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CD8 T Cell-Mediated Lung Damage in Response to the Extracellular Pathogen Pneumocystis Is Dependent on MHC Class I Expression by Radiation-Resistant Lung Cells

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Pneumocystis, a fungal, extracellular pathogen causes a life-threatening pneumonia in patients with severe immunodeficiencies. In the absence of CD4 T cells, Pneumocystis infection results in vigorous CD8 T cell influx into the alveolar and interstitial spaces of the lung. This response results in lung damage characterized by low pO2 and albumin leakage into the bronchoalveolar lavage fluid similar to other CD8 T cell-mediated interstitial lung diseases. How this extracellular pathogen elicits a CD8 T cell response is not clear, and it was the aim of our study to determine the Ag specificity of the recruited CD8 T cells and to determine whether MHC class I expression was necessary to initiate lung damage. Using an adoptive T cell-transfer model with either polyclonal wild-type CD8 T cells or transgenic influenza virus-specific CD8 T cells we found that CD8 T cell recruitment is Ag-specific and requires the continuous presence of the Pneumocystis pathogen. Bone marrow chimera experiments using Rag-1 and β2-microglobulin-deficient mice as hosts demonstrated a requirement for MHC I expression on nonbone marrow-derived cells of the lung. This suggests either direct processing of Pneumocystis Ags by nonbone marrow-derived cells of the lung or the induction of lung damage triggered by a lung-specific autoantigen. Using perforin-, Fas-, and IFN-γ-deficient animals, we showed that these molecules are not directly involved in the CD8-mediated lung damage. However, CD8 T cell-mediated lung damage is Ag-specific induced by a MHC I-expressing nonbone marrow-derived cell in the lung and is dependent on the continued presence of live Pneumocystis.

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Pneumocystis rarely causes disease in the general population, but it is a frequent cause of morbidity and mortality in persons who are immune compromised. Especially in patients with AIDS the risk of infection increases drastically with CD4 T cell counts below 200 cells/μl (1).

Although Pneumocystis is considered to be a strictly extracellular fungal pathogen (2, 3), CD4 T cell-deficient humans and mice infected with Pneumocystis species mount a vigorous CD8 T cell response, resulting in the influx of these cells into the lung tissue and alveolar spaces. This CD8 T cell influx results in increased albumin leakage into the alveolar spaces and decreased arterial pO2 levels, indicating that the integrity of the epithelial/endothelial cells of the lung has been compromised and that the lung no longer functions optimally (4).

We recently found that the increased CD8 T cell influx during the course of Pneumocystis pneumonia (PCP) in CD4-depleted mice is the result of a type I IFN-dependent signaling pathway. CD4 T cell-depleted IFNαR-deficient mice (IFNαR KO) show decreased CD8 T cell influx into the lung in response to infection with Pneumocystis and improved clinical course of disease as measured by weight loss and arterial pO2 levels. Furthermore, the animals had reduced lung damage as measured by albumin leakage and reduced secretion of proinflammatory cytokines into the bronchoalveolar lavage fluid (BALF) when compared with wild-type animals (5).

Pneumocystis is an extracellular pathogen; therefore a vigorous CD8 T cell influx into the lung in response to this infection is surprising. Mechanisms that lead to CD8 T cell recruitment, their Ag specificity as well as subsequent effector mechanisms involved resulting in lung damage are not yet clear.

Most APCs present peptides from cytosolic proteins in the context of MHC class I (MHC I) to CD8 T cells and the classical CD8 T cell effector function is to kill the target cells presenting specific foreign Ags in association with MHC I molecules (6). In this regard, CD8 T cells play a major role in the clearance of virus-infected cells as well as tumor cells via either granule-mediated killing (perforin/granzyme pathway) or the engagement of the Fas/Fas ligand pathway (7). Furthermore, CD8 T cells produce cytokines such as TNF-α and IFN-γ that function to recruit and activate other effector cells including macrophages and neutrophils (7, 8). Because of their presence in inflammatory lesions, CD8 T cells have been implicated in autoimmune phenomena including type-1 diabetes and rheumatoid arthritis, where they are thought to contribute significantly to tissue damage (9, 10). However, their activation mechanisms and the Ag they recognize in these contexts are not clear.

PCP, particularly in CD4-depleted animals is described as an interstitial pneumonia and presents with CD8 T cell accumulations as similarly observed in some interstitial pneumonias associated with manifestations of idiopathic and rheumatic diseases of the lung (4, 11–15). However, little is known about the mechanisms by which CD8 T cells cause damage in CD4-depleted animals infected with Pneumocystis. Therefore to better understand the

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mechanisms of this damage, we used a CD4-depleted mouse model to determine the Ag specificity, dependence on Ag persistence of the CD8-mediated lung damage and to determine the dependence of this damage on MHC I expression and on cytotoxic T cell effectors molecules. Our results indicate that in the CD4 T cell-depleted model of PCP, CD8 T cell-mediated lung damage is Ag-specific and is driven by the presence of *Pneumocystis* pathogen. Furthermore, the expression of MHC I on nonbone marrow-derived cells in the lung tissue is required. Perforin, Fas, or IFN-γ alone were not involved as effectors of lung damage, suggesting either a concerted action of multiple effector molecules or novel mechanisms yet to be elucidated.

