Type-I IFN Signaling Suppresses an Excessive IFN- γ Response and Thus Prevents Lung Damage and Chronic Inflammation During *Pneumocystis* (PC) Clearance in CD4 T Cell-Competent Mice

Nicole Meissner,* Steve Swain,*
Kate McInerney,[†] Soo Han,*
and Allen G. Harmsen*

From the Department of Veterinary Molecular Biology,* and the
Microbiology Department,[†] Montana State University, Bozeman,
Montana

Immune-reconstitution after highly active antiretroviral therapy (HAART) is often incomplete, and some HIV-infected individuals fail to regenerate type-I interferon (IFN)-producing pDCs. We recently demonstrated that during *Pneumocystis* (PC) infection in CD4 T cell-competent mice the absence of type-I IFN signaling results in chronic pulmonary inflammation and fibrosis despite clearance. Because the mechanisms involved are poorly understood, we further characterized the role of type-I IFN signaling in immune responses to PC. We show that type-I IFN signaling around day 7 postinfection is critical to the outcome of inflammation. Microarray analysis of pulmonary CD11c⁺ cells revealed that at day 7 post infection, wild-type cells up-regulated type-I IFN-responsive genes as well as SOCS1, which is a critical negative-regulator of type-I IFN and IFN- γ signaling. This was associated with an eosinophilic lung inflammation, PC clearance, and complete restitution. However, pulmonary CD11c⁺ cells from IFNAR^{-/-} mice demonstrated increased tumor necrosis factor (TNF)- α production and lacked SOCS1-induction at day 7. This was followed by a transient lymphocytic and IFN- γ response before switching to a chronic eosinophilic inflammation of the lung. Early neutralization of TNF- α did not prevent chronic inflammation in IFNAR^{-/-} mice, but treatment with an anti-IFN- γ antibody did. We propose that during PC lung infection type-I IFNs induce SOCS1-associated regulatory mechanisms, which prevent excessive IFN- γ -mediated responses that cause chronic lung damage. Therefore, partial immune-reconstitution in AIDS, attributable to reduced type-I IFN

actions, might disrupt regulatory aspects of inflammation, causing unexplained chronic pulmonary complications as seen in some patients during HAART. (*Am J Pathol* 2010, 176:2806–2818; DOI: 10.2353/ajpath.2010.091158)

The advent of highly active antiretroviral therapy (HAART) has greatly improved immune functions in HIV-infected individuals and has led to a rapid reduction of opportunistic infections such as *Pneumocystis pneumonia*.^{1,2} However, there is increasing recognition of other, non-infectious chronic pulmonary sequelae such as chronic obstructive lung diseases, asthma, pulmonary hypertension, and pulmonary neoplasms.³ The causes of these diseases are likely multifactorial, including immune-mediated mechanisms possibly because of lack of regulation.

Immune reconstitution after HAART can be incomplete, as HIV infection not only causes the loss of CD4⁺ T cells, but results in a decrease of dendritic cell (DC) subpopulations such as type-I interferon (IFN)-producing plasmacytoid DC (pDC).^{4,5} In some patients reconstitution of CD4 T cells may be satisfactory, but they may not adequately reconstitute DCs and specifically type-I IFN-producing pDC numbers.^{6,7} The resulting lack of type I IFN production likely affects effector functions of cells of both innate and acquired immunity.⁸ How this may in addition influence immune regulatory aspects is not clear.

Dendritic cells (DCs) and macrophages are important initiators and coordinators of both the innate and acquired immune responses. They can release large amounts of tumor necrosis factor (TNF)- α and/or type-I IFNs and responds to it.^{9–11} In addition, cross regulatory mechanisms

Supported by National Institute of Health grants RO1HL5502, RO1HL090488, and NIH COBRE grant 2P20RR020185-06.

Accepted for publication February 18, 2010.

Address reprint requests to Nicole Meissner, M.D., Veterinary Molecular Biology Department, Montana State University, 960 Technology Blvd., Bozeman, MT 59718. E-mail: nicolem@montana.edu.

during inflammation are simultaneously initiated in these cells to prevent the unnecessary expansion of immune activation and tissue damage. Cytokine signaling also induces the expression of "suppressor of cytokine signaling molecules" (SOCS). These molecules function as negative regulators of inflammation by interfering with cytokine-initiated JAK-STAT-signaling pathways.^{12,13} SOCS1 expression, for example, is initiated by and negatively regulates both type-I IFN and IFN- γ , and it has a specific and nonredundant role in the negative regulation of IFN- γ .

We recently demonstrated in a mouse model of PC lung infection in CD4-competent mice that absence of IFNAR (type-I IFN receptor) results in a delayed but exacerbated TH2-mediated immune response, followed by lung fibrosis by day 35 post infection despite pathogen clearance when compared with wild-type mice in which the inflammation results in complete restitution.¹⁴ Here we further characterized the role of type-I IFN-signaling in regulating the immune response to PC lung infection in CD4-competent mice. We found that activation events near day 7 postinfection are critical in determining the outcome of inflammation. At this time CD11c⁺ alveolar macrophages and/or pulmonary CD11c⁺ DCs were the predominant inflammatory cells present in the lung. Microarray analysis of pulmonary CD11c⁺ cells at day 7 post-PC infections showed a transient type-I IFN-mediated gene expression signature in cells derived from wild-type mice, but not from IFNAR^{-/-} mice. This was associated with a concomitant upregulation of SOCS1 in pulmonary CD11c⁺ cells from wild-type mice which preceded a transient eosinophilic immune response at day 14 postinfection. In contrast, CD11c⁺ cells from IFNAR^{-/-} mice demonstrated a unique up-regulation of TNF- α at day 7 and also lacked SOCS1 expression at day 7. This was followed by a transient lymphocytic and IFN- γ -dominated inflammation by day 14 post infection before switching to an exacerbated and persisting eosinophilic inflammation. Neutralization of TNF- α during the course of infection did not prevent the chronic inflammation in IFNAR^{-/-} mice. However, treatment with neutralizing anti-IFN- γ antibody resulted in complete resolution of inflammation.

The lack of a timely induction of negative regulators such as SOCS1 by type I IFN signaling in pulmonary CD11c⁺ cells may be responsible for a transient but excessive and damaging IFN- γ response in IFNAR^{-/-} mice after PC infection. Partial immune reconstitution during HAART caused by reduced type-I IFN activity may also influence regulatory aspects of inflammation, which could promote chronic IFN- γ -mediated inflammatory sequelae.

Materials and Methods

Mice

C.B17 SCID mice, as a source for *Pneumocystis* (PC) organisms, were bred and maintained at Montana State University Animal Resource Center. A breeder pair of IFNAR KO mice on a 129/SvEv background,¹⁵ which share the same major histo-compatibility antigens with C57BL/6 mice, was kindly provided by Prof. D.E. Levy

(New York University, School of Medicine, New York, NY), and a colony has been established and maintained at the Montana State University Animal Resource Center. C57BL/6 wild-type mice were bred in house and used as control animals. Previous experiments using wild-type mice with BALB/c, C57BL/6, 129/SvEv, and mice with 129/C57BL/6 mixed background have not demonstrated differences in timing of clearance, as well as the extent and phenotype of inflammation.^{14,16} Experimental groups consisted of five mice each, and each experiment was performed at least twice.

PC Infection and Enumeration

Lung homogenates from PC-infected mice were used as a source of PC and animals were infected, under deep isofluorane anesthesia, intratracheally with 10⁷ PC nuclei in 100 μ l of lung homogenate diluted in PBS-buffer. PC-burden of infected animals was assessed microscopically by enumeration of trophozoid nuclei count in lung homogenates in 10–50 oil immersion fields as previously described.¹⁷ The limit of detection for this technique is log₁₀ 4.43.

