Respiratory Reovirus 1/L Induction of Intraluminal Fibrosis, a Model of Bronchiolitis Obliterans Organizing Pneumonia, Is Dependent on T Lymphocytes

Elizabeth I. Majeski,* Manjeet K. Paintlia,*
Andrea D. Lopez,* Russell A. Harley,[†]
Steven D. London,* and Lucille London*

From the Departments of Microbiology and Immunology,* and
Pathology and Laboratory Medicine,[†] Medical University of South
Carolina, Charleston, South Carolina

Bronchiolitis obliterans organizing pneumonia (BOOP) is a clinical syndrome characterized by perivascular/peribronchiolar leukocyte infiltration leading to the development of intraalveolar fibrosis. We have developed an animal model of BOOP where CBA/J mice infected with 1×10^6 plaque-forming units (PFU) reovirus 1/L develop follicular bronchiolitis and intraalveolar fibrosis similar to human BOOP. In this report, we demonstrate a role for T cells in the development of intraluminal fibrosis associated with BOOP. Corticosteroid treatment of reovirus 1/L-infected mice both inhibited the development of fibrotic lesions when administered early in the time-course and promoted the resolution of fibrotic lesions when corticosteroid administration was delayed. Further, the depletion of either CD4⁺ or CD8⁺ T cells before reovirus 1/L infection also inhibited fibrotic lesion development. Both corticosteroid treatment and depletion of CD4⁺ or CD8⁺ T cells also resulted in decreased expression of the proinflammatory and profibrotic cytokines, interferon (IFN)- γ and monocyte chemoattractant protein-1 (MCP-1). Further, treatment of mice with a neutralizing monoclonal antibody to IFN- γ also significantly inhibited the development of fibrosis. Taken together, these results suggest a significant role for T cells in the development of reovirus 1/L-induced BOOP fibrotic lesions in CBA/J mice and suggests that T_H1-derived cytokines, especially IFN- γ , may play a key role in fibrotic lesion development. (*Am J Pathol* 2003, 163:1467–1479)

Bronchiolitis obliterans organizing pneumonia (BOOP), first described in 1985, is a pattern of injury characterized histologically as “patchy plugs of fibrous tissue (Masson bodies) filling bronchiolar lumens (bronchiolitis obliterans) and alveolar ducts and spaces (organizing pneu-

monis).”^{1–3} This patchy fibrosis may begin as focal lesions within the alveoli and the terminal bronchioles of the lung and progress bilaterally over time.¹ Other histological features include clusters of mononuclear inflammatory cells, chronic inflammation in the walls of the surrounding alveoli with reactive type II cells, increased numbers of foamy macrophages in the alveoli, and preserved lung architecture.^{2,4} The development of BOOP is often of unknown etiology (idiopathic BOOP) but BOOP has also been associated as a consequence of lung injury due to environmental toxins, bacterial infections, viral infections, and lung or bone marrow transplantation.^{3,5} BOOP is responsive to corticosteroids and treatment with prednisone continues to be the primary treatment for patients with symptomatic and progressive disease.^{2–4}

Since infiltrating lymphocytes are associated with the initiation of BOOP lesions,^{6,7} it is possible that these cells play an active role in the progression of inflammatory foci into lesions that are progressively dominated by fibroblasts. In patients with BOOP, there is an increase of activated bronchoalveolar lavage (BAL) lymphocytes with up to 80% to 95% of this cellular infiltrate being comprised of cytotoxic/suppressor CD8⁺ T cells.^{6–9} These cells may be involved in the inflammation and subsequent fibrosis occurring in BOOP patients.^{2,5,7,10–12} Several studies have shown that the infiltration of T lymphocytes may be important in the development of other forms of pulmonary fibrosis, although the data from both animal models and patients has been equivocal.^{13–18} Although these existing models of experimental pulmonary fibrosis have been useful for histopathological and functional investigations of other types of fibrotic events in the lung, the process of fibrotic lesion development in these models may be distinct from BOOP lesion development. Thus, differences in the phenotype of the inflammatory cell infiltrate, expression of soluble mediators, and response to various treatments may be different and,