**Materials and Methods**

**Mice**

C.B17 SCID mice were used as *Pneumocystis* source mice as well as recipient animals in some CD8 T cell transfer experiments and were bred and maintained at Montana State University Animal Resource Facility. Transgenic mice expressing the Vβ8.2/Vα10 chains in the TCR of CD8 T cells specific for the influenza virus HA2 transmembrane peptide at residues 518–528 (Y1STVASSLL) are in a BALB/c background and were provided by Tradex International (Saranac Lake, NY) and maintained in the Montana State University Animal Resource Facility (16).

Homozygous mice with targeted mutations for β2m-microglobulin (β2m)−/− (B6.129P2-Tnfrsf6 tm1Osa/J stock: 001178), CD1d1/d2 (C57BL/6J stock: 000531), IFN-γ (C57BL/6J stock: 000472), perforin (B6.129P2-Tnfrsf6 tm1Osa/J stock: 000531), Fas (C57BL/6J stock: 000472), CD8α (CD8αtm1Mak stock: 002665), CD1d1/d2 (C57BL/6J stock: 003814), Rag1 (B6.129S7-Rag1tm1Mom/J stock: 002216), perforin (C57BL/6J stock: 000531), and CD45.1 congeneric C57BL/6 mice (B6.SJL-Ptprc Pep 3/Boy/J stock: 002014) were purchased from Jackson ImmunoResearch Laboratories. Wild-type animals (C57BL/6J stock: 000651 and C57BL/6J stock: 000664) were obtained from The Jackson Laboratory and were used as donors for CD8 T cell reconstitution experiments into *Pneumocystis*-infected SCID mice, and wild-type control animals. All animals were housed at the Montana State University Animal Resource Facility under germfree conditions in ventilator cages with sterile food and water. In each experiment except for the radiation bone marrow chimeras, animals were sex matched and injected at 6–8 wk of age.

To eradicate *Pneumocystis* in some CD4-depleted animals, mice were fed trimethoprim/sulfamethoxazole (TMP/SMX) chocolate-flavored food pellets (Bio-Serv) 7 or 14 days postinfection till day 28 postinfection when pathogen lung burden was assessed. Animal experiments were approved by the Institution Animal Care and Use Committee and strictly followed the animal care and handling procedures as required by the Animal Welfare Act as well as National Institutes of Health and U.S. Department of Agriculture guidelines.

**CD4 T cell depletion**

For in vivo CD4 cell depletion, animals were injected with 300 μg of purified GK1.5 rat α-mouse CD4 Ab i.p 2–4 days before infection with *Pneumocystis* and subsequently repeated twice per week until the endpoint of the experiment (17). The GK1.5 hybridoma cell line was obtained from the American Type Culture Collection, with cells grown according to the instructions and Abs produced in ascites.

**Infection of mice with *Pneumocystis***

Lung homogenates from *Pneumocystis murina* species infected C.B17 SCID mice were used as a source and either SCID mice or CD4-depleted recipient animals were infected with 10^5 *Pneumocystis* nuclei in a 100 μl volume previously described via intratracheal instillation under deep isoflurane gas anesthesia (18). Because the *Pneumocystis* pathogen is species specific, it will be referred to as *Pneumocystis* throughout the article. Previously published experiments repeatedly showed that CD4-depleted animals cleared the infection with complete restitution of lung function and histology within 28 days postinfection and that injection of an irrelevant isotype Ab as controls did not have an effect on lymphocyte cell populations and lung pathology (4, 5). Thus, experimental control animal groups in this study consisted of uninfected wild-type animals.

**CD8 T cell isolation from spleens of donor animals for adoptive transfer experiments**

To increase yield and purity of CD8 T cell isolation, donor animals were in vivo CD4 T cell-depleted 2 days before they were used as donors and then euthanized by CO2 narcosis. Spleens were removed and transferred into ice-cold Hank’s salt solution. Single-cell suspensions were obtained by passing spleens through a steel screen and were then pelleted by centrifugation. RBCs were lysed for 5 min using ACK lysis buffer (0.15 M NH4Cl, 1 mM KHCO3, and 0.1 mM Na2 EDTA) (5 ml/spleen), and cells were counted using a hemocytometer. CD8<sup>+</sup> T cells for the adoptive transfer were isolated using a Murine T cells CD8 Subset Column kit (R&D Systems) designed to prepare mouse CD8 T cells via high-affinity negative selection with cells prepared according to the manufacturer’s protocol. The purity of isolated CD8 T cells was generally between 85 and 90% with no contaminating CD4 T cells. Cells were resuspended in Hank’s buffer at a concentration of 1.25 × 10^6 cells/ml and 5 × 10^6 cells were then injected by i.v. injection into the tail vein. In all adoptive transfer experiments, recipient mice were *Pneumocystis* infected 2 days before adoptive transfer of CD8 T cells.

