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Role of Type I IFNs in Pulmonary Complications of Pneumocystis murina Infection

Nicole N. Meissner, Steve Swain, Mike Tighe, Ann Harmsen, and Allen Harmsen

Despite the advent of highly active antiretroviral therapy, pulmonary complications in AIDS are a common clinical problem. Pneumocystis jiroveci infection causes a life-threatening pneumonia, especially in individuals with CD4 T cell deficiencies as occurs in AIDS. Although Pneumocystis sp. is an extracellular fungal pathogen, CD8 T cells are the predominant lymphocyte recruited to the lung in CD4-deficient humans and mice during Pneumocystis pneumonia, and we have found that these CD8 T cells are responsible for subsequent lung damage in CD4 T cell-depleted mice. Comparing CD4 T cell-depleted IFN-α receptor knockout (KO) mice to wild-type mice, we found that this CD8 T cell recruitment and lung damage is type I IFN (IFN-αβ) dependent. However, in both CD4 competent, wild-type and IFN-α receptor (IFNAR) KO mice, Pneumocystis infection leads to an eosinophilic granulocyte influx with bronchial epithelial changes as seen in asthma. This response is delayed in IFNAR KO mice, as is pathogen clearance. Although the inflammation is transient in wild-type animals and resolves upon Pneumocystis clearance, it is more severe and persists through day 35 postinfection in IFNAR KO mice, leading to fibrosis. In addition, IFNAR KO, but not wild-type, mice mount a Pneumocystis-specific IgE response, an indicator of allergic sensitization. Thus, in the absence of IFNAR signaling and CD4 T cells, Pneumocystis infection leads to fibrosis. In addition, IFNAR KO, but not wild-type, mice mount a Pneumocystis-mediated lung damage does not occur, whereas in CD4-competent animals, the absence of IFNAR signaling results in an exacerbated Th2 response, asthma-like symptoms, and fibrosis. Therefore, both CD4 T cell- and type I IFN-mediated mechanisms can determine pulmonary complications from Pneumocystis infection. The Journal of Immunology, 2005, 174: 5462–5471.

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2 Abbreviations used in this paper: IFNAR, IFN-α receptor; BAL, bronchoalveolar lavage; KO, knockout; PCP, Pneumocystis pneumonia; penh, enhanced pause.
Because of its important immune regulatory function, especially its influence on Ag presentation to CD8 T cells and CD8 T cell activation, we examined the role of IFNAR signaling in the induction of CD8 T cell-mediated lung damage during *Pneumocystis* pneumonia (PCP) in CD4-depleted mice as well as the role of IFNAR in clearance of the infection in CD4-competent (undepleted) mice. We found in IFNAR KO mice a significant reduction of CD8 T cell recruitment into the lung during PCP. This was associated with reduced lung damage, improved arterial oxygen saturation, and reduced proinflammatory cytokine secretion into the bronchoalveolar lavage (BAL) fluid compared with CD4 T cell-depleted wild-type animals with the same level of *Pneumocystis* infection. These findings indicate a critical role of IFNAR signaling in the activation and recruitment of CD8 T cells in CD4-depleted animals and, thus, in the development of immune-mediated damage in PCP.

In the CD4-competent host, in both wild-type and IFNAR KO mice, a strong Th2-mediated eosinophilic inflammation occurred; however, in the absence of IFNAR, the Th2 response to *Pneumocystis* was exacerbated. This was characterized by more intense and persistent eosinophil accumulation, mucus cell metaplasia, and IgE production, as well as beginning lung fibrosis. Thus, signaling through the IFNAR results in exacerbation of the Th2 response to *Pneumocystis* when CD4 T cells are present, but results in amelioration of CD8 T cell-mediated lung damage when CD4 T cells are absent.

### Materials and Methods

#### Mice

C.B17 SCID mice, as a source of *Pneumocystis* organisms, were bred and maintained at Montana State University’s Animal Resource Center. All animal experiments were approved by the institutional animal care and use committee and strictly followed the animal care and handling procedures required by the Animal Welfare Act as well as National Institutes of Health and U.S. Department of Agriculture guidelines. A breeder pair of IFNAR KO mice (animals are on a B6/129 mixed background) was provided by Prof. D. E. Levy (New York University School of Medicine, New York, NY) and were bred and maintained at Montana State University’s Animal Resource Center. Mixed background control mice were purchased from The Jackson Laboratory (B6129PF2J Stock: 100903).