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Address reprint requests to Lucille London, Ph.D., Department of Microbiology and Immunology, Medical University of South Carolina, P.O. Box 250504, 173 Ashley Avenue, Charleston, South Carolina 29425. E-mail: londonl@musc.edu.

therefore, may fail to accurately reflect the intraluminal and fibroblastic nature of the bronchoalveolar obliteration observed in BOOP lesions.^{19–21}

We have described a spectrum of inflammatory lung diseases after respiratory infection with reovirus serotype 1, strain Lang (reovirus 1/L), which is dependent on the strain of mice used.^{22–27} In this spectrum CBA/J mice infected with 1×10^6 PFU reovirus 1/L develop a histologically severe inflammation characterized by an infiltration of lymphocytes organized adjacent to the pulmonary vasculature of the lung.²³ This pattern of mononuclear cell organization without the involvement of intraluminal fibrosis results in lesions histopathologically consistent with the non-fibrotic human syndrome termed follicular bronchiolitis (FB). However, accompanying the development of FB in CBA/J mice are the presence of foamy macrophages and the elicitation of a non-specific fibrotic response of the lung characteristic of BOOP fibrotic lesions.²³ To investigate the role of the inflammatory cell infiltrate, especially T cells, in the development of reovirus 1/L-induced fibrotic lesions, the effect of either corticosteroid treatment or the depletion of CD4⁺ or CD8⁺ T cells before reovirus 1/L infection was determined. Our results indicate that corticosteroid treatment of reovirus 1/L-infected mice both inhibited the development of fibrotic lesions when administered early in the time-course and promoted the resolution of fibrotic lesions when corticosteroid administration was delayed. In addition, the depletion of either CD4⁺ or CD8⁺ T cells before reovirus 1/L infection also inhibited fibrotic lesion development. Both corticosteroid treatment and depletion of CD4⁺ or CD8⁺ T cells also resulted in decreased expression of the proinflammatory and profibrotic cytokines, interferon (IFN)- γ and monocyte chemoattractant protein-1 (MCP-1). Finally, treatment of mice with a neutralizing monoclonal antibody to IFN- γ also significantly inhibited the development of fibrosis. Taken together, the results suggest a significant role for T cells in the development of reovirus 1/L-induced BOOP fibrotic lesions in CBA/J mice and that T_H1 derived cytokines, especially IFN- γ , may play a key role in fibrotic lesion development.

Materials and Methods

Animals

Four- to 5-week-old female CBA/J mice (The Jackson Laboratory, Bar Harbor, ME) were maintained in microisolator cages under specific pathogen-free conditions in a BL-2 facility. Cages were housed in an HEPA-filtered animal isolator clean room (Nuaire Inc., Plymouth, MN). All animal manipulations were performed in class II biological safety cabinets. Virally primed mice were kept physically isolated from all other mice.

Virus

Reovirus 1/L was originally obtained from Dr. W. Joklik (Duke University School of Medicine, Durham, NC). Third-passage gradient-purified stocks were obtained by

re-cloning and amplifying parental stocks on L-929 fibroblast cells [American Type Culture Collection (ATCC), Rockville, MD] as previously described.²³ Following the purification of new stocks, infectious viral titers were obtained by limiting dilution on L-929 monolayers.²³

Inoculation Protocol

Animals were lightly anesthetized with an i.p. injection of 0.08 ml of 20% ketamine (Vetalar 100 mg/ml; Fort Dodge Laboratories, Inc., Fort Dodge, IA) and 2% PromAce (acepromazine maleate 10 mg/ml; Ayerst Laboratories, New York, NY) before immunization. Animals were infected by the intranasal (i.n.) application of 1×10^6 PFU of reovirus 1/L in 30 μ l (15 μ l in each nostril) in sterile injectable grade 0.9% NaCl (Baxter Healthcare Corp., Deerfield, IL). Control animals were inoculated with 30 μ l (15 μ l in each nostril) of sterile injectable grade 0.9% NaCl. After the indicated timepoints, animals were sacrificed with an i.p. injection of 0.2 ml sodium Nembutal (50 mg/ml; Abbott Laboratories, North Chicago, IL).