**Bone-marrow chimera generation between RAG KO and β<sub>m</sub> KO mice: irradiation, reconstitution, screening and CD8 T cell transfer**

To obtain mice that lacked MHC I expression specifically on nonbone marrow-derived cells, we generated radiation bone marrow chimeras using the method previously described (19). Briefly, recipient β<sub>m</sub> KO and Rag-1 KO mice were lethally irradiated with 9.5 Gy from a 140Cs source in a split dose and 10<sup>5</sup> bone marrow cells from Rag-1 KO donor animals were then injected. After 8 wk of engraftment, the peripheral blood of the chimeras was screened by FACS to determine whether any remaining T or B cells were present in the chimeric animals. As expected, no T or B cells were found in the blood of either group of mice. At 9 wk after bone marrow-engraftment, the chimeric mice were infected with *Pneumocystis* species as described above. Two days later, 5 × 10<sup>6</sup> naive wild-type congenic CD45.1<sup>+</sup> CD8 T cells were adoptively transferred into the chimeric mice and lung function and lung damage were examined 26 days later.

**Body weight and lung function assessment**

Body weights of all animals were recorded before *Pneumocystis* infection and on day 28 of infection. Respiratory rates were assessed on day 28 of infection and compared with the uninfected control group, using a whole body plethysmograph. Mice were confined within the chamber so that respiration per minute could be calculated from steady strip chart recordings. For blood gas analysis arterial blood was obtained from the tail artery of each animal and analyzed at Deaconess Hospital on an AVL Omni3 Blood gas autoanalyzer (AVL Medical Instruments) for pO<sub>2</sub>, pCO<sub>2</sub>, and pH (4).

**Assessment of cellular infiltrates into the lungs using bronchoalveolar lavage**

Bronchoalveolar lavages were performed as previously described (18). Briefly, mice were given a lethal i.p. injection of 90 mg/kg sodium pentobarbital solution and exsanguinated. Lung lavages were performed by intratracheal cannulation with a total volume of 5 ml HBSS + 0.5%EDTA. A 100-μl BALF cell aliquot was spun onto glass slides and stained with Diff-Quick<sup>®</sup> (Dade-Behring) for leukocyte differentiation. Cell counts of BALF samples were performed using a hemocytometer and 10<sup>3</sup> cells per sample used to assess lymphocyte subset distribution via flow cytometry using Abs to CD4 and 10<sup>6</sup> cells per sample used to assess neutrophil subset distribution. Cells were acquired using an LSR II and analyzed by FACSAria (BD Biosciences). Before staining, cells were incubated with unlabeled anti-mouse FcR Abs to block FcR-mediated nonspecific staining and then incubated with an appropriately diluted mixture of the above Abs for 30 min. Cells were washed three times with PBS with 2%FCS and analyzed on a FACSCalibur (BD Biosciences).

**Enumeration of *Pneumocystis* nuclei**

*Pneumocystis* burden was assessed by enumeration of its nuclei as previously described (17). Briefly, lungs of exsanguinated animals were removed and the right lobe was transferred into 5 ml of HBSS (Invitrogen Life Technologies) and homogenized through mesh screens. A 100-μl aliquot of a 1/20 diluted homogenate was spun onto a glass slide using a Shandon Cytospin 3 centrifuge, smears were stained with Diff-Quik (Dade-Behring), and the number of *Pneumocystis* nuclei in 10–50 oil immersion fields was counted. The limit of detection for this technique is log<sub>10</sub> 4.43 for right lobe homogenates.

**Albumin quantification in BALF**

Albumin concentrations in BALF were determined as an indicator for inflammatory exudates and as a measure of lung damage. Albumin reagent (baccillus Calmette-Guérin) from Sigma-Aldrich and an albumin standard
solution was used for this quantitative colorimetric test. Briefly, standards were prepared as 1-fold serial dilutions in HBSS/3 mM EDTA ranging from 0.1 to 2 mg/ml. One hundred microliters of the standard or sample was added to individual wells in a 96-well plate and mixed with 50 µl of albumin reagent (bacillus Calmette-Guerin). Absorbance (OD) of the reaction was determined using a Thermo max microplate reader (Molecular Devices) at 628 nm, and the amount of albumin present in the samples was determined using Softmax software.

**Cytokine analysis using bead arrays in BALFs and tissue culture supernatants**

TNF-α, IFN-γ, IL-5, IL-4, IL-2, and cytokine secretion into the BALF was assessed using the Mouse Th1-Th2 and Mouse Inflammation Cytometric Bead Array assay kits (BD Biosciences) according to the manufacturer’s instructions. Assays were read on a BD FACScan flow cytometer and data analyzed using the BD Cytometric Bead Array Software.