#### CD4 T cell depletion

For in vivo CD4 T cell depletion, animals were injected with 300 μg of purified GK1.5 rat anti-mouse CD4 Ab i.p. 2–4 days before infection with *Pneumocystis* and subsequently injected twice per week until the end of the experiment as previously described (9, 26, 27). The GK1.5 hybridoma cell line was obtained from American Type Culture Collection, and the cells were grown according to the instructions provided and with Abs produced in ascites.

#### Infection of mice with *Pneumocystis*

Lung homogenates from *Pneumocystis*-infected C.B17 SCID mice were used as a source of *Pneumocystis* nuclei, and CD4-depleted or CD4-undepleted (CD4 T cell-competent) recipient animals were infected with 10⁷ *Pneumocystis* nuclei in a 100-μl volume as previously described via intratracheal instillation with mice under deep isoflurane anesthesia (26, 28).

#### Blood gas analysis

As a measure of lung function, arterial blood was obtained from the tail artery of each animal into capillary tubes and analyzed at Deaconess Hospital (Bozeman, MT) on an AVL Omi3 Blood gas autoanalyzer (AVL Medical Instruments) for pO₂, pCO₂, and pH as we have previously described (9).

#### Assessment of cellular infiltrates into lungs and lymphocyte differentiation using FACS analysis

BALs were performed as previously described (28). Briefly, mice were lethally injected with 90 mg/kg sodium pentobarbital solution i.p. and exsanguinated. Lung lavages were performed by intratracheal cannulation with a total volume of 5 ml of HBSS and 0.5% EDTA. A cell aliquot was spun onto glass slides and stained with Diff-Quik (Dade-Behring) for leukocyte differentiation. Cell counts of BALs were performed with a hemocytometer, and 10⁶ cells/sample were used to assess lymphocyte subset distribution via flow cytometry. For this, a four-color immunofluorescence stain was performed using directly conjugated allophycocyanin-anti-mouse CD4 (L3T4), CyChrome-anti-mouse CD8α (Ly2), PE-anti-mouse CD43 (B11) and FITC-anti-mouse CD44 (Ly-2, clone IM7) Abs. All Abs were obtained from BD Pharmingen. Before staining, cells were incubated with unlabeled anti-mouse FcR Abs to block FcR-mediated unspecific staining, then incubated with a mixture of the appropriate dilutions of the above Abs for 30 min. Cells were washed three times with PBS/2% FCS and analyzed on a FACS Calibur (BD Biosciences).

#### Histological analysis of lung tissue

Left lung lobes were inflated with 10% neutral buffered formalin through a tracheal cannula, removed, and immersed in formalin for at least 24 h, as previously described, before paraffin embedding (29). Sections were cut 5 μm thick on a Leica RM2155 microtome and stained with H&E to assess tissue morphology, with Alcin Blue and periodic acid-Schiff to detect mucus production, and with Masson Tri-Chrome to detect collagen deposition. All reagents were purchased from Richard Allen Scientific and stained according to the manufacturer’s instructions.

#### Enumeration of *Pneumocystis* nuclei

*Pneumocystis* burden was assessed by enumeration of its nuclei as previously described (26). 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 Cytopsin 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 log₁₀ 4.43 for right lobe homogenates.

#### Albumin quantification in BAL fluids

Albumin concentrations in BAL fluids were determined as readout for inflammatory exudates and a measure for the extent of lung damage. Albumin reagent (BCG) from Sigma-Aldrich and an albumin standard solution were used for this quantitative colorimetric test. Briefly, standards were prepared in 1/2 dilutions in HBSS/3 mM EDTA ranging from 0.1–2 mg/ml. One hundred microliters of standard per sample was added to the appropriate well in a 96-well plate and mixed with 50 μl of albumin reagent (BCG). Production of green-blue color was immediately read on a Thermomax microplate reader (Molecular Devices) at 628 nm and analyzed using Softmax software (Molecular Devices).