Methylprednisolone Administration

As an initial dosing regimen either 10 mg/kg or 20 mg/kg methylprednisolone (MPS) ($\sim$ 0.1 to 0.2 mg/mouse) (Sigma Chemicals, St. Louis, MO) dissolved in PBS was administered i.p. to mice beginning on either days 0, 5, 10, or 14 post-reovirus 1/L infection and given daily until the completion of the time-course. Since these initial studies indicated that treatment with MPS (20 mg/kg) either beginning at day 0 or day 5 post infection or administration of MPS (10 mg/kg) before day 5 post reovirus 1/L infection (beginning at day 0) resulted in an increased mortality rate as compared to that observed in untreated, reovirus 1/L-infected mice (Table 1), all additional studies were performed using MPS at a concentration of 10 mg/kg beginning 5 days post-reovirus 1/L infection unless otherwise noted.

CD4 and CD8 Depletion

Adult CBA/J mice were treated i.p. with either 0.5 mg of purified GK1.5 monoclonal antibody (mAb)²⁸ for depletion of CD4⁺ lymphocytes or 0.25 mg of purified 53–6.72 mAb²⁹ for depletion of CD8⁺ lymphocytes for three consecutive days. Depleted mice were then infected i.n. with 1×10^6 PFU of reovirus 1/L in 30 μ l (15 μ l in each nostril) in sterile injectable grade 0.9% NaCl. Control, depleted animals were inoculated with 30 μ l (15 μ l in each nostril) of sterile injectable grade 0.9% NaCl. The depleted state was maintained by treating with either 0.5 mg purified GK1.5 or 0.25 mg of purified 53–6.72 mAb every 6 days. Depletion of the appropriate subset of T cells was verified by flow cytometry of cells obtained from the lymph node and spleen before infection with reovirus 1/L on day 0 and on days 7 and 14 postinfection. Depleted mice were evaluated for the development of BOOP fibrotic lesions at day 21 post-reovirus 1/L infection.

Table 1. Modulation of Reovirus 1/L-Induced BOOP Fibrotic Lesions in CBA/J Mice

Day*	Dose†	% Mortality‡	FB§	Fibrosis¶
Untreated	0.0 mg/kg	20%	+++	++++
Day 0	20 mg/kg	60%	+++	ND
Day 0	10 mg/kg	40%	+++	ND
Day 5	20 mg/kg	40%	++	+
Day 5 ^{**}	10 mg/kg	20%	++	+
Day 10 ^{††}	10 mg/kg	20%	+++	++
Day 14 ^{††}	10 mg/kg	20%	+++	++
Untreated ^{‡‡}	0.0 mg/kg	0%	+ ^{§§}	+
CD4 ⁻				
Untreated ^{‡‡}	0.0 mg/kg	0%	++ ^{§§}	+
CD8 ⁻				

*Day treatment with methylprednisolone was begun postinfection with 1×10^6 PFU reovirus 1/L.

†Dose of methylprednisolone administered daily i.p. in 100 μ l PBS.

‡Percent mortality on day 14 postreovirus 1/L infection.

§Follicular bronchiolitis severity on day 14 postreovirus 1/L infection.

¶Fibrotic lesion severity on day 21 postreovirus 1/L infection.

^{||}Experiment performed once with two mice per timepoint.

^{**}Experiment performed five times with two mice per timepoint.

^{††}Experiment performed twice with two to four mice per timepoint.