**Statistical analysis**

Data from the two experimentally relevant chimera groups were analyzed using an unpaired, two-tailed t-test. A one-way ANOVA was performed on data when more than two groups were involved. When a significant F-statistic was detected ($p < 0.05$) then means were compared using the post hoc Tukey test.

**Results**

IFN-γ, perforin, and Fas-dependent mechanisms alone do not appear to be responsible for the occurring lung damage but reveal regulatory functions

We have previously shown that the immune response to *Pneumocystis* is dominated by CD8 T cells in animals that lack CD4 T cells (4). In fact, 28 days after infection of CD4 T cell-depleted mice with *Pneumocystis*, CD8 T cells account for 70–80% of the lymphocytes recruited into the lungs (Fig. 1A). Furthermore, CD8 T cell recruitment into the BALF is strongly associated with deterioration of lung function as measured by decreased arterial pO2 levels (Fig. 1B) and resulting lung damage as indicated by albumin leakage into the BALF (Fig. 1C). Both of these pathologic changes are commonly observed in CD4 T cell-deficient patients infected with *Pneumocystis* (20).

A major functional attribute of CD8 T cells is their ability to specifically kill target cells via recognition of Ag in the context of MHC I-peptide complexes (6, 7). This cytotoxic response is mediated by two distinct molecular pathways: the granule exocytosis pathway, which is dependent on expression of the pore-forming molecule perforin by the cytotoxic T cell, and the Fas/Fas ligand (CD95/CD95L) pathway which is controlled by the up-regulation of CD95L on the effector T cell (7). To determine whether the CD8 effector T cells recruited to the lungs of CD4-depleted, *Pneumocystis*-infected mice induce lung damage via perforin or CD95/CD95L-dependent pathways, we CD4-depleted perforin KO and Fas KO mice and then infected these mice with *Pneumocystis*. We then determined the extent of lung damage induced by the CD8 T cells. As shown in Fig. 2, neither perforin nor Fas-mediated mechanisms alone appear to be responsible for the induction of CD8 T cell-mediated lung damage as the course of disease was not improved in either group of mice when compared with CD4-depleted wild-type animals. In fact, CD4-depleted perforin KO animals had significantly lower arterial pO2 levels (Fig. 2A) and slightly increased albumin levels (Fig. 2B) when compared with CD4-depleted wild-type animals. Furthermore, CD8 T cell influx (Fig. 2C) and absolute CD8 T cell numbers (data not shown) were not significantly different between the three groups. Thus, these data argue that CD8 effector T cells mediate lung damage in the CD4 T cell-depleted PCP model independently of either perforin or CD95/CD95L.

IFN-γ secretion by Ag-specific CD8 T cells can initiate the recruitment and cytotoxic activity of other cells such as macrophages and neutrophils and can also directly cause tissue damage (7, 21). In previous experiments it was demonstrated that IFN-γ was consistently elevated during the course of *Pneumocystis* infection (5, 22, 23). Therefore, we hypothesized that IFN-γ produced by the CD8 T cells recruited to the lungs of *Pneumocystis*-infected, CD4-depleted hosts may be responsible for the lung damage seen in these animals. However, on comparison of *Pneumocystis*-infected, CD4 T cell-depleted wild-type animals to CD4 T cell-depleted IFN-γ KO animals, we saw no improvement in lung function (pO2 levels, Fig. 2D) in the CD4-depleted IFN-γ KO mice and lung damage (albumin levels, Fig. 2E) was equivalent between the CD4-depleted IFN-γ KO and wild-type animals. In fact, arterial pO2 was significantly lower in the CD4-depleted IFN-γ KO mice (Fig. 2D) when compared with CD4-depleted wild-type animals while albumin concentrations in BALF fluid were not different (Fig. 2E) and the percentage of CD8 T cell present in the BALF was similar to wild-type animals (Fig. 2F). However, the absolute number of CD8 T cells was significantly higher in BALF of the CD4-depleted IFN-γ KO animals with an increase in the number of CD44highCD43low expressing CD8 T cells (data not shown), possibly causing the increased impairment of gas exchange and explaining the decreased pO2 levels. Regardless, the data showed that CD8 T cells do not mediate lung damage in the CD4-depleted PCP model via an IFN-γ-dependent pathway.

**CD8 T cell recruitment into the lung in response to Pneumocystis infection is Ag specific and is responsible for lung damage**

To assess whether CD8 T cells recruited to the lung during *Pneumocystis* infection are Ag specific or are recruited as a result of a

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**FIGURE 1.** During the course of *Pneumocystis* pneumonia in CD4-depleted mice, CD8 T cell influx is associated with increased albumin leakage into the BALF and decreased lung function. The percentage of CD8 T cells present in the BALF 28 days after *Pneumocystis* infection was determined by FACS analysis (A). Arterial pO2 levels were assessed as a measure of lung function (B) and albumin concentrations in BALF were determined as a measure of lung damage (C). Significant differences between the groups are indicated by *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. 