#### Cytokine analysis using bead arrays in BALs and tissue culture supernatants

TNF-α, IFN-γ, IL-5, IL-4, IL-2, IL-6, MCP-1, and IL-10 cytokine secretion into the BAL fluid was assessed using the Mouse Thi-Th2 and Mouse Inflammation Cytometric Bead Array assay kit (BD Biosciences) according to the manufacturer’s instructions. Assays were read on a FACSScan flow cytometer (BD Biosciences), and data were analyzed using cytometric bead array software (BD Biosciences). In addition, IL-10, IL-13, and TGF-β1 concentrations in BAL fluid were measured using a commercially available mouse specific sandwich ELISA (R&D Systems).

#### *Pneumocystis*-specific serum IgG and IgE analysis

*Pneumocystis*-specific IgG and IgE responses were determined using a sandwich ELISA technique. Briefly, 96-well, flat-bottom plates were coated with 10 μg/ml *Pneumocystis* Ag isolated from lungs of heavily infected source mice as previously described (30) in 0.05 M sodium carbonate buffer, pH 9.6, for 3 h. Plates were washed five times with PBS/0.05% Tween 20 (wash buffer) and blocked overnight with 5% nonfat dry milk in PBS/0.05% Tween 20 (blocking buffer). Plates were incubated with 1/100 in sample buffer (1% BSA/PBS) for *Pneumocystis*-specific IgG and 1/25 for *Pneumocystis*-specific IgE, and 100 μl/well was incubated at 37°C for 2 h. Plates were washed six times; 100 μl/well of alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma-Aldrich) at 1/2500 in sample buffer was added to plates for *Pneumocystis*-specific IgG detection, and 2 μg/ml biotinylated anti-mouse IgE (BD Biosciences; clone R5-118) was added for *Pneumocystis*-specific IgE detection, and incubation was performed for 1 h at room temperature, then plates were washed six times. Avidin-HRP (Vector Laboratories) was diluted 1/10,000 in sample buffer, and 100 μl/well was incubated with 1% H₂O₂ in PBS/0.05% Tween 20 (substrate buffer), and 1 mg/ml PNP (Sigma-Aldrich) was added to each well. Color was developed with 2 mg/ml fast blue (Sigma-Aldrich) and stopped by adding 1 M NaOH. Abs were read on a ThermoMax microplate reader (Molecular Devices) at 628 nm and analyzed using Softmax software (Molecular Devices).
was added to plates for IgE detection, then incubation was performed for 30 min. Plates were washed again six times, then 50 µl/well ABTS buffer (Sigma-Aldrich) was added to plates for IgE detection, and 50 µl/well p-nitrophenyl-phosphate (Sigma-Aldrich) in dietholamine buffer, pH 9.8 (Sigma-Aldrich), at 1 mg/ml was added to plates for IgG detection, and incubation proceeded for 30 min. Plates were read at 405 nm wavelength on a VersaMax plate reader (Molecular Devices). Samples were read for OD, and positive and negative control sera were included in the assay.

Total serum IgE was measured using a mouse IgE capture and detection Ab pair plus mouse IgE standard from BD Biosciences, and the assay was performed according to the manufacturer’s protocol.

Assessment of airway hyperreactivity in response to methacholine challenges using a whole-body plethysmograph

Airway hyperresponsiveness was measured by induction of the enhanced pause (penh) in the airflow upon exposure to increasing dosages of aerosolized methacholine (0, 2.5, 5, 10, and 20 mg/ml) in a whole-body plethysmograph for rodents (Buxco). Between each methacholine dosage, animals were allowed to rest for up to 15 min to return to baseline values. The lowest dosage of methacholine that induced significant differences in penh values between the groups was compared.

Statistical analysis

For statistical analysis, a one-way ANOVA was performed, and pairwise comparisons were made using the post-hoc Tukey test. Data were assumed to be significantly different at \( p < 0.05 \). Data are shown as the mean ± SEM.