^{‡‡}Mice were depleted of CD4⁺ or CD8⁺ cells prior to reovirus 1/L infection as described in Materials and Methods.

^{§§}Follicular bronchiolitis severity on day 21 post-reovirus 1/L infection.

ND, not determined.

In Vivo Interferon- γ Depletion

An anti-IFN- γ mAb (R4-6A2, rat IgG1, ATCC HB170) was obtained from ATCC³⁰⁻³² and ascites fluid was generated for *in vivo* use (Strategic Biosolutions, Newark, NJ). Reovirus 1/L-infected (10^6 PFU BOOP) CBA/J mice were treated i.p. every 3 days beginning on day 3 postinfection with either 100 μ g anti-IFN- γ antibody in PBS or 100 μ g normal rat IgG (Sigma) in PBS. Mice were evaluated on days 14 and 21 postinfection for the development of fibrotic lesions by hematoxylin and eosin (H&E) and Mason's trichrome stain.

Bronchoalveolar Lavage (BAL)

BAL was performed *in situ* by injecting and withdrawing a 0.5 ml aliquot of Hank's balanced salt solution (HBSS) twice through an intubation needle (21 gauge). A total of 1.5 ml of HBSS was used. BAL fluid was frozen at -70°C until use. Cells collected by BAL were washed three times with HBSS containing 5% fetal calf serum (FCS) and 0.05% azide, and resuspended at 1×10^6 cells/ml.

Histology

Lungs were inflated *in situ* with 10% neutral buffered formalin (0.5 mls) (Richard-Allan Scientific, Kalamazoo, MI) by intratracheal (i.t.) intubation, removed, and suspended in an additional 10% neutral buffered formalin overnight before being embedded in paraffin. H&E stain and Mason's trichrome stain, which was used to visualize collagen deposition, were performed on 4- μ m sections. Inflammatory infiltration with the development of FB, which is defined as a mononuclear cell infiltrate that

condenses into prominent peribronchiolar lymphoid accumulations, was blindly evaluated. FB was scored on a scale of 0 to 3: 0, normal; 1, mild (< 4 follicles per lobe); 2, moderate (between 5 and 8 follicles per lobe); 3, severe (> 8 follicles per lobe). Fibrosis was scored on a scale of 0 to 4: 0, normal; 1, mild; 2, moderate; 3, severe; 4, very severe.

Hydroxyproline (HP) Assay

The extent of pulmonary fibrosis was also determined by estimating total lung collagen as reflected by the measurement of the HP content of the lung as previously described.^{25,26,33} Mice were sacrificed at various intervals after infection with reovirus 1/L and the lungs were removed, lyophilized, and weighed. Differences between groups were examined for statistical significance using two-tailed Student's *t*-test. A *P* value less than 0.05 was considered significant.

Antibodies

The following monoclonal antibodies were used in this study: Cy-Chrome-conjugated rat anti-mouse CD45 (30-F11, leukocyte common antigen, Ly-5); fluorescein isothiocyanate (FITC)-conjugated hamster anti-mouse CD3 (145-2C11, CD3 ϵ chain); FITC-conjugated rat anti-mouse CD8a (53-6.7, Ly-2); R-phycoerythrin (PE)-conjugated rat anti-mouse CD4 (GK1.5, L3T4) (Caltag, Burlingame, CA); R-PE-conjugated rat anti-mouse Pan-NK cells (DX5); FITC-conjugated rat anti-mouse CD45R/B220 (RA3-6B2); R-PE-conjugated rat anti-mouse CD11b (M1/70, integrin α_m chain, Mac-1 α chain); and FITC-conjugated rat anti-mouse Ly6G (RB6-8C5, Gr-1, neutrophils) (Pharmingen, San Diego, CA); hamster anti-rat CD3 (ϵ -chain, 48-2B) (Santa Cruz Biotechnology, Santa Cruz, CA); rat anti-mouse CD11b (Mac-1 α chain) (Serotec, Westbury, NY); and rat anti-mouse neutrophil (MCA 771F) (Serotec).