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random bystander effect in response to a vigorous ongoing inflammatory response, BALB/c SCID mice were infected with *Pneumocystis* and 2 days later either reconstituted with $5 \times 10^6$ polyclonal CD8 T cells isolated from spleens of naive wild-type animals or with CD8 T cells specific for the influenza virus HA2518–528 peptide isolated from naive HA TCR transgenic mice (16). The course of disease in these CD8 T cell reconstituted groups was compared with unreconstituted, infected SCID mice. At day 28 postinfection arterial blood gases and albumin levels were measured and FACS analysis of BALF was performed as described above.

As expected, no CD4 T cells were detected in BALF of any the reconstituted SCID mice (data not shown). Body weights and respiratory rates did not differ significantly between the three infected groups, but were decreased when compared with the uninfected group (data not shown). However, while all three infected animal groups had reduced pO2 levels when compared with the uninfected control group, SCID mice reconstituted with wild-type CD8 T cells had significantly lower pO2 levels (Fig. 3A) when compared with both unreconstituted but infected SCID mice and HA-specific CD8 T cell reconstituted and infected SCID mice. Albumin concentrations in the BALF (Fig. 3B) were significantly increased in SCID mice reconstituted with wild-type CD8 T cells, yet no differences could be detected between uninfected SCID mice, unreconstituted but *Pneumocystis*-infected SCIDs, and infected SCID mice reconstituted with HA-specific CD8 T cells (Fig. 3B). *Pneumocystis* lung burden was equal in animals receiving HA-specific CD8 T cells compared with unreconstituted SCID mice, and it was slightly higher in animals receiving wild-type CD8 T cells (Fig. 3C). However, in our experience, increased PC burden on its own does not lead to increased albumin leakage.

Interestingly, leukocyte accumulation in the BALF revealed a 10 times higher influx of lymphocytes into the lungs of wild-type CD8 T cell-reconstituted animals, as compared with both unreconstituted SCID mice and HA-specific CD8 T cell reconstituted animals (data not shown). In agreement with this result, FACS analysis revealed a ten times higher influx of CD8 T cells into the BALF in animals that received polyclonal wild-type CD8 T cells, as opposed to those mice receiving influenza HA-specific CD8 T cells (Fig. 4A). Furthermore, the CD8 T cells present in the BALF...
CD8 T cell-mediated lung damage is not dependent on expression of the nonclassical class I molecule, CD1d

β2m is not only utilized in classical MHC I molecules, but is also a subunit of the nonpolymorphic MHC I-like molecule CD1d (CD1d1 and CD1d2). CD1d1 presents glycolipids to a subset of T cells termed NKT cells and is required for their development (26). When NKT cells are activated by CD1d, they secrete perforin and IFN-γ and are associated with the activation of CD8⁺ T cells (27). To rule out the involvement of CD1d and NKT cells in CD8-mediated damage in response to Pneumocystis infection, we infected CD4 T cell-depleted CD1d (CD1d1 and CD1d2) KO mice with Pneumocystis and followed their course of disease in comparison to CD4-depleted wild-type animals. Animals were sacrificed at day 28 postinfection, and data were collected as described.

FIGURE 4. Increased lung damage in Pneumocystis-infected SCID mice that were wild-type CD8 T cell transferred is associated with increased CD8 T cell influx displaying an effector-cell phenotype. Total CD8 T cell numbers as well as numbers of CD4⁺CD8⁻ CD8 effector-like T cells were assessed by differential cell count of cells in BALF and subsequent FACS analysis of lymphocyte subsets and are shown in A. The cytokine secretion profile in BALF is shown in B and was assessed using a Th1/Th2 cytokine bead array assay. Significant differences between the groups are indicated by *, p < 0.05; **, p < 0.01; and ***, p < 0.001.

CD8 T cell recruitment and lung damage are dependent on the expression of β₂m

As recruitment of CD8 T cells to the lungs of CD4 depleted Pneumocystis-infected mice appears to be dependent on Ag-specific CD8 T cells, we wanted to determine whether CD8 T cell-mediated lung damage is dependent on MHC I. β₂m KO mice express little if any MHC I and are severely CD8 T cell deficient, although some CD8 T cells may be detectable in peripheral tissues (24). In contrast, CD8α-chain KO mice are deficient in functional cytotoxic CD8 T cells, despite MHC I expression on all tissue cells (25). To assess the role of MHC I, we performed a CD8 T cell adoptive transfer experiment into Pneumocystis-infected, CD4 T cell-depleted β₂m KO mice as well as Pneumocystis-infected, CD4 T cell-depleted CD8α-chain KO mice. The course of disease progression in both groups was followed to day 28 postinfection. Pneumocystis lung burden in both animal groups at day 28 post-infection was similar (Fig. 5A). pO₂ levels were slightly lower in CD4-depleted CD8α KO mice receiving wild-type CD8 T cells as compared with CD4-depleted β₂m KO mice that received wild-type CD8 T cells (Fig. 5B). Moreover, albumin leakage was significantly higher in the CD8α KO mice that received CD8 T cells as compared with β₂m KO mice that received CD8 T cells (Fig. 5C). Again, the increased albumin leakage in the CD4-depleted CD8α-KO mice that received wild-type CD8 T cells was associated with significantly higher CD8 T cell influx into the BALF (Fig. 5D). Together these data indicated that the lung damage observed in PC infected CD4-depleted animals is dependent on CD8 T cells and suggested that MHC I-expressing cells are also necessary.
above. No differences were found between CD4-depleted CD1d KO animals and CD4-depleted wild-type animals infected with *Pneumocystis*, and on day 28 postinfection albumin leakage into the BALF was measured (A), *Pneumocystis* burden in the lung was determined (B), and the percentage (C) and total number (D) of CD8 T cells present in the BALF were determined.