Results

Type I IFNs signaling is required in the induction of immune-mediated lung damage during the course of PCP in CD4-depleted mice

To assess the role of type I IFNs in the activation of CD8 T cells in the course of PCP, IFNAR KO mice were CD4 T cell-depleted and Pneumocystis-infected via intratracheal instillation of \( 10^7 \) nuclei of Pneumocystis. The course of disease progression and lung damage was assessed on day 28 postinfection and compared with the course of disease in CD4 T cell-depleted wild-type animals. Day 28 postinfection was chosen because we have previously shown that lung damage in Pneumocystis-infected, CD4 T cell-depleted animals is well established by this time, although the animals are not yet terminally sick (9). Each experiment included a control group consisting of uninfected wild-type animals, because previous comparative analysis of uninfected lungs from IFNAR KO mice and wild-type mice showed no differences with regard to lung structure and cellular composition of BAL cells (>95% alveolar macrophages). Each experimental group consisted of five animals, and each experiment was repeated three times. Arterial blood gases were taken from all groups, animals were then killed, and Pneumocystis lung burden, lung histopathology, and airway inflammatory responses were determined. Blood gas analysis revealed a significantly better arterial oxygenation of IFNAR KO mice on day 28 postinfection compared with that in wild-type animals (Fig. 1A), and albumin levels, as a measure for vascular leakage, were significantly reduced in IFNAR KO mice compared with the infected wild-type animals (Fig. 1B). Pneumocystis burden was not different in the two groups and cannot account for the differences in measures of lung function and damage (Fig. 1C).

Leukocyte populations recruited into the BAL fluids were as assessed by differential cell count and revealed a similar lung accumulation of neutrophils and macrophages in wild-type and IFNAR KO mice, with a slightly reduced accumulation of lymphocytes in IFNAR KO mice (data not shown). Lymphocyte subset analysis via FACS confirmed depletion of CD4 T cells using an anti-CD4 Ab (clone L3T4) that does not compete with the GK1.5 mAb epitope. Furthermore, CD8 T cells were the predominant lymphocyte population recruited to the lung, with a similar activation profile in the two groups as assessed by the percentage of CD44- and CD43high-expressing CD8 T cells (data not shown). However, total lymphocyte numbers were significantly lower in BAL fluid of IFNAR KO mice compared with that of wild-type mice due to a reduced influx of activated CD8 T cells (Fig. 2). In agreement with

![Figure 1](http://www.jimmunol.org/)
these findings, cytokine analysis in the BAL fluid showed significantly reduced secretion of the proinflammatory cytokines TNF-α, IFN-γ, IL-6, and MCP-1 (Fig. 3), because they can be secreted by activated CD8 T cells and other cells. The levels of the anti-inflammatory cytokines IL-10 and TGF-β were not significantly different between the groups of mice (data not shown).

**FIGURE 2.** Lymphocyte numbers are reduced in BAL fluids of CD4-depleted IFNAR KO mice compared with wild-type animals during the course of PCP due to reduced influx of activated CD8 T cells. Lymphocyte numbers were assessed by performing total leukocyte count using a hemocytometer and differential cell counts of leukocytes in BAL fluid using Diff-Quik-stained cytospin slides. Lymphocyte differentiation and their activation profile were performed via FACS analysis staining for CD4, CD8, CD44, and CD43 surface markers. A, Comparative analysis of absolute lymphocyte numbers assessed in BAL fluid in IFNAR KO mice compared with wild-type mice. Total CD8 T cell (B) and activated CD44/CD43 CD8 T cell numbers (C) were calculated based on the percentage of total lymphocytes. As no lymphocytes were detectable in BAL fluid of uninfected control animals, absolute lymphocyte numbers as well as subset values were zero. Statistical differences between infected IFNAR KO and wild-type animals are indicated above the bars of the wild-type group: *, p < 0.05; **, p < 0.01; ***, p < 0.001.

**Lack of IFNAR signaling leads to a delayed, but complete, clearance of Pneumocystis infection in CD4-competent animals**

We have previously found that in immune-competent, wild-type animals, *Pneumocystis* infection is cleared by days 14–21, with minimal or no residual inflammation of lung tissue by day 28 post-infection. Because of the reduced inflammation seen in CD4-depleted IFNAR KO mice in response to *Pneumocystis* infection, we determined whether clearance of the infection would be impaired in CD4 T cell-competent (undepleted) mice. Therefore, we infected both CD4-competent, wild-type and IFNAR KO mice with *Pneumocystis* intratracheally and evaluated *Pneumocystis* clearance, *Pneumocystis*-specific serum immune globulin levels, and the inflammatory response induced on days 14, 21, and 28 post-infection.