Assessment of Pulmonary Infiltrate and Lymphocyte Differentiation

Broncho-alveolar lavages (BAL) were performed as previously described.¹⁸ Briefly, mice were intraperitoneally (i.p.) injected with 90 mg/kg sodium pentobarbital solution and exsanguinated. BALs were performed by intratracheal cannulation, and instillation and extraction of 5 $\times$ 1 ml HEPES buffered saline solution containing 3 mmol/L EDTA. Lung lavage cells were enumerated using a hemocytometer, spun onto glass slides and stained with Diff-Quick (Siemens) for differential counting. Lymphocyte differentiation was performed via specific antibody staining with anti-CD4 FITC (Pharmingen, San Jose, CA), anti-CD8-APC (Pharmingen), anti-CD25-PE and anti-FoxP3 Cychrome (eBiosciences, San Diego, CA). For intracellular detection of FoxP3, surface marker staining was performed before cell fixation and permeabilization using the Cytofix/Cytoperm kit from Beckton Dickinson (San Jose, CA), following the manufacturer's protocol, followed by staining for FoxP3. Subsequent analysis was performed via fluorescence activated cell sorting analysis using a FACS Calibur (Beckton Dickinson). Gates were placed on lymphocytes based on their forward and sideward scatter profile. For subset analysis additional gates were placed either on all CD4 or CD8⁺ lymphocyte subsets using the histogram setting.

Albumin Quantification in BAL Fluid

Albumin concentrations were measured in BAL fluids as an indicator for lung damage using a quantitative colorimetric test with reagents from Eagle Diagnostics (Cedar Hill, TX) and albumin standard solution ranging from 0.1–2 mg/ml. 100 μ l sample was mixed with 50 μ l of albumin reagent (BCG) and production of green-blue

color changes read on a Thermo-Max microplate reader (Molecular Device, Silicon Valley, CA) at 628 nm and analyzed using Softmax software (Molecular Devices).

Cytokine Protein Analysis in BAL Fluids and Lung Homogenates

Cytokine concentrations in BAL fluids for TNF- α , interleukin (IL)-10, IFN- γ , MCP-1, and IL-6 were measured using the Mouse Inflammation Cytokine Bead Array (Beckton Dickinson) according to the manufacturer's protocol. Assays were read on a FACS *Calibur* machine (Beckton Dickinson) and analyzed using cytometric bead array software (Beckton Dickinson).

IFN- α and IFN- β concentrations in lung homogenates were assessed using commercially available anti-mouse IFN- α and IFN- β ELISAs through R&D Systems (Minneapolis, MN) from PBL Biomedical Laboratories. The IFN- α assay is based on the international reference standard for mouse interferon α (Mu-IFN- α) provided by the National Institutes of Health. For the assay, lungs were excised from each animal at day 1, 3, 5, and 7 post PC infection, homogenized in 5 mls of HEPES buffered saline solution, and frozen at -20°C . Once all data points were collected, frozen samples were thawed, and the cell debris were spun down at 2000 rpm in a Sorvall RT6000 centrifuge. Supernatant was collected and was used in the ELISA assay according to the manufacturer's instructions. Protein concentrations of each supernatant ranged between 220–250 $\mu\text{g/ml}$.

Monoclonal Antibody Treatment of Mice

All antibody depletion studies were performed using established protocols and depletions initiated one day before PC infection. For *in vivo* CD8 T cell-depletion, animals were twice weekly intraperitoneally injected with 300 μg of purified TIB210 rat anti-mouse monoclonal CD8 antibody.¹⁹ For *in vivo* neutralization of TNF- α , mice were intraperitoneally injected once per week with 250 μg of hamster anti-mouse TNF- α antibody (clone TN3-19.12, Leinco Technologies, Inc., St. Louis, MO).²⁰ For *in vivo* neutralization of IFN- γ , mice were injected once weekly with 250 μg of purified rat anti-mouse neutralizing IFN- γ antibody (clone R4-6A2, available through ATCC HB170 R4-6A2) following recommendations of previous publications.²¹

Histological Analysis of Lung Tissue

Right lung lobes were tied off and removed for PC enumeration, and left lung lobes were inflated with 10% neutral buffered formalin through a tracheal cannula, removed *en bloc*, and immersed in formalin for at least 24 hours, before paraffin embedding. Sections were cut 5- μm -thick on a Leica RM2155 microtome and an H&E staining was performed to assess tissue morphology and infiltrate using reagents from Richard Allen Scientific Thermo, Kalamazoo, MI and following the manufacturer's protocol. Light microscopy was performed using a Zeiss Axiostar microscope

(Zeiss, Jena, Germany). The extent of the cellular infiltrate in lung sections was assessed based on a subjective scale from 1 to 5, in which grade 1 defined no infiltrate and grade 5 defined consolidation.

Isolation of Pulmonary CD11c⁺ Cells and RNA Preparation

CD11c⁺ cells were isolated from the lungs of three individual PC-infected IFNAR^{-/-} and wild-type mice at day 7 and 14 post infection using CD11c (N418) magnetic microbeads from Miltenyi, Auburn, CA (MACS). RNA was isolated using RNeasy spin columns (Qiagen, Valencia, CA). For this, lungs of infected animals were removed, minced, and digested in 25 mls of RPMI media containing 50 U/ml collagenase IV (Sigma, St. Louis, MO) and 5 U/ml DNase (Promega, Madison, WI) for 1 hour at 37°C to obtain a single cell suspension. Lung digests were then carefully strained through a 100- μm nitex cell strainer (Fisher Scientific, Pittsburgh, PA). Cells were washed twice in PBS and the cell pellet suspended in 400 μl MACS buffer (0.5% BSA/PBS/2 mmol/L EDTA) containing 10 μg of anti-Fc receptor antibody (Pharmingen) to block unspecific Fc-receptor-mediated binding. 100 μl of MACS CD11c-beads were added to each sample and incubated for 15 minutes at $4-10^{\circ}\text{C}$, washed according to the manufacturer's instructions, and run over LS separation columns (Miltenyi). Magnetic bead-labeled cells were positively selected after several wash steps and the purity of a cell aliquot confirmed by FACS analysis using a PE-labeled rat anti-mouse CD11c antibody (eBiosciences, San Diego, CA). Cell samples with a purity of greater than 90% were used for RNA isolation using RNeasy spin columns (Qiagen) including additional on-column DNase treatment. The integrity of the RNA was confirmed using an Agilent bioanalyzer, and samples with a RIN (RNA Integrity Number) of >8 were used for further analysis.

Gene Array Analysis of Pulmonary CD11c⁺ Cells

Total RNA from the 12 samples (three biological replicates of wild-type and IFNAR^{-/-} cells at day 7 and day 14 postinfection) was amplified, biotin-labeled, and hybridized to Affymetrix GeneChip Mouse Genome Array 430 2.0 (Affymetrix, Santa Clara, CA) as described in the users' manual (Affymetrix GeneChip Expression Analysis Technical Manual, November 2004), using the GeneChip (eukaryotic) Expression 3' Amplification One-Cycle Target Labeling and Control Reagents kit (#900493). Briefly, total RNA (approximately 4 μg per sample) was reverse transcribed to cDNA using a T7-oligo(dT) primer. After second-strand cDNA synthesis, the double-stranded cDNA was purified as a template for the subsequent *in vitro* transcription (IVT) reaction. Linearly amplified biotin-labeled complementary RNA (cRNA) was synthesized in the presence of a biotinylated nucleotide analog/ribonucleic acid mix. The labeled cRNA was purified, fragmented, and hybridized to the arrays at 45°C for 16 hours with constant rotational mixing at 60 rpm. Washing and staining of the arrays was performed using the Affymetrix

Gene Chip Fluidics Station 450. Arrays were scanned using an Affymetrix GeneChip Scanner 7G and GCOS software version 1.4.

Microarray data were analyzed using GeneSpring software version 7.3 (Agilent Technologies, Palo Alto, CA). The Affymetrix CEL files were imported and normalized using Robust Multichip Averaging with median polishing,²² and genes were filtered for present calls in at least 3 of the 12 arrays (equivalent to one replicate set). The gene list was further trimmed to identify genes with fold-change differences of at least 1.5 in any comparison. Statistically significant differentially expressed genes were identified through a two-way analysis of variance using a Student *t* test ($P \leq 0.02$) and Benjamini and Hochberg multiple test correction. Hierarchical trees were generated for conditions and genes using a Pearson Correlation and an average linkage clustering algorithm. Raw data of this study were deposited into the GEO data base with the accession number: GSE 18607 <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE18607>.