**FIGURE 6.** CD1d and NKT cells are not required for CD8 T cell-mediated lung damage during the course of PCP. CD4-depleted CD1d KO and wild-type mice were infected with *Pneumocystis*, and on day 28 postinfection albumin leakage into the BALF was measured (A), *Pneumocystis* burden in the lung was determined (B), the percentage (C) and total number (D) of CD8 T cells present in the BALF were determined.

CD8 T cell recruitment and lung damage in response to *Pneumocystis* infection is dependent on MHC I expression by nonbone marrow-derived cells in the lung

The presented data indicate that CD8 T cell-mediated lung damage during *Pneumocystis* infection is dependent on MHC I expression on bone marrow-derived professional APCs like DCs or on nonbone marrow derived cells present in the lung (i.e., lung epithelial or endothelial cells). We therefore transferred bone marrow from Rag-1 KO donors (all cells express MHC I) into lethally irradiated Rag-1 KO mice (MHC I expression by all cell types in chimeric mice; referred to as stroma/BM and β2m KO mice (MHC I expression only on bone marrow derived cells in chimeric mice; referred to as stroma/β2m/BM). Eight weeks postengraftment, the chimeric mice were infected with *Pneumocystis*. CD8 T cells isolated from wild-type donor mice were adoptively transferred 1 day later into half of the infected chimeric animals. Animals were sacrificed at day 28 postinfection and assessed for disease progression and evidence of lung damage.

**FIGURE 7.** CD8 T cell recruitment and lung damage in response to *Pneumocystis* infection is dependent on MHC I expression on nonbone marrow-derived lung cells. Radiation bone marrow chimeric mice that were either deficient in MHC I expression on all nonbone marrow-derived stromal cells but were able to express MHC I on bone marrow-derived cells (stroma/BM into Rag into β2m) or were able to express MHC I on both bone marrow-derived as well as nonbone marrow-derived cells (stroma/BM into Rag into Rag) were generated as described in Materials and Methods. Both groups of mice were then infected with *Pneumocystis*. Two days later, half of the mice were injected with 5 × 10^6 wild-type CD8 T cells (A–D) or sham treated (E–H), and the course of disease progression followed until day 28 postinfection when the extent of lung damage was assessed. Albumin leakage was significantly higher (A) and pO2 levels significantly lower (B) and CD8 T cell influx significantly higher (D) in CD8 T cell reconstituted animals that were able to express MHC I on nonbone marrow-derived lung tissue compared with animals negative for MHC I expression on nonbone marrow-derived cells. The extent of lung damage was minimal and similar in both groups when mice were not CD8 T cell reconstituted (E–H). Significant differences between the groups are indicated by *, p < 0.05; **, p < 0.01; and ***, p < 0.001.
animals that received wild-type CD8 T cells but expressed MHC I on bone marrow-derived APCs but not on lung stromal cells (stroma\textsuperscript{e}BM\textsuperscript{+}). These findings were associated with significantly higher (approximately four times higher) CD8 T cell influx into the BALF of stroma\textsuperscript{+}BM\textsuperscript{+} animals when compared with stroma\textsuperscript{e}BM\textsuperscript{+} mice (Fig. 7D), even though Pneumocystis lung burden was not distinctly different between the infected groups (Fig. 7C). Furthermore, no difference between the two groups with regard to disease progression and signs of lung damage were found when CD8 T cells were not adoptively transferred to the chimera to disease progression and signs of lung damage were found when CD8 T cells were not adoptively transferred to the chimera (Fig. 7, E–H). Moreover, albumin levels in BALF as well as arterial blood gases were not different between the groups that did not receive CD8 T cells and the stroma\textsuperscript{e}BM\textsuperscript{+} mice that did receive CD8 T cells (compare Fig. 7, A and E). This confirms that lung damage during the course of Pneumocystis pneumonia is CD8 T cell-mediated, Ag-specific and is dependent on expression of MHC I by a radiation-resistant, stromal-like cell in the lung.