Flow Cytometric Analysis

Cells collected by BAL were washed three times with HBSS containing 5% FCS and 0.05% azide, and resuspended at 1×10^6 cells/ml. Cells were stained for cell surface marker expression as previously described except that all cells were also stained with anti-CD45 (30-F11), leukocyte common antigen Ly-5, and only anti-CD45-positive cells were acquired for analysis.^{24,26} Isotype-matched controls were run for each sample (Caltag and Pharmingen). The total number of PMNs was obtained by adding the anti-Gr-1 (Ly6G) single-positive cells and the anti-Gr-1/anti-Mac-1 (integrin α_m chain) double-positive cells. The total number of B cells was obtained by adding the anti-B220 (CD45R) single-positive cells plus the anti-B220/anti-Mac-1 double-positive cells. The total number of macrophages was obtained by enumerating those cells stained only with anti-Mac-1. Flow cytometric analysis was performed using a dual-laser FACS Caliber flow cytometer and the Cell Quest acqui-

sition and analysis software program (BD Biosciences, San Jose, CA).

RNase Protection Assay

Total cellular RNA was isolated from whole lungs by guanidium denaturation using TRI-reagent (Molecular Research Center, Cincinnati, OH). Riboquant multiprobe ribonuclease protection assay (RPA) mouse template sets mCK-1b, mCK-2b, mCK-3b and mCK-5 were purchased from Pharmingen. Template set mCK-1b contained probes for Interleukin (IL)-2 - 5, -9, -10, -13, -15, and IFN- γ . Template set mCK-2b contained probes for IL-1 α , -1 β , -1Ra, -6, -10, -12, IFN- γ inducing factor (IGIF), IFN- γ , and migration inhibitory factor (MIF). Template set mCK-3b contained probes for tumor necrosis factor (TNF)- β , lymphotoxin (LT)- β , TNF- α , IL-6, IFN- γ , IFN- β , transforming growth factor (TGF)- β 1, TGF- β 2, TGF- β 3, and MIF. Template set mCK-5 contained probes for the chemokines, lymphotactin (Ltn), regulated on activation normal T cells expressed and secreted (RANTES), eotaxin, macrophage inflammatory protein (MIP)-1 β , MIP-1 α , MIP-2, interferon inducible protein (IP)-10, monocyte chemoattractant protein (MCP)-1, and T-cell activation factor (TCA)-3. All template sets also contained probes for the control genes GAPDH and L32. RPA analysis was performed as previously described²⁶ using radiolabeled RNA transcripts from the multiprobe sets generated by *in vitro* transcription (Pharmingen) following the manufacturer's instructions. Gels were dried and exposed to Fuji RX film at -70°C with DuPont Cronex Quanta III intensifying screens for 1 to 5 days. Band intensities on scanned RPA gels were analyzed using the public domain NIH Image program developed at the U.S. National Institutes of Health. Specific cytokine or chemokine band intensities were normalized to L32 controls to account for differences in total RNA loading in each sample. The mean $\pm$ SD of the densitometric measurements from two independent experiments with two mice per time point (four independent autoradiographs) over the indicated timepoints were determined. Differences in expression level between uninfected controls and reovirus 1/L-infected groups were examined for statistical significance using a two-tailed Student's *t*-test. A *P* value less than 0.05 was considered significant.

ELISA

100 μL of BAL fluid was analyzed for mouse IFN- γ and MCP-1 in duplicate using the R&D Systems Quantikine M immunoassay systems (R&D Systems, Minneapolis, MN). The results were expressed as the mean $\pm$ SD. Differences between groups were examined for statistical significance using a two-tailed Student's *t*-test. A *P* value less than 0.05 was considered significant.