TaqMan RT-PCR

To confirm regulated genes identified in our gene array analysis, semiquantitative TaqMan RT-PCR was performed using one-step RT-PCR Master mix reagents from Applied Biosystems and mouse SOCS1 specific probe and primers designed using the Primer Express software: SOCS1 forward: 5'-CCGTGGGTCGCGAGAAC-3'; SOCS1 reverse: 5'-AAGGAAGTCAGGTAGTCACGGAGTA-3'; SOCS1 probe: 6FAM-5'-TGGCGCGCATCCCTCTTAAC-CC-3'-TAMRA. As an endogenous control the TaqMan mouse GAPDH control reagents were used. Samples were set up following a standard protocol by the manufacturer including no RT controls for both GAPDH and SOCS1 in all samples as well as no template controls and analyzed on a 7500 Fast Real-Time PCR System Thermal cycler (Applied Biosystems, Carlsbad, CA).

Statistical Analysis

For statistical analysis of multiple groups either a one-way analysis of variance or two-way analysis of variance was performed. Pair-wise comparisons were made using either the post hoc Tukey test or Bonferroni test. Data were asserted to be significantly different at $P < 0.05$. For comparison of only two groups a Student *t* test was performed. *P* values < 0.05 were marked as *, *P* values < 0.01 were marked as **, and *P* values < 0.001 were marked as ***.

Results

IFN- α Production Is Induced Within the First 7 Days Post PC Lung Infection in Wild-Type Mice

In our experimental set up, CD4 T cell competent wild-type mice clear PC infection commonly between day 14

Figure 1. IFN- α production in lung homogenates of wild-type mice after PC lung infection. IFN- α concentrations were determined by ELISA in lung homogenates of wild-type mice after PC lung infection at day one, three, five, and seven post infection. Significant differences between the samples were determined by one-way analysis of variance and * $P < 0.05$ when compared with concentrations at day 1.

and 21 post infection.¹⁴ However, we were unable to detect either IFN- α or β in either BAL fluid or lung homogenates of wild-type mice at days 14 through 28 post PC lung infection by ELISA. As type I IFNs are considered early mediators of immune responses,⁸ we then determined both IFN- α and IFN- β concentrations in lung homogenates of wild-type mice early in the course of infection at days 1, 2, 3, 5, and 7. Although low, we found IFN- α concentrations significantly increased at day 7 post PC infection when compared with the earlier time points (Figure 1), however no IFN- β could be detected in the same lung homogenates (data not shown). We therefore chose day 7 as our earliest time point to further analyze the immune response elicited after PC lung infection. Subsequent time points of analysis were at days 14, 21, and 28 post infection as previously described.¹⁴

CD4-Competent IFNAR^{-/-} Mice Show a Biphasic Polarization of the Immune Response During PC Clearance

Previous analysis revealed that IFNAR^{-/-} mice responded with a delayed but then exacerbated TH-2-mediated immune response after PC lung infection when compared with wild-type mice.¹⁴ We found here that both mouse groups had similar cellular infiltrates in BAL fluid at day 7 post infection, consisting predominantly of macrophage-type cells (Figure 2A). After day 7, wild-type mice then rapidly progressed to a Th-2-type immune response with greater than 50% eosinophils present in BAL fluid at day 14 post infection^{14,16} (Figure 2B). However, we show here that in IFNAR^{-/-} mice the progression of inflammation is not delayed but results first in a predominantly lymphocytic response at day 14 (80% of all BAL cells, Figure 2C), before switching to an eosinophilic response. This was associated with a transient threefold increased albumin level (Figure 2D) and a 100-fold increased IFN- γ concentration (Figure 2E) in BAL fluid of IFNAR^{-/-} mice. Although total BAL cell numbers and the cellular composition was very similar in wild-type and IFNAR^{-/-} mice at day 7 post infection, IFNAR^{-/-} mice demonstrated a 50-fold higher TNF- α concentration in BAL fluid (Figure 2F). This remained elevated through-

Figure 2. IFNAR^{-/-} mice but not wild-type mice demonstrate a biphasic polarization of the immune response during PC clearance, which is associated with lung damage. The inflammatory responses of IFNAR^{-/-} mice (white circles) and wild-type mice (black squares) were compared during the course of PC clearance at day 7, 14, 21, and 28 post infection by examining BAL fluid. Leukocyte differentiation was performed microscopically. Demonstrated are % macrophages (A), % eosinophils (B), and % lymphocytes (C) present in the BAL fluid, as well as albumin (D), IFN-γ (E), and TNF-α concentrations (F) over the course of infection and clearance in both animal groups. PC clearance was monitored by microscopic nuclei count displayed in LOG 10 (G). Significant differences between the samples were determined by two-way analysis of variance and are indicated with **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

out the assessed time course. In wild-type mice TNF-α secretion was only transiently increased at day 14 post infection, coinciding with maximal inflammation. Although we previously reported that IL-10 concentrations were increased in wild-type BAL fluid at day 14 post infection, we found this already elevated at day 7 when compared with IFNAR^{-/-} mice¹⁴ (data not shown). We previously reported that PC clearance is slightly delayed in CD4-competent IFNAR^{-/-} mice when compared with wild-type mice.¹⁴ In present results, PC clearance also appears slightly delayed in IFNAR^{-/-} mice, however, differences between the groups were not large enough to be statistically significant (Figure 2G).

Lymphocyte subset characterization was performed via antibody staining of BAL cells for FACS analysis with CD4, CD8, CD25, and FoxP3. The percentages of CD4⁺

Figure 3. Lymphocyte differentiation in BAL fluids over the course of PC clearance in IFNAR^{-/-} and wild-type mice reveal an increased influx of both CD4 and CD8 T cells in IFNAR^{-/-} mice. Lymphocyte differentiation was performed over the course of PC clearance in BAL fluid of wild-type and IFNAR^{-/-} mice by FACS analysis. The percentage and absolute numbers of CD4⁺ (A and B) and CD8⁺ lymphocytes (C and D) was plotted from wild-type (black square) and IFNAR^{-/-} mice (white circle). Significant differences between the samples were determined by two-way analysis of variance and are indicated with **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

and CD8⁺ T cells shown in Figure 3, A and C reflect the proportion of these cells among the gated lymphocyte population. Whereas at day 7 lymphocyte subset numbers were slightly higher in BAL fluid of wild-type mice, by day 14 relative (expressed in %) and absolute (expressed as total cell numbers) CD4 and CD8⁺ T cell numbers were significantly higher in IFNAR^{-/-} mice than wild-type mice (Figure 3, A–D). Whereas relative and absolute CD4 T cell numbers continued to rise over the course of PC clearance in IFNAR^{-/-} mice (Figure 3, A and B), the influx of CD8 T cells peaked in BAL fluid at day 14 post infection (Figure 3, C and D). This coincided with peak albumin levels in this group at the same time point (Figure 2D). The percentage of CD25⁺CD4⁺ T cells in both groups ranged from 5 to 30% of all CD4⁺ cells through the course of infection (data not shown). Intracellular expression of FoxP3 in CD25⁺CD4⁺ cells was used as a marker to identify regulatory T cells.^{23,24} Our analysis revealed that at day 14 post infection wild-type mice appeared to have a relatively higher percentage of CD25⁺CD4⁺ T cells that also expressed FoxP3 (approximately 75% of all CD25⁺CD4⁺ T cells in wild-type) when compared with IFNAR^{-/-} mice (approximately 50% of all CD25⁺CD4⁺ T cells). However, based on the higher numbers of total CD4 T cells in IFNAR^{-/-} mice at this time point, the calculation of absolute cell numbers still resulted in more FoxP3⁺ CD4 cells in BAL fluid of IFNAR^{-/-} mice than in wild-type mice (approximately 1 × 10⁴ cells in wild-type versus 1 × 10⁵ cells in IFNAR^{-/-} mice; data not shown).

