infection is critically dependent upon the relative proportions of these cell types available for recruitment into lung tissue. These data further support vaccination strategies against *Pneumocystis* that focus on increasing precursor frequency within circulating lymphocytes of Tc1 $CD8^+$ T lymphocytes and/or decreasing precursor frequency of non-Tc1 $CD8^+$ T lymphocytes.

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