**FIGURE 3.** Proinflammatory cytokine levels are significantly lower in BAL fluid of IFNAR KO mice compared with wild-type animals during the course of PCP. Cytokine concentrations as a measure of inflammatory activity were assessed in BAL fluids of CD4 T cell-depleted IFNAR KO and wild-type mice suffering from PCP using the inflammation bead array (BD Biosciences). TNF-α (A), IFN-γ (B), IL-6 (C), and MCP-1 (D) were significantly lower in IFNAR KO mice. Cytokine concentrations in the BAL fluid of uninfected control animals were between 0 and 5 pg/ml for all cytokines and may appear as undetectable in the appropriate graphs. Statistical differences between infected IFNAR KO and wild-type animals are indicated above the data bars of the wild-type group: *, p < 0.05; **, p < 0.01; ***, p < 0.001.
in IFNAR KO mice, the cellular influx into lung airways did not peak until day 21 (Fig. 4 C).

Pneumocystis infection of CD4 T cell-sufficient mice leads to transient eosinophilic immune response in wild-type animals, resulting in pathological changes similar to those of asthma. This response is prolonged and exaggerated in IFNAR KO animals and leads to pulmonary fibrosis. BAL fluid leukocyte differentiation revealed that clearance of the pathogen in both wild-type and IFNAR KO animals was associated with a strong eosinophil granulocyte influx. This influx was again delayed in IFNAR KO mice to day 21 (Fig. 5 A). Because eosinophilic airway inflammation can be associated with increased airway hyperreactivity as in allergic asthma, animals were exposed to increasing dosages of methacholine, and airway resistance was assessed using a whole-body plethysmograph for rodents. On day 14 postinfection, wild-type animals showed transiently increased penh values in response to low dose methacholine (2.5 mg/ml), whereas IFNAR KO mice did not. However, on day 28 postinfection, IFNAR KO animals demonstrated increased penh values (Fig. 5 B), whereas wild-type animals had returned to baseline levels. BALB/c as well as C57BL/6 wild-type mice were tested in the same experimental setting, and similar transient airway eosinophilia as well as airway hyperreactivity were found (data not shown) (S. Swain, and A. Harmsen, manuscript in preparation) during the course of clearance of the infection, indicating a general immune phenomenon rather than a strain-specific response. To further exclude that eosinophilic influx was not due to an Ag incompatibility reaction due to the instillation of Pneumocystis-infected mouse lung homogenate derived from SCID mice on a BALB/c genetic background into animals with a mixed (B6/129) genetic background, B6/129 mixed background wild-type animals were either intratracheally instilled with lung homogenate from an uninfected allogeneic B6/129 mouse or an uninfected BALB/c SCID mouse and compared with mice receiving the instillation of Pneumocystis-infected lung homogenate from BALB/c SCID mice used in the experimental settings described above. Intratracheal instillation of uninfected lung homogenates from BALB/c SCID mice or allogeneic B6/129 wild-type mice did not lead to any detectable eosinophilic cell response in BAL fluids on day 14 postinstillation, whereas the instillation of Pneumocystis-infected lung homogenates did, indicating that the eosinophilic response was due to the presence of Pneumocystis pathogen in the lung homogenate (data not shown).

Histological analysis of formalin-fixed lung tissue (Fig. 6) in wild-type mice on day 14 revealed an intense Th2-like immune response and confirmed recruitment of eosinophils and lymphocytes into the lung airways and interstitium as well as giant cell formation and epithelial and goblet cell metaplasia of the bronchi resembling an asthmatic inflammatory response. In contrast, only mild inflammation was detected on day 14 postinfection in lungs of IFNAR KO mice, with no eosinophil accumulation detectable, but by this time, some lymphocyte recruitment had occurred as well as the formation of some giant cells (Fig. 6, top row). On day 21, however, lungs of IFNAR KO mice showed an even more...
severe inflammation in response to the infection than that on day 14 in wild-type animals with massive eosinophil and lymphocyte infiltration, giant cell formation, and mucus cell hyperplasia with almost complete consolidation of the lung tissue. In contrast, wild-type animals were already resolving the response, with only minor peribronchial eosinophil and lymphocyte infiltrate remaining, and the bronchial epithelia height had nearly returned normal (Fig. 6, second row). On day 28 postinfection, wild-type animals showed only minimal residual inflammation, whereas IFNAR KO mice still showed severe asthmatic inflammation similar to that on day 21 (Fig. 6, third row). Because the intense Th2-like response in IFNAR KO mice persisted, we repeated the experiments and chose day 35 instead of day 28 as our latest time point. The time course of the inflammation in the second experiment mirrored the previous findings and showed a still vigorous eosinophilic inflammation of the lung present through day 35 postinfection, with prominent thickening of the bronchial epithelium, goblet cell hyperplasia, and peribronchial as well as alveolar cellular infiltrate (Fig. 6, bottom row). In addition to the H&E stains, we performed Alcin Blue/periodic acid-Schiff stains, which stained mucus glycoprotein and confirmed mucus cell hyperplasia as a hallmark of asthma throughout day 35 in both wild-type and IFNAR KO animals (Fig. 7, rows 1 and 2). Chronic inflammation of the lung can lead to irreversible changes in the form of tissue fibrosis with loss of tissue elasticity and reduced gas exchange. Masson Tri-Chrome staining of formalin-fixed lung tissue revealed prominent interstitial as well as peribronchial collagen deposition as a sign of lung fibrosis in IFNAR KO mice on day 35 postinfection, whereas lung tissue of wild-type animals at the same time point looked like that in uninfected controls (Fig. 7, row 4).