Figure 4. Antibiotic treatment initiated at day seven but not at day 14 post PC-infection can ameliorate development of chronic lung inflammation in $IFNAR^{-/-}$ mice. Antibiotic treatment of $IFNAR^{-/-}$ mice with SMX/TMP was initiated either at day seven or at day 14 post infection for two consecutive weeks and the outcome of inflammation assessed at day 35 post infection. **A:** PC nuclei counts as a measure of PC lung burden and PC-specific serum antibody concentration (**B**) in treated and untreated animals were measured. The extent of lung inflammation was assessed by H&E staining of lung tissue sections in some animals before initiation of antibiotic treatment at day seven and day 14 post infection (**C**) and in four animals per group after antibiotic treatment at day 35 post infection (**D**). Pictures were taken at $\times 4$ magnification, and picture insets are $\times 40$ magnification with an additional $\times 100$ magnification in **D** from the same section demonstrating eosinophilic infiltrate. The extent of infiltrate was subjectively graded on an arbitrary scale from one to five, with grade one defining no to minimal infiltrate and grade five defining complete consolidation (summarized in **E**). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Outcome of the Immune Response in $IFNAR^{-/-}$ Mice Is Determined by Day 7 Post Infection

We wanted to determine whether a critical time existed during the early immune response to PC lung infection, at which, if infection was cleared, progression of inflammation could be halted in $IFNAR^{-/-}$ mice. We therefore initiated antibiotic treatment of PC-infected $IFNAR^{-/-}$ mice with TMP/SMX-antibiotic feed at day 7, or day 14 post infection for two consecutive weeks. The extent of remaining lung inflammation was assessed at day 35 post infection to allow for enough time to initiate resolution of inflammation, should it be induced, after pathogen clearance and was compared with PC-infected but untreated $IFNAR^{-/-}$ mice. As demonstrated, antibiotic treatment eliminated PC infection in both treatment groups efficiently (Figure 4A). In the untreated group only one animal had minimal residual trophozooid counts indicating natural clearance of the infection. This was associated with the highest PC-specific antibody production in this group (Figure 4B). Histological analysis of lung tissue was performed by H&E staining and graded. Lung tissue of representative examples from infected animals before antibiotic treatment demonstrated that at day 7 and 14 post infection the inflammatory infiltrate was still minor in $IFNAR^{-/-}$ mice (Figure 4C) and was graded as 1.5 and 2.5, respectively. At day 35 post infection (Figure 4D), despite complete clearance of the pathogen in both treatment groups, only those animals that received anti-

biotic treatment starting at day 7 through 21 post PC infection had minimal remaining inflammation of the lung (graded 1.5). Indeed, in $IFNAR^{-/-}$ mice that had not started antibiotic treatment until day 14 post infection, inflammation progressively increased throughout day 35 from grade 2.5 to grade 4. As expected, $IFNAR^{-/-}$ mice that had not received treatment exhibited the most intense inflammation, graded as 5, with significant remaining eosinophilic infiltrate (see $\times 40$ or $\times 100$ magnification inset in Figure 4D). The grades were shown to be significant between the treatment groups (Figure 4E).

Pulmonary $CD11c^{+}$ Cells from Wild-Type and $IFNAR^{-/-}$ Mice Show a Unique Inflammatory Gene Expression Profile During Early Responses to PC Infection

Because immunological events around day 7 post infections appear to be critical in determining the outcome of inflammation during PC clearance, we further investigated immune responses at day 7. At day 7 post PC infection, the cellular infiltrate found in BAL fluid consisted predominantly of alveolar macrophage/DC-type cells (Figure 2A). Alveolar macrophages, as well as pulmonary dendritic cells (DCs), are characterized by variable expression of $CD11c$.^{25,26} Characterization of pulmonary $CD11c^{+}$ cells from wild-type and $IFNAR^{-/-}$ mice by FACS analysis revealed variable co-expression of MH-

Figure 5. Gene and conditioning tree of differentially regulated genes expressed in pulmonary CD11c⁺ cells from IFNAR^{-/-} and wild-type mice as determined by gene array analysis. Gene array analysis was performed with RNA from pulmonary CD11c⁺ cells isolated from wild-type and IFNAR^{-/-} mice at day seven and 14 post PC infection. RNA from three individual animals per group and time point was assessed. Displayed is a gene and conditioning tree of significantly regulated genes between the comparison groups that demonstrates high reproducibility.

CII, CD40, B220, and CD11b between the groups and time points suggesting functional heterogeneity of these cells (data not shown). To determine whether type-I IFN signaling in pulmonary CD11c⁺ cells of wild-type mice was induced after PC lung infection, we performed an extensive gene expression analysis of enriched pulmonary CD11c⁺ cells isolated from lung digests of both wild-type and IFNAR^{-/-} mice at day seven and 14 post infection using the Affymetrix Gene Chip Mouse Genome Array 430 2.0.

This analysis revealed that approximately 18,000 of 35,000 genes were called present in at least one condition. Of these genes 3100 were at least 1.5-fold differentially regulated between the groups ($P < 0.02$). Figure 5 shows a Gene and Condition Tree of the data demonstrating good reproducibility between the replicates in each group. A K-means analysis identified eight distinct sets of gene expression patterns between the comparison groups (K-means analysis not shown). We were especially interested in those genes that were differentially regulated at day 7 post infection between the groups and found that: (1) 178 genes were uniquely up-regulated in wild-type animals at day 7 post infection; (2) 501 genes

Figure 6. Gene expression analysis of pulmonary CD11c⁺ cells from IFNAR^{-/-} and wild-type mice after PC lung infections reveal a unique inflammatory profile between the groups. Demonstrated are the expression profiles of some of the regulated genes identified between the comparison groups. Expression intensity is displayed as normalized ratio to allow for multiple chip comparison. **A:** Expression level of genes coding for cell surface markers characterizing the analyzed cell population. **B:** Expression level of various type I IFN-regulated genes between the groups. **C:** Expression of various SOCS (suppressor of cytokine signaling molecules) between the groups. **D:** Differential expression level of various cytokines and growth factors. **E:** Expression of various chemokines. All genes displayed in **B-E** are significantly regulated between the groups with a P value < 0.02 as identified through a two-way analysis of variance using a Student t test and Benjamini and Hochberg multiple test correction.

were uniquely up-regulated in IFNAR^{-/-} mice at day 7; and (3) 185 genes were uniquely up-regulated only in wild-type animals at day 7 and 14 post infection.

Many of the highly regulated genes were not annotated, however; in Figure 6, A-E is a small selection of annotated genes that are regulated between the groups.

Gene expression levels are demonstrated as normalized ratio. RNA expression for various genes representing surface markers that characterize the cell population analyzed is depicted in Figure 6A. Although the expression level for the marker CD11c was equal throughout the time points and between the comparison groups (white bar), various other activation and differentiation markers, including CD40, DC-Sign, CD83, and CD86 varied in their expression in wild-type and IFNAR^{-/-} mice and between the time points.

At day 7 post infection, we found a multitude of type-I IFN-responsive genes uniquely up-regulated in pulmonary CD11c⁺ cells from wild-type mice but not IFNAR^{-/-} mice (Figure 6B). The genes included Mx1, OAS1 and 2, IRF7, ISG54, IL-15, and others. Most of these genes were already down-regulated by day 14 post infection to levels seen in IFNAR^{-/-} mice. In conjunction with the up-regulation of effector-molecules of the type-I IFN pathway, specific regulatory molecules of this response were concomitantly up-regulated. Coinciding with the type-I IFN response in wild-type CD11c⁺ cells was the upregulation of molecules of the Suppressor of Cytokine Signaling (SOCS) family. Of particular interest was the eightfold higher expression of SOCS1 (Figure 6C, red bar) and a threefold higher expression of SOCS3 (white bar) in wild-type animals at day 7 when compared with IFNAR^{-/-} mice. In IFNAR^{-/-} mice, a comparable level of SOCS1 expression was not seen until day 14 post infection.

In contrast, a unique proinflammatory gene expression profile was detected in pulmonary CD11c⁺ cells isolated from IFNAR^{-/-} mice (Figure 6D) especially at day 7 post infection. We found, consistent with the protein analysis in BAL fluid (see Figure 2F), a unique up-regulation of TNF- α expression (Figure 6D: black bar). We also found a unique up-regulation of pro-inflammatory cytokines such as IL-18, IL-16, Follistatin-like 1, and the profibrotic CTGF (Connective Tissue Growth Factor). Many of these factors were not only expressed at lower levels in wild-type mice at day 7, but were even more dramatically down-regulated at day 14 post infection when compared with IFNAR^{-/-} mice.