The presence of Pneumocystis Ag is required for the maintenance of CD8 T cell influx into the BALF and the resulting lung tissue damage. CD4 T cell-depleted wild-type animals were Pneumocystis-infected, and cohorts of the animals were started on a Pneumocystis-eradicating antibiotic treatment regimen with TMP/SMX at day 7 or 14 postinfection and continued until day 28. At day 28, Pneumocystis burden (A), albumin leakage (B), and CD8 T cell influx (C) was assessed. Significant albumin leakage was only detected in the untreated animal group and was associated with high CD8 T cell influx. Significant differences between the groups are indicated by *, p < 0.05; **, p < 0.01; and ***, p < 0.001.

Discussion

CD8 T cells recruited to the lung during the course of Pneumocystis pneumonia in the CD4 T cell-deficient host show a classical effector phenotype with up-regulation of CD44/CD43 as well as the secretion of IFN-\gamma and TNF-\alpha and other proinflammatory cytokines (28, 29). Classical CD8 T cell-effector functions are typically induced in response to signals delivered via the interaction of Ag-loaded MHC I molecules on the APC and the TCR on the T cells. This interaction results in the formation of immunological synapses (6) and direct damage to the APC via perforin and granzyme release by the effector CD8 T cells or by Fas/Fas ligand interactions between the effector T cell and its target (7). Indeed, these mechanisms are involved in the destruction of the endothelial cell layer in an animal model of vascular leak syndrome (30). Though albumin leakage in CD4-depleted Pneumocystis-infected mice appears to be the result of direct damage to the vascular/epithelial integrity by CD8 T cells, our studies using either CD4-depleted perforin KO mice or CD4-depleted Fas KO mice did not reveal any improvement in the course of disease in these mice when compared with CD4-depleted wild-type animals. These results are consistent with previous findings by Liu et al. (31) showing that CD8 T cell-mediated damage to alveolar epithelial cells in an in vitro model is also perforin and FAS independent; however, they could demonstrate mediation of lung damage through TNF-\alpha. TNF-\alpha can be directly cytotoxic via signaling through its specific receptors, and recently, Wright et al. (32) showed an improved course of disease and less lung damage during Pneumocystis pneumonia in TNFRII-deficient animals compared with wild-type mice. However, these authors found that the influx of total CD8 T cells into the lung of TNFRII KO animals was significantly reduced compared with wild-type animals indicating that either CD8 T cells mediate damage via TNF-\alpha secretion or CD8 T cell recruitment is supported by TNF-\alpha secretion of other cells and signaling through its receptors. IFN-\gamma secretion, as seen in Pneumocystis pneumonia, is a hallmark of Ag-specific CD8 T cell activation. IFN-\gamma can induce the up-regulation of MHC I, MHC II, and costimulatory molecules on APCs to promote cell-mediated immunity. Furthermore, IFN-\gamma activates and increases the antimicrobial and tumoricidal activity of monocytes, macrophages, neutrophils, and NK cells (33). With these pleiotropic effects, IFN-\gamma is another potential candidate in the mediation of lung damage during the course of PCP. However, using CD4-depleted IFN-\gamma KO mice in our animal model of PCP, we found no improvement in the course of disease when compared with CD4-depleted wild-type animals. In fact, in infected IFN-\gamma KO animals the total cell numbers in BALF were significantly higher and this influx correlated
with even more impaired gas exchange. These data indicate that neither perforin nor IFN-γ are responsible for the CD8-mediated lung damage; however, these molecules may play a regulatory role during the course of CD8-mediated lung damage in the CD4-depleted Pneumocystis infection model. In this regard, both, IFN-γ as well as perforin have been shown recently to be critical in the maintenance of CD8 T cell homeostasis and induction of apoptosis of the effector cell pool to restore “normal” CD8 T cell numbers after Ag specific stimulation (34, 35). Furthermore, in an animal model for Listeria monocytogenes infection, the absence of either perforin or IFN-γ leads to an enhanced CD8 T cell-mediated immune response and further supports their roles as modulators of a CD8 T cell-mediated immune response (36).

Using adoptive transfer of CD8 T cells into Pneumocystis-infected SCID mice, we determined that CD8 T cell recruitment into the lung, as well as the resulting lung damage was not a random bystander effect, but rather Ag-specific. Only those animals that received polyclonal wild-type CD8 T cells showed significant CD8 T cell recruitment, signs of damage to the epithelial/endothelial integrity, and significantly lower arterial PO2 levels when compared with animals that received CD8 T cells specific to an irrelevant influenza virus protein. Since Ag-specific activation of CD8 T cells generally requires MHC I molecules, we transferred wild-type CD8 T cells into MHC I-deficient β2m KO recipient mice and compared lung damage to CD8 KO-recipient mice that also received CD8 T cells. These experiments showed that CD8 T cell recruitment and the induction of lung damage require the presence of β2m molecules and most likely the formation of classical MHC I molecules as additional experiments with mice deficient in expression of the non-classical MHC I molecule CD1d did not show any differences in the clinical course of disease progression and induction of lung damage.