FIGURE 6. Eosinophilic inflammation was apparent in both CD4-competent IFNAR KO and wild-type animals, with lung changes similar to those seen in asthma. However, the response was delayed, but exacerbated and prolonged, in IFNAR KO mice. Shown are photomicrographs of formalin-fixed and H&E-stained lung sections taken at ×10 and ×200 magnifications. Eosinophilic lung inflammation with bronchial cell hypertrophy (+) and giant cell formation (<) can be seen in all sections. Delayed onset of inflammation in IFNAR KO mice on day 14 (first row) was noticed and was more severe on day 21 in IFNAR KO mice compared with that on day 14 in wild-type animals. Although the inflammation in wild-type animals waned to almost complete resolution on days 28 and 35 (rows 3 and 4), inflammation remained almost unchanged in IFNAR KO mice through day 35 despite clearance of the pathogen by day 28 (rows 2–4).

Pneumocystis exposure in CD4-competent mice leads to Pneumocystis-specific allergic sensitization

Because of the transient histological changes in wild-type animals and the prolonged inflammation in IFNAR KO animals resembling allergic inflammation, we determined whether Pneumocystis could induce a specific IgE response as found during allergic sensitization. Total IgE was detectable in both wild-type and IFNAR KO animals (Fig. 8A), but only in IFNAR KO mice did total IgE increase over 28 days, although this was not statistically significant compared with the wild-type group. However, a dramatic Pneumocystis-specific IgE response was only induced in the IFNAR KO group beginning on day 21 postinfection, with highest readings on day 28 (Fig. 8B). Therefore, a delayed clearance of the pathogen in IFNAR KO mice may have been responsible for the induction of the Pneumocystis-specific IgE response changing Pneumocystis from an immunological Ag to an allergen.

Cytokine profile analysis of BAL fluids from both groups over the above time course revealed a Th2 pattern (Fig. 9, A–C) with increased production of IL-5, IL-13, and TGF-β. Highest cytokine concentrations were measured on day 14 in wild-type animals and
on day 21 in IFNAR KO animals, and the induction of these cytokines was comparable between the two groups at their individual peak time points. IFN-γ concentrations were unchanged during the time course of clearance in both groups (Fig. 9D). A second, independent experiment showed similar results. However, in this experiment, IL-10 was also measured, because it has been identified to down-regulate specific immune responses. Wild-type mice produced significant amounts of IL-10 on day 14 postinfection, but no IL-10 was produced on day 14, and little was produced on day 21 postinfection in IFNAR KO mice (row 4, panel A).

**Discussion**

In humans and mouse models of PCP, it has been shown that in the absence of CD4 T cells, a vigorous CD8 T cell influx into the infected lung occurs during the course of the disease, which mediates the occurring lung damage (9).