In addition, a variety of chemokines involved in eosinophil-recruitment, such as MCP-3 (CCL7), Eotaxin-2 (CCL24), and MCP-2 (CCL8) were in wild-type pulmonary CD11c⁺ cells, already up-regulated at day 7 post infection but were not up-regulated until day 14 in pulmonary CD11c⁺ cells from IFNAR^{-/-} mice (Figure 6E). This is congruent with the delayed eosinophilic influx seen in IFNAR^{-/-} mice.

Early and Continuously High Secretion of TNF- α Is Not the Cause of Chronic Inflammation in IFNAR^{-/-} Mice After PC Lung Infection

Responses initiated at day 7 post PC infection appeared instrumental in predicting the outcome of inflammation in IFNAR^{-/-} mice. At day 7, IFNAR^{-/-} mice had distinct and high TNF- α concentrations in their BAL fluid (Figure 2F), at least in part derived from pulmonary CD11c⁺ cells (Figure 6D). We wanted to assess whether neutralization

of early TNF- α could have beneficial effects on the outcome of lung inflammation. We also wanted to determine whether CD8 T cells recruited into the lung by day 14 post infection were effector cells responsible for the extent of inflammation and lung damage in CD4-competent IFNAR^{-/-} mice (see Figures 3 and 5). Therefore, CD4 T cell-competent IFNAR^{-/-} mice were either treated with neutralizing anti-TNF- α antibody; or CD8 T cell-depleted via anti-CD8 T cell antibody injection. The effects of treatment were compared with infected but untreated IFNAR^{-/-} mice at day 28 post infection. H&E staining of lung tissue demonstrated no significant difference in the extent of remaining inflammation in any treatment group (Figure 7A). The extent of infiltrate was graded as 5 for the untreated and anti-CD8-treated mouse group and 4.5 for the anti-TNF- α treated group as compared with uninfected IFNAR^{-/-} mice, graded 1 (Figure 7B). Despite infiltrate levels, TNF- α concentrations in BAL fluid from anti-TNF- α -treated mice were below the detection limit of the assay (Figure 7C) and CD8 T cells were absent in BAL fluid of all IFNAR^{-/-} mice in the anti-CD8-treated group (data not shown). Interestingly, PC lung burden in both untreated and anti-TNF- α -treated IFNAR^{-/-} mice were only slightly above the detection limit but much reduced over the initial infectious dose given. However, CD8 T cell-depleted IFNAR^{-/-} mice showed a significantly increased PC lung burden (Figure 7D), although antibody titers were comparable with the untreated mouse group and was higher than in the anti-TNF- α -treated group (Figure 7E).

Neutralization of IFN- γ in IFNAR^{-/-} Mice Prevents the Course of Chronic Lung Inflammation

IFNAR^{-/-} mice demonstrated a transient but greatly increased production of IFN- γ at or around day 14 post PC lung infection, which coincided with high albumin leakage suggesting lung damage (Figure 2, D and E). To determine whether IFN- γ caused the chronic inflammation, we treated IFNAR^{-/-} mice with neutralizing anti-IFN- γ antibody and compared the degree of inflammation to untreated IFNAR^{-/-} mice at day 28 post infection. Neutralization of IFN- γ did not interfere with PC clearance (Figure 8A). However, total BAL cell numbers and albumin concentrations were significantly lower in the anti-IFN- γ -treated group when compared with the untreated IFNAR^{-/-} group (Figure 8, B and C), which was associated with a reduced proportion of eosinophils in the BAL fluids (Figure 8D) of the anti-IFN- γ -treated group. In accordance with the BAL data, histological analysis of lung tissue demonstrated significantly reduced infiltrate in anti-IFN- γ -treated mice in which the extent of infiltrate was graded 1.5–2.5 compared with untreated IFNAR^{-/-} mice, graded at 4.5–5 (Figure 8, E and F). These data demonstrated that the transient but excessive IFN- γ response in IFNAR^{-/-} mice is the mediator of chronic inflammation during PC clearance in IFNAR^{-/-} mice. To determine the cellular source of IFN- γ production we infected four groups of IFNAR^{-/-} mice with PC, and each

Figure 7. Neither the neutralization of TNF- α nor the depletion of CD8⁺ T cells prevents chronic inflammation in IFNAR^{-/-} mice. Three groups of IFNAR^{-/-} mice were PC-infected and received either no additional treatment, were CD8 T cell-depleted by antibody injection (clone TIB210), or received neutralizing anti-TNF- α antibody treatment (clone TN3-19.12). The extent of remaining pulmonary inflammation was assessed by lung histology and H&E staining at day 28 post PC infection and was compared with uninfected mice (**A**). The extent of infiltrate was subjectively graded on an arbitrary scale of one-five, with grade one defining no to minimal infiltrate and grade five defining complete consolidation and results are summarized in **B**. TNF- α -concentrations (**C**), total BAL cell numbers, PC lung burden (**D**), and PC specific serum IgG (**E**) were also measured at day 28 post infection. Significant differences between the groups were assessed by one-way analysis of variance analysis and are indicated with * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. NS, not significant.

group was either CD4 T cell-depleted, or CD8 T cell-depleted or CD4 and CD8 T cell-depleted. IFN- γ levels in BAL fluids were determined at day 16 post infection and compared with infected but untreated IFNAR^{-/-} mice. These data demonstrated that only those mice that received anti-CD4 T cell treatment had low (only CD4 T cell-depleted) or no (anti-CD4 and anti-CD8 T cell-depleted) detectable IFN- γ concentrations present in their BAL fluid at this time point indicates that the production of IFN- γ was CD4-T cell-dependent (Figure 8G).

To understand how immunological events at day 7 could lead to the detrimental IFN- γ response at day 14 post PC lung infection in IFNAR^{-/-} mice we specifically searched our array data for genes known to be involved in the regulation of IFN- γ . Type-I IFNs and IFN- γ induce, and are negatively regulated by SOCS1 in a feedback regulatory mechanism.¹³ The lack of SOCS1 expression, particularly in DCs, has been demonstrated to be responsible for the excessive IFN- γ -mediated hyperinflammatory response in SOCS1^{-/-} mice.²⁷ Pulmonary CD11c⁺ cells of IFNAR^{-/-} mice lack the expression of SOCS1 at day 7 post infections, and it was only delayed induced at day 14 (Figure 6C). To confirm that SOCS1 expression was indeed reduced in pulmonary CD11c⁺ cells at day 7 post PC infection we performed quantitative Taqman PCR from RNA isolated from three independent samples of enriched pulmonary CD11c⁺ cells from wild-type and IFNAR^{-/-} mice. As demonstrated in Figure 9, SOCS1 expression was indeed eightfold reduced in pulmonary CD11c⁺ cell derived from infected IFNAR^{-/-} mice as compared with cells from wild-type animals. This analysis

confirmed our array data in independent samples and could potentially connect the transient high secretion of IFN- γ in IFNAR^{-/-} mice to the lack of SOCS1 during the early phase of the immune response to PC.

Discussion

The mechanism underlying chronic pulmonary complications such as chronic obstructive lung diseases, asthma, and pulmonary hypertension in patients under HAART is poorly understood.^{3,28} We recently demonstrated in CD4 T cell-competent mice that lack of type-I IFN signaling results in a chronic inflammatory lung disease with tissue fibrosis after PC lung infection, despite subsequent pathogen clearance.¹⁴ We now further characterized how type-I IFN signaling functions to facilitate resolution of inflammation during PC clearance.

Here we established that in IFNAR^{-/-} mice, immunological events around day 7 post PC infection determined whether inflammation would be resolved or not, regardless of whether PC clearance was subsequently achieved. At day 7, the predominant inflammatory cells in BAL fluid of both IFNAR^{-/-} and wild-type mice were CD11c⁺ alveolar macrophage and DC-type cells. This was associated with peak IFN- α concentrations in lung homogenates of wild-type mice whereas IFNAR^{-/-} mice, and not wild-type mice, had high TNF- α concentrations in BAL fluids. These data suggested that pulmonary immune sentinel cells of IFNAR^{-/-} mice responded to PC

Figure 8. Anti-IFN- γ treatment prevents chronic inflammation after PC lung infection in IFNAR^{-/-} mice. Two groups of IFNAR^{-/-} mice were PC infected and either treated with neutralizing anti-IFN- γ antibody or left untreated. PC lung burden (**A**), total BAL cell numbers (**B**), BAL albumin concentrations (**C**), and % eosinophils in BAL fluids (**D**) were determined at day 28 post infection. The extent of residual lung inflammation at day 28 post infection was assessed by lung histology in four animals per group by H&E staining (**E**). The extent of infiltrate was subjectively graded on an arbitrary scale of one–five, with grade one defining no to minimal infiltrate and grade five defining complete consolidation and is summarized in **F**. In a separate experiment, IFN- γ concentrations were measured in BAL fluid in CD4 T cell–depleted, CD8 T cell–depleted, CD4/CD8 T cell–depleted, and untreated IFNAR^{-/-} mice and wild-type mice at day 16 post infection (**G**). Statistical differences were assessed between the two comparison groups using a one-way analysis of variance and are indicated with * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

lung infection equally early but had a distinct activation profile.