These results still did not address which cells in the lung interact with Pneumocystis-specific CD8 T cells to cause damage in the lung during PCP. Therefore, using bone marrow chimera mice, we assessed whether CD8 T cell Ag recognition occurs in the context of MHC I expression on nonbone marrow–derived stromal lung tissue cells, as opposed to bone marrow–derived APCs. Data from these experiments confirmed that those changes in lung tissue permeability for albumin as well as the exacerbation of lost lung function resulting in decreased PO2 levels during PCP are due to CD8 T cell influx. Most importantly, these results indicated that both maximum recruitment of CD8 T cells as well as the induction of lung damage is dependent on MHC I expression on radiation resistant cells in the lung and suggest that the Ag is presented by either lung epithelial or endothelial cells and that MHC I expression on bone marrow–derived cells is not sufficient to induce lung damage.

As Pneumocystis is not an intracellular pathogen, these findings could suggest that the CD8-T cell induced lung damage is in response to presentation of a self-Ag by the lung cells possibly induced in response to the presence of Pneumocystis. In this regard it has been suggested that CD8 T cell-mediated autoimmune diseases such as type I diabetes mellitus, occur due to a type I IFN-dependent mechanism and can, in fact, be abrogated using neutralizing Abs to IFN-α (37, 38). However, the aggravating agent inducing the initial type I IFN response in diabetes has not been identified. Therefore, we wanted to determine whether the initial infection with Pneumocystis could induce a continuing CD8 T cell influx and lung damage even after the pathogen is cleared. By allowing the CD8 T cell response to develop, then clearing the pathogen with SMP/TMX treatment, we found that the CD8 T cell response was driven only in the presence of Pneumocystis. Thus, it appears unlikely that the CD8 T cell response is directed against a cryptic self Ag unless it requires constant interaction of the pathogen with the epithelial/endothelial lining.

As of yet there is no evidence that Pneumocystis Ags are processed in the intracellular MHC I pathway by epithelial or endothelial cells which could be used to explain CD8 T cell-mediated lung tissue damage due to direct presentation of pathogen-specific peptides. However, Pneumocystis pathogen does grow in close proximity to the alveolar lining and has been shown to directly bind to alveolar epithelial cells (39, 40). Wang et al. (41) reported recently that Pneumocystis can activate the NF-κB signaling pathway in vitro when cocultured with a mouse lung epithelial cell line leading to the secretion of proinflammatory cytokines and chemokines. Furthermore, Hahn et al. (42) demonstrated increased production of MIP-2 by rat alveolar epithelial cells in response to the interaction with Pneumocystis cell wall β-glucan. Other studies investigating the induction of CD8 T cell-mediated apoptosis of lung epithelial cells in acute respiratory distress syndrome and interstitial pneumonia suggested that the activation of CD8 T cells may occur via superantigen stimulation (43, 44). Though Pneumocystis is not known to express any superantigen-like molecules it is a possibility that Pneumocystis cross-links and activates CD8 T cells and epithelial cells in a nonprocessed manner similar to superantigens.

Although it is clear that CD8 T cell-mediated lung damage is Ag-specific, requires the Pneumocystis pathogen to drive the CD8 response and requires MHC I expression on non-bone marrow–derived lungs, our data using perforin, Fas, and IFN-γ KO mice indicated that the direct effector mechanisms involved in mediating the damage cannot easily be attributed to one single pathway, but suggest either an array of effector molecules or novel mechanisms that still need to be elucidated. In this regard, we found that lung damage was significantly improved in the course of PCP in CD4 T cell-depleted IFNαR KO mice (deficient in type I IFN signaling pathway), when compared with CD4-depleted wild-type animals and that CD8 T cell influx into the BALF was reduced and pro-inflammatory cytokines such as IFN-γ, TNF-α, IL-6, and MCP-1 were also lower in the lungs of these mice (5). Furthermore, in other interstitial pneumonia diseases of unknown etiology and in some autoimmune diseases like rheumatoid arthritis, the release of type I inflammatory cytokines by infiltrating CD8 T cells is thought to play an important role in pathogenesis, although the mode of cellular activation is not understood (13, 14, 45). Thus, it is possible that IFN-γ production specifically by CD8 T cells also contributes to the lung damage seen in Pneumocystis-infected CD4-depleted mice. Taken together we show that CD8 T cell recruitment during the course of PCP in the absence of CD4 T cells results in direct lung damage due to Ag recognition in the context of MHC I expressed on stromal lung tissue and requires the presence of the pathogen. Although Pneumocystis is an extracellular pathogen, damage to the epithelial/endothelial integrity may be disrupted in a CD8 T cell-mediated and MHC I-dependent manner due to either direct presentation of Pneumocystis Ag itself by these cells or the induction of a self-Ag expressed on the endothelium or epithelium due to direct interaction of the pathogen with this cell barrier. In this regard, the disease does resemble other CD8 T cell-associated interstitial lung diseases of unknown origin and as some CD8-associated autoimmune diseases such as rheumatoid arthritis. Therefore, further understanding of the mechanism of CD8 T cell activation and effector functions and their interplay with other effector cells such as alveolar macrophages may lead to more effective treatment options and the prevention of permanent lung damage.