We found that CD4-depleted IFNAR KO mice with disrupted signaling for both IFN-α and IFN-β showed significantly better lung function as well as less lung damage compared with wild-type animals, although the *Pneumocystis* burden of both groups was identical. This was associated with significantly reduced influx of activated CD8 T cells into the lungs of infected IFNAR KO mice. The mechanisms of CD8 T cell-mediated lung damage during the course of PCP are not clear, but CD8 effector cells have been reported to secrete proinflammatory cytokines/chemokines such as IFN-γ, TNF-α, and RANTES (31, 32), which are involved in effector functions and can induce target cell death. Wright et al. (33) suggested a role for TNF-α receptor signaling in the mechanisms of CD8 T cell-mediated lung damage during the course of PCP by influencing the secretion of other proinflammatory cytokines and chemokines, such as TNF-α, keratinocyte-derived cytokine, RANTES, and MCP-1. We found in IFNAR KO mice, compared with wild-type mice, significantly reduced concentrations of proinflammatory cytokines, including TNF-α, IFN-γ, MCP-1, and IL-6, all of which could be produced by activated CD8 T cells. This occurred despite vigorous *Pneumocystis* growth in the lungs of both groups. These findings indicate that the induction of these cytokines and recruitment of CD8 T cells to the lung require type I IFN-dependent mechanisms during the course of PCP.

CD8 T cell-mediated mechanisms of damage have been implicated in other lung diseases, such as extrinsic allergic alveolitis or hypersensitivity pneumonitis (34). The immune mechanisms by which CD8 T cells are activated and what Ag they detect in these diseases is unclear; however, similar pathological sequelae can be
seen in interstitial lung diseases, that occur in the context of autoimmune diseases such as rheumatoid arthritis (35). Furthermore, type I IFN-dependent mechanisms have been implicated in the pathogenesis of other autoimmune diseases, such as systemic lupus erythematosus and diabetes mellitus type I, and treatment of these patients with anti-IFN-α mAbs has been suggested (36, 37). That lung damage in PCP in the absence of CD4 T cells is dependent on IFNAR signaling suggests that a therapeutic approach of neutralizing IFN-α signaling of classic PCP could alleviate lung damage in this disease. Thus, we determined whether a deficiency in IFNAR signaling would also affect host resistance to Pneumocystis in a CD4-competent animal. Previous pathogenesis studies revealed that IFNAR KO mice are highly susceptible to infections by a broad array of different viruses (e.g., Poxviridae, Arenaviridae, Rhabdoviridae, and Togaviridae), yet have normal resistance to the microbial pathogen Listeria monocytogenes, and it has been established that the type I IFN system (IFN-αβ) and the type II IFN system (IFN-γ) are nonredundant (12, 38). In a study using influenza virus infection, the inflammation that occurred in both wild-type animals and IFN-γ KO mice was predominantly lymphocytic, whereas in IFNAR KO mice, it was predominantly eosinophilic (39). Interestingly, when we compared Pneumocystis clearance in CD4-competent IFNAR KO mice to that in wild-type mice, we found that both animal groups responded with a severe Th2-like immune response, with massive recruitment of eosinophils into the lung tissue as well as bronchial epithelia cell hyperplasia with mucus production and giant cell formation. However, IFNAR KO mice showed a delayed onset of inflammation (day 21 vs day 14 in wild-type animals), followed by a prolonged and augmented Th2-like immune response, resulting in exacerbated lung pathology. This was associated with delayed clearance of the pathogen due to delayed induction of a Pneumocystis-specific IgG Ab response (complete clearance in wild-type animals by day 14; in IFNAR KO mice by day 28). These data indicate that a type I IFN response is not necessary for pathogen clearance, yet it is necessary for the timing and modulation of induction of the protective immune response. Why the Pneumocystis-specific IgG response is delayed in IFNAR KO mice is not clear; however, the absence of IFNAR signaling could lead to delayed maturation of professional APCs and, therefore, specific Th cells. Furthermore, the importance of IFN-α in conjunction with IL-6 in the induction of plasma cell differentiation and immune globulin secretion has recently been described (40), and the loss of IFNAR signaling could directly influence and delay plasma cell differentiation and Ab secretion in response to Pneumocystis infection.