Both type-I IFNs and TNF- α are important coordinators and effector molecules of the innate and acquired immune response and their production is efficiently elicited, e.g., following Toll-like receptor–signaling in specific macrophages and DC subsets.²⁹ Using a *Pneumocystis* pneumonia mouse model Zhang et al demonstrated that TLR2 signaling is important in the induction of the innate response to PC lung infection and results in TNF- α secretion.^{30,31} However, other TLRs such as TLR4 and TLR3 must also be involved to elicit type-I IFN production.³² Type-I IFN signaling is critical for the maturation of professional antigen presenting cells (APCs).^{33–35} After PC infection, absence or presence of early type-I IFN–

signaling is likely to activate pulmonary DCs and alveolar macrophages shaping the acquired immune response and thus the outcome of inflammation. In support of this we found transient but distinct differences during the initiation of the acquired immune response between the comparison groups. In IFNAR^{-/-} mice the acquired immune response to PC infection was not delayed, as initially thought,¹⁴ but was first predominated by a TH1–mediated lymphocyte response by day 14 post infection, which developed into a persistent TH2–mediated eosinophilic response by day 21. This early lymphocytic response was associated with transient but high IFN- γ production coupled with high albumin leakage, suggesting lung damage.³⁶ In wild-type mice, and not in IFNAR^{-/-} mice, the immediate progression to a TH2–

Figure 9. Quantitative PCR confirms reduced SOCS1 expression in pulmonary CD11c⁺ cells isolated from IFNAR^{-/-} versus wild-type mice at day seven post *Pneumocystis* infection. Relative quantification of SOCS1 expression in pulmonary CD11c⁺ cells isolated from three individual wild-type and IFNAR^{-/-} mice was performed using TaqMan technique with gene-specific probes and primers for mouse SOCS1 as the target gene and GAPDH as the endogenous control gene. Expression levels are demonstrated in Log₁₀ relative quantification with the expression level in wild-type mice as reference. Statistical differences were assessed between the two comparison groups using a Student *t* test and are indicated with **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

mediated response was not only associated with an early induction of the immune regulatory cytokine IL-10 at day 7¹⁴ (data not shown), but mice also exhibited minimal evidence for lung damage during the maximal immune response at day 14. Qureshi et al,³⁷ and Ruan et al,³⁸ have demonstrated a role of IL-10 in the modulation of the immune response to PC during clearance and in the prevention of lung damage during the course of *Pneumocystis* pneumonia in CD4 T cell-depleted mice, which is likely also true in CD4-competent mice. Although type I IFNs are known as potent inflammatory mediators,^{39,40} Chang et al⁴¹ recently demonstrated that LPS-induced TLR-4 activation in bone marrow-derived macrophages results in a type I IFN-dependent high secretion of IL10, which modulates the extent of the production of proinflammatory mediators such as TNF- α , IL-1, and IL-6. Based on these findings, a novel anti-inflammatory role of type-I IFNs was proposed. This is also suggested by our model of chronic lung inflammation in the absence of type-I IFN signaling after PC lung infection despite subsequent clearance.

Regulatory T cells modulate inflammatory responses elicited by effector T cells via cognate as well as cytokine-mediated mechanisms.^{42,43} CD25⁺FoxP3⁺ regulatory CD4 T cells dampen the inflammatory immune response to PC infection of the lung,⁴⁴ and we found a decreased percentage of FoxP3⁺ cells within the CD25⁺ CD4⁺ T cell subset present in BAL fluid of IFNAR^{-/-} mice when compared with wild-type mice during peak lymphocyte influx (data not shown). However, absolute numbers of this subset were still higher in IFNAR^{-/-} mice compared with wild-type mice. Therefore, it is not clear whether this relative difference is biologically relevant in our system. However, decreased and/or delayed IL-10 secretion combined with a relative decrease of CD4 T

cells with a potential regulatory phenotype during peak inflammation in IFNAR^{-/-} mice could point to an imbalance of immune regulatory mechanisms concomitantly induced during PC clearance. Thus, lack of type-I IFN signaling during early initiation of inflammation may not necessarily interrupt mechanisms of pathogen clearance but could have profound effects on inflammation control.

By performing gene array studies we were able to demonstrate that type I IFN signaling is indeed induced after PC infection in pulmonary CD11c⁺ cells of wild-type mice, but not in cells derived from IFNAR^{-/-} mice. Type-I IFN responsive genes were transiently but highly expressed at day 7 and not at day 14 post infection. This indicated a temporal effect of type-I IFNs on wild-type pulmonary CD11c⁺ cells and a role of type-I IFNs in their priming during the course of PC infection. Furthermore, regulatory molecules such as the suppressor of cytokine signaling molecules SOCS1 and SOCS3 were simultaneously induced in wild-type pulmonary CD11c⁺ cells. This supported the hypothesis that immune regulatory and activating mechanisms to PC infection are concomitantly induced in wild-type mice. In contrast, at day 7 post infection pulmonary CD11c⁺ cells from IFNAR^{-/-} mice demonstrated a unique proinflammatory cytokine profile including up-regulation of TNF- α , IL-1, IL-6, and Follistatin-like 1 (FSL1), which is an inducer of TNF- α ⁴⁵ and lacked induction of the above mentioned SOCS molecules. These proinflammatory cytokines were not only significantly reduced in expression in CD11c⁺ cells from wild-type mice at day 7 but were even more dramatically down-regulated at day 14 post infection indicating their tight control in the presence of type I IFN-signaling. These data demonstrated that absence of type-I IFN-signaling results in an alternative early activation pathway of pulmonary sentinel cells such as CD11c⁺ DCs and alveolar macrophages after PC lung infections. This is likely to have caused the distinct immune activation pattern of the acquired immune response in IFNAR^{-/-} mice.

Both TNF- α and type-I IFNs are associated with chronic inflammatory disease when deregulated. Bancherau et al³³ proposed that a dichotomy in DC activation exists depending on whether they differentiate in the presence of type-I IFNs (IFN-DCs) or TNF- α (TNF-DCs). An imbalance between these two DC-types could lead to excessive inflammation.^{33,46} However, *in vivo* neutralization of continuously high TNF- α levels in IFNAR^{-/-} mice over the course of PC infection and clearance did not improve the chronic lung inflammation. These findings demonstrated that although TNF- α is a "usual suspect" in the cause of many chronic inflammatory diseases, other factors must be driving the progression of inflammation in this model. In this regard we noticed in CD4-competent IFNAR^{-/-} mice, a significant influx of additional CD8 T cells into the lungs at day 14 post infection, which did not occur to this extent in wild-type mice. This raised the question whether the transient albumin peak at day 14 in CD4-competent IFNAR^{-/-} mice was caused by CD8 T cell-mediated damage³⁶ and whether this was a precondition for chronic inflammation. However, depletion of CD8 T cells from CD4-competent IFNAR^{-/-} mice also did not improve the outcome of inflammation after PC infection. Instead it interfered with pathogen clearance. In

other studies Steele et al,⁴⁷ as well as Kolls et al,⁴⁸ demonstrated an additional role of CD8 T cells in increased resistance to PC infection as a result of IFN- γ -mediated mechanisms. On the contrary, Garvy et al⁴⁹ demonstrated that CD4-competent IFN- γ -deficient mice had normal pathogen clearance, but that IFN- γ had a regulatory role in the inflammatory response. These findings suggest a great flexibility in the immunological mechanisms required for PC clearance but that the mechanism used may determine the extent of overall lung damage induced and whether complete restitution of organ functions follows pathogen clearance.

At day 14 post PC infection, CD4 T cell-competent IFNAR^{-/-} mice demonstrated a transient peak of IFN- γ secretion and high albumin concentration in BAL fluid indicating lung damage. Neutralization of this IFN- γ response via treatment of IFNAR^{-/-} mice with anti-IFN- γ antibody indeed prevented the development of the chronic inflammation without negatively affecting PC clearance. This clearly linked excessive IFN- γ secretion in IFNAR^{-/-} mice to the induction of chronic inflammation during the course of PC clearance. Our results indicate that the source of IFN- γ production was CD4 T cell-dependent, as depletion of CD8 T cells does not improve the outcome of inflammation, and depletion of CD4 T cells resulted in elimination of IFN- γ at day 16 post infection in BAL fluid. Whether CD4 T cells produce the IFN- γ directly or a small subset of potentially IFN- γ -producing NK cells is currently under investigation.

It is currently not clear how this transient IFN- γ response in IFNAR^{-/-} mice at day 14 post infection is connected to immunological events at day 7 that appear to determine the outcome of inflammation in IFNAR^{-/-} mice. However, as a powerful proinflammatory cytokine, signaling and extent of IFN- γ secretion is known to be tightly regulated. In this regard Nguyen et al⁵⁰ demonstrated in a mouse model for LCMV infection that type I IFNs negatively regulate IFN- γ -responses via STAT1-mediated pathways. It is now also known that SOCS1 interferes with this pathway and has a critical and nonredundant role in the negative regulation of IFN- γ functions.^{51,52} Our gene expression analysis of pulmonary CD11c⁺ cells demonstrated that the early type-I IFN response at day 7 post infection was associated with significant upregulation of SOCS1 in pulmonary CD11c⁺ cells from wild-type but not in IFNAR^{-/-} mice. This was confirmed by Taqman RT-PCR in independent samples. SOCS1 is induced by, and negatively regulates, an array of cytokines and growth factors including type I IFNs and IFN- γ and interferes with TLR-signaling. SOCS1^{-/-} macrophages also produce greater amounts of TNF- α and IL-12 compared with wild-type cells in response to LPS.⁵³ Lack of SOCS1 in knock-out mice results in an IFN- γ -mediated, ubiquitous, and spontaneous immune pathology resulting in death of the animals.^{51,52} By using conditional null mice, it was demonstrated that SOCS1 expression specifically in DCs, and not T cells, is necessary to prevent this pathology.⁵⁴ Further experiments demonstrated that early induction of type-I IFNs also induce SOCS1 expression which may prevent downstream IFN- γ production and signaling and thus the immune pathology seen in these SOCS1^{-/-} mice.⁵⁵ It is therefore possible that the lack of early (day 7) induction of regulatory mechanisms such as SOCS1 by

type-I IFNs in pulmonary CD11c⁺ cells of IFNAR^{-/-} mice could result in deviated immune activation with excessive induction and signaling of IFN- γ in T cells, causing lung damage, which requires healing by fibrosis. Induction of SOCS1 expression in pulmonary CD11c⁺ cells from IFNAR^{-/-} mice at day 14 post infection may be initiated by IFN- γ signaling, which then stimulates a negative feed-back regulatory mechanism and allows for the progression to a delayed TH-2 response by day 21.

Regardless of the exact mechanisms involved, the data in this mouse model demonstrate an important role of type I IFNs in controlling inflammation. This may also point to the importance of complete immune reconstitution of both the type-I IFN system and CD4 T cell functions in the prevention of opportunistic infection, and induction of aberrant immune responses leading to tissue damage. It is possible that lack of sufficient recovery of type-I IFN-producing cells in HIV-infected individuals under HAART could result in the lack of immune regulatory mechanisms, which in the lung may result in unexplained inflammatory complications despite pathogen clearance. Low-dose type-I IFN therapy may be a consideration in those patients under HAART who do not demonstrate good recovery of the type-I IFN production to prevent chronic inflammatory diseases.

Acknowledgments

We thank Dr. Mark Jutila, Dr. David Pascual, David Taylor, and Michelle Wilkison for critical reading of the manuscript.

References

1. Sepkowitz KA: Opportunistic infections in patients with and patients without Acquired Immunodeficiency Syndrome. *Clin Infect Dis* 2002, 34:1098–1107
2. Thomas CF Jr, Limper AH: Pneumocystis pneumonia. *N Engl J Med* 2004, 350:2487–2498
3. Hull MW, Phillips P, Montaner JS: Changing global epidemiology of pulmonary manifestations of HIV/AIDS. *Chest* 2008, 134:1287–1298
4. Marchetti G, Franzetti F, Gori A: Partial immune reconstitution following highly active antiretroviral therapy: can adjuvant interleukin-2 fill the gap? *J Antimicrob Chemother* 2005, 55:401–409
5. Siegal FP, Fitzgerald-Bocarsly P, Holland BK, Shodell M: Interferon-alpha generation and immune reconstitution during antiretroviral therapy for human immunodeficiency virus infection. *AIDS* 2001, 15:1603–1612
6. Siegal F: Interferon-producing plasmacytoid dendritic cells and the pathogenesis of AIDS. *Res Initiat Treat Action* 2003, 8:10–13
7. Soumelis V, Scott I, Gheysa F, Bouhour D, Cozon G, Cotte L, Huang L, Levy JA, Liu YJ: Depletion of circulating natural type 1 interferon-producing cells in HIV-infected AIDS patients. *Blood* 2001, 98: 906–912
8. Levy DE, Marie I, Prakash A: Ringing the interferon alarm: differential regulation of gene expression at the interface between innate and adaptive immunity. *Curr Opin Immunol* 2003, 15:52–58
9. Lanzavecchia A, Sallusto F: Regulation of T cell immunity by dendritic cells. *Cell* 2001, 106:263–266
10. Liu YJ, Kanzler H, Soumelis V, Gilliet M: Dendritic cell lineage, plasticity and cross-regulation. *Nat Immunol* 2001, 2:585–589
11. Longman RS, Braun D, Pellegrini S, Rice CM, Darnell RB, Albert ML: Dendritic-cell maturation alters intracellular signaling networks, enabling differential effects of IFN-alpha/beta on antigen cross-presentation. *Blood* 2007, 109:1113–1122
12. Hanada T, Yoshimura A: Regulation of cytokine signaling and inflammation. *Cytokine Growth Factor Rev* 2002, 13:413–421