**Pneumocystis** infection in IFNAR KO mice, compared with that in wild-type mice, resulted in exacerbated, asthma-like responses. Interestingly, despite concerns about side effects, IFN-α treatment has been shown to be a successful option in the treatment of steroid-resistant asthma, and its mechanisms of actions are thought to involve the promotion of a Th1, rather than a Th2, immune response, thus disrupting the cycle of eosinophilic inflammation and airway hyperreactivity (24, 25). Furthermore, it was shown that histamine, released during allergic inflammation, inhibits the secretion of IFN-α from plasmacytoid dendritic cells, which could promote a strong Th2 response, as in allergic inflammation. This might be an explanation for the decreased type I IFN levels found in atopic patients compared with healthy individuals (41). These findings strongly suggest a role of type I IFN signaling in modulating allergic inflammation in humans. The importance of type I IFN in allergic sensitization has previously been demonstrated in a mouse model of allergic sensitization to OVA comparing wild-type animals to animals lacking the IFN-β gene (42). In the absence of IFN-β, animals showed a more severe pulmonary eosinophilic inflammation, with goblet cell hypertrophy and increased production of Th2 cytokines as well as OVA-specific IgE in response to the sensitization protocol, compared with wild-type or control mice.
heterozygous littermates. Also, increased OVA-specific T cell proliferation indicated an important role for type I IFN in the modulation and inhibition of allergic sensitization. Although both wild-type and IFNAR KO mice responded with a strong Th2-mediated immune response to *Pneumocystis* infection with changes resembling allergic (type I hypersensitivity) reactions, this was only transient in wild-type animals and waned fast. However, in animals with a defect in the IFNAR signaling pathway, a prolonged asthmalike inflammatory response of the lungs was noted, with signs of beginning fibrotic tissue remodeling, as seen in chronic asthma (43); only in this group was *Pneumocystis*-specific IgE production detectable. This can be regarded as evidence for allergic sensitization to the pathogen, which could, in turn, promote an IgE-mediated allergic inflammatory response upon re-exposure to the pathogen in IFNAR KO mice. Why allergic sensitization occurred in IFNAR KO mice and not in wild-type mice is unknown. However, significant secretion of IL-10 was only seen in wild-type mice before resolution of the inflammation. IL-10 has been shown to be secreted by specific regulatory T cells, which have been described to play a key role in the specific down-regulation of Th2 responses and have been considered important in the down-regulation of allergic inflammations (44–46). Therefore, the absence of IFNAR signaling may have affected the induction of regulatory T cells because of impaired maturation of regulatory dendritic cells (47). However, additional characterization of T cell and APC subsets in the lung during the course of *Pneumocystis* infection needs to be performed.

In summary, we found that during the course of PCP, in the absence of CD4 T cells, a type I IFN-dependent, vigorous CD8 T cell response is induced. This suggests that type I IFN could be a therapeutic target to modulate the CD4 T cell-independent inflammatory response during the course of PCP and possibly other extrinsic allergic pneumonitis-type responses. However, in a CD4-competent host, the immune response induced during the course of clearance of *Pneumocystis* resembles histological lung changes that occur during a classical asthmatic inflammation. Although IFNAR signaling does not appear to be critical for the clearance of the infection, it modulates and down-regulates this inflammation upon clearance of the pathogen, leading to complete recovery of the lung without residual inflammation. However, the absence of IFNAR signaling in a CD4-competent animal leads to a prolonged, Th2-mediated, eosinophilic lung pathology as well as an allergic sensitization to the pathogen with *Pneumocystis*-specific IgE production, possibly due to the loss of IL-10-producing regulatory T cells. Because mechanisms of allergic sensitization are still poorly understood, yet genetic as well as environmental factors are thought to contribute to the onset of the disease (48, 49), we propose that exposure to *Pneumocystis* can induce allergic sensitization to the pathogen in the presence of impaired type I IFN signaling, which may induce a continuous aggravation during re-exposure. Furthermore, based on the induction of a strong Th2-mediated immune response during the natural course of clearance of *Pneumocystis* in the lung, exposure of an individual with pre-existing asthma to *Pneumocystis* may lead to severe exacerbation of asthma or other obstructive lung diseases. Interestingly, a study by Morris et al. (50) found an association of chronic obstructive pulmonary lung disease severity and *Pneumocystis* colonization of the lung in patients with chronic obstructive pulmonary lung disease. Furthermore, there are increasing reports of pulmonary complications in AIDS patients, such as emphysema, pulmonary hypertension, noninfectious inflammatory disease, and asthma, as well as the clinical presentation of PCP as asthma (51–53). It is likely that depending on the immune status of a patient infected with HIV, the clinical manifestation of *Pneumocystis* infection is variable, and chronic colonization (that may or may not be detectable) in a partially immune-reconstituted patient may lead to pulmonary complications other than classical PCP.

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**Disclosures**

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**References**