13. Yoshimura A, Mori H, Ohishi M, Aki D, Hanada T: Negative regulation of cytokine signaling influences inflammation. *Curr Opin Immunol* 2003, 15:704–708
14. Meissner NN, Swain S, Tighe M, Harmsen A: Role of type I IFNs in pulmonary complications of *Pneumocystis murina* infection. *J Immunol* 2005, 174:5462–5471
15. Muller U, Steinhoff U, Reis LF, Hemmi S, Pavlovic J, Zinkernagel RM, Aguet M: Functional role of type I and type II interferons in antiviral defense. *Science* 1994, 264:1918–1921
16. Swain SD, Meissner NN, Harmsen AG: CD8 T cells modulate CD4 T-cell and eosinophil-mediated pulmonary pathology in pneumocystis pneumonia in B-cell-deficient mice. *Am J Pathol* 2006, 168:466–475
17. Harmsen AG, Stankiewicz M: Requirement for CD4+ cells in resistance to *Pneumocystis carinii* pneumonia in mice. *J Exp Med* 1990, 172:937–945
18. Harmsen AG: Role of alveolar macrophages in lipopolysaccharide-induced neutrophil accumulation. *Infect Immun* 1988, 56:1858–1863
19. Wright TW, Gigliotti F, Finkelstein JN, McBride JT, An CL, Harmsen AG: Immune-mediated inflammation directly impairs pulmonary function, contributing to the pathogenesis of *Pneumocystis carinii* pneumonia. *J Clin Invest* 1999, 104:1307–1317
20. Ferran C, Sheehan K, Schreiber R, Bach JF, Chatenoud L: Anti-TNF abrogates the cytokine-related anti-CD3 induced syndrome. *Transplant Proc* 1991, 23:849–850
21. Kawakami K, Qureshi MH, Zhang T, Koguchi Y, Shibuya K, Naoe S, Saito A: Interferon-gamma (IFN-gamma)-dependent protection and synthesis of chemoattractants for mononuclear leucocytes caused by IL-12 in the lungs of mice infected with *Cryptococcus neoformans*. *Clin Exp Immunol* 1999, 117:113–122
22. Bolstad BM, Irizarry RA, Astrand M, Speed TP: A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* 2003, 19:185–193
23. Pillai V, Ortega SB, Wang CK, Karandikar NJ: Transient regulatory T-cells: a state attained by all activated human T-cells. *Clin Immunol* 2007, 123:18–29
24. Fontenot JD, Rudensky AY: A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. *Nat Immunol* 2005, 6:331–337
25. Holt PG, Stumbles PA: Regulation of immunologic homeostasis in peripheral tissues by dendritic cells: the respiratory tract as a paradigm. *J Allergy Clin Immunol* 2000, 105:421–429
26. Stumbles PA, Upham JW, Holt PG: Airway dendritic cells: co-ordinators of immunological homeostasis and immunity in the respiratory tract. *Apmis* 2003, 111:741–755
27. Hanada T, Yoshida H, Kato S, Tanaka K, Masutani K, Tsukada J, Nomura Y, Mimata H, Kubo M, Yoshimura A: Suppressor of cytokine signaling-1 is essential for suppressing dendritic cell activation and systemic autoimmunity. *Immunity* 2003, 19:437–450
28. Grubb JR, Moorman AC, Baker RK, Masur H: The changing spectrum of pulmonary disease in patients with HIV infection on antiretroviral therapy. *AIDS* 2006, 20:1095–1107
29. Stetson DB, Medzhitov R: Type I interferons in host defense. *Immunity* 2006, 25:373–381
30. Zhang C, Wang SH, Lasbury ME, Tschang D, Liao CP, Durant PJ, Lee CH: Toll-like receptor 2 mediates alveolar macrophage response to *Pneumocystis murina*. *Infect Immun* 2006, 74:1857–1864
31. Zhang C, Wang SH, Liao CP, Lasbury ME, Durant PJ, Tschang D, Lee CH: Toll-like receptor 2 knockout reduces lung inflammation during *Pneumocystis pneumonia* but has no effect on phagocytosis of *Pneumocystis* organisms by alveolar macrophages. *J Eukaryot Microbiol* 53 Suppl 2006, 1:S132–133
32. Barton GM, Medzhitov R: Toll-like receptor signaling pathways. *Science* 2003, 300:1524–1525
33. Banchereau J, Pascual V, Palucka AK: Autoimmunity through cytokine-induced dendritic cell activation. *Immunity* 2004, 20:539–550
34. Rutella S, Bonanno G, Pierelli L, Mariotti A, Capoluongo E, Contemi AM, Ameglio F, Curti A, De Ritis DG, Voso MT, Perillo A, Mancuso S, Scambia G, Lemoli RM, Leone G: Granulocyte colony-stimulating factor promotes the generation of regulatory DC through induction of IL-10 and IFN-alpha. *Eur J Immunol* 2004, 34:1291–1302
35. Beignon AS, Skoberne M, Bhardwaj N: Type I interferons promote cross-priming: more functions for old cytokines. *Nat Immunol* 2003, 4:939–941
36. Meissner NN, Lund FE, Han S, Harmsen A: 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. *J Immunol* 2005, 175:8271–8279
37. Qureshi MH, Harmsen AG, Garvy BA: IL-10 modulates host responses and lung damage induced by *Pneumocystis carinii* infection. *J Immunol* 2003, 170:1002–1009
38. Ruan S, Tate C, Lee JJ, Ritter T, Kolls JK, Shellito JE: Local delivery of the viral interleukin-10 gene suppresses tissue inflammation in murine *Pneumocystis carinii* infection. *Infect Immun* 2002, 70:6107–6113
39. Belardelli F, Ferrantini M, Proietti E, Kirkwood JM: Interferon-alpha in tumor immunity and immunotherapy. *Cytokine Growth Factor Rev* 2002, 13:119–134
40. Samuel CE: Antiviral actions of interferons. *Clin Microbiol Rev* 2001, 14:778–809, table of contents
41. Chang EY, Guo B, Doyle SE, Cheng G: Cutting edge: involvement of the type I IFN production and signaling pathway in lipopolysaccharide-induced IL-10 production. *J Immunol* 2007, 178:6705–6709
42. von Boehmer H: Mechanisms of suppression by suppressor T cells. *Nat Immunol* 2005, 6:338–344
43. Taylor A, Verhagen J, Blaser K, Akdis M, Akdis CA: Mechanisms of immune suppression by interleukin-10 and transforming growth factor-beta: the role of T regulatory cells. *Immunology* 2006, 117:433–442
44. McKinley L, Logar AJ, McAllister F, Zheng M, Steele C, Kolls JK: Regulatory T cells dampen pulmonary inflammation and lung injury in an animal model of pneumocystis pneumonia. *J Immunol* 2006, 177:6215–6226
45. Miyamae T, Marinov AD, Sowders D, Wilson DC, Devlin J, Boudreau R, Robbins P, Hirsch R: Follistatin-like protein-1 is a novel proinflammatory molecule. *J Immunol* 2006, 177:4758–4762
46. Banchereau J, Pascual V: Type I interferon in systemic lupus erythematosus and other autoimmune diseases. *Immunity* 2006, 25:383–392
47. Steele C, Zheng M, Young E, Marrero L, Shellito JE, Kolls JK: Increased host resistance against *Pneumocystis carinii* pneumonia in gamma delta T-cell-deficient mice: protective role of gamma interferon and CD8(+) T cells. *Infect Immun* 2002, 70:5208–5215
48. Kolls JK, Habetz S, Shean MK, Vazquez C, Brown JA, Lei D, Schwarzenberger P, Ye P, Nelson S, Summer WR, Shellito JE: IFN-gamma and CD8+ T cells restore host defenses against *Pneumocystis carinii* in mice depleted of CD4+ T cells. *J Immunol* 1999, 162:2890–2894
49. Garvy BA, Ezekowitz RA, Harmsen AG: Role of gamma interferon in the host immune and inflammatory responses to *Pneumocystis carinii* infection. *Infect Immun* 1997, 65:373–379
50. Nguyen KB, Cousens LP, Doughty LA, Pien GC, Durbin JE, Biron CA: Interferon alpha/beta-mediated inhibition and promotion of interferon gamma: sTAT1 resolves a paradox. *Nat Immunol* 2000, 1:70–76
51. Marine JC, Topham DJ, McKay C, Wang D, Parganas E, Stravopodis D, Yoshimura A, Ihle JN: SOCS1 deficiency causes a lymphocyte-dependent perinatal lethality. *Cell* 1999, 98:609–616
52. Alexander WS, Starr R, Fenner JE, Scott CL, Handman E, Sprigg NS, Corbin JE, Cornish AL, Darwiche R, Owczarek CM, Kay TW, Nicola NA, Hertzog PJ, Metcalf D, Hilton DJ: SOCS1 is a critical inhibitor of interferon gamma signaling and prevents the potentially fatal neonatal actions of this cytokine. *Cell* 1999, 98:597–608
53. Nakagawa R, Naka T, Tsutsui H, Fujimoto M, Kimura A, Abe T, Seki E, Sato S, Takeuchi O, Takeda K, Akira S, Yamanishi K, Kawase I, Nakanishi K, Kishimoto T: SOCS-1 participates in negative regulation of LPS responses. *Immunity* 2002, 17:677–687
54. Hanada T, Tanaka K, Matsumura Y, Yamauchi M, Nishinakamura H, Aburatani H, Mashima R, Kubo M, Kobayashi T, Yoshimura A: Induction of hyper Th1 cell-type immune responses by dendritic cells lacking the suppressor of cytokine signaling-1 gene. *J Immunol* 2005, 174:4325–4332
55. Fenner JE, Starr R, Cornish AL, Zhang JG, Metcalf D, Schreiber RD, Sheehan K, Hilton DJ, Alexander WS, Hertzog PJ: Suppressor of cytokine signaling 1 regulates the immune response to infection by a unique inhibition of type I interferon activity. *Nat Immunol* 2006, 7:33–39