Results

Methylprednisolone Modulates the Development of Fibrosis After i.n. Infection with Reovirus 1/L

To determine the efficacy of treatment with MPS on the development of reovirus 1/L-induced BOOP fibrotic lesions, CBA/J mice were infected i.n with 1×10^6 PFU reovirus 1/L and treated with various doses of MPS daily at various timepoints post-reovirus 1/L infection. Mice inoculated with saline and treated with MPS beginning either on day 0, 5, 10, or 14 postinoculation did not develop any abnormalities and 100% of treated animals survived (data not shown). In reovirus 1/L-infected mice, when treatment with MPS was begun simultaneously to infection with reovirus 1/L at day 0, a three-fold increase in mortality of reovirus 1/L-infected animals (60% *versus* 20%) was observed when animals were treated with 20 mg/kg MPS daily (Table 1). Even when the dose of MPS was reduced to 10 mg/kg daily, a two-fold increase in mortality of reovirus 1/L-infected animals (40% *versus* 20%) was observed as compared to untreated, reovirus 1/L-infected animals (Table 1). Due to the high degree of mortality associated with these treatment regimens, we were unable to evaluate fibrotic lesion development at day 21 postinfection (Table 1). However, FB formation, which is characterized as a mononuclear cell infiltrate that condenses into peribronchiolar lymphoid accumulations, was prominent.

To prevent the increase in mortality of MPS-treated, reovirus 1/L-infected mice that was observed, and to determine whether MPS treatment effects fibrotic lesion development, the administration of MPS was delayed until day 5 postinfection. Mice treated with 20 mg/kg MPS daily still exhibited an increase in mortality (40% *versus* 20%) (Table 1). However, mice treated with 10 mg/kg MPS daily exhibited a similar mortality rate of 20% as compared to untreated, reovirus 1/L-infected mice (Table 1). As determined by H&E (Figure 1, A and C) and Mason's trichrome staining (Figure 1, B and D), a significant inhibition of fibrotic lesion development was observed in MPS-treated, reovirus 1/L-infected mice (Figure 1, C and D), as compared to untreated, reovirus 1/L-infected mice (Figure 1, A and B). However, both FB and foamy macrophages are still prominently observed in the lungs of MPS-treated reovirus 1/L-infected mice at days 14 and 21 postinfection (Figure 1, C and D). In addition, to support the histological evaluation, total lung collagen was estimated by the biochemical measurement of HP content of the lungs on days 14 and 21 from reovirus 1/L-infected mice to evaluate the extent of pulmonary fibrosis. Values were expressed as the percentage of that obtained in control mice. As shown in Figure 2, a two-fold (day 14) to three-fold (day 21) increase in HP accumulation in the lungs was observed postinfection with reovirus 1/L as compared to saline, inoculated controls. In contrast, less than a 1.5-fold increase in HP content was observed in MPS-treated, reovirus 1/L-infected mice as compared to untreated, reovirus 1/L-infected mice on

Figure 1. Inhibition of fibrosis associated with BOOP after corticosteroid treatment or after depletion of CD4⁺ or CD8⁺ T cells. Normal (A, B, I, J), MPS-treated (C, D, K, L), CD4-depleted (E, F), and CD8-depleted (G, H) CBA/J mice were i.n. infected with reovirus 1/L and paraffin-embedded lung sections were stained with H&E (A, C, E, G, I, K) or Mason's trichrome (B, D, F, H, J, L) for determination of collagen deposition on days 14 (J) or 21 (B, D, F, H, L) post-reovirus 1/L infection. With Mason's trichrome the nuclei stain a dark blue/purple, muscle stains red, and collagen stains blue. Reovirus 1/L-infected CBA/J lung on day 21 postinfection (A and B); Reovirus 1/L-infected CBA/J mice treated daily with MPS (beginning on day 5 postinfection) on day 21 postinfection (C and D); CD4-depleted, reovirus 1/L-infected CBA/J lung (E and F); CD8-depleted, reovirus 1/L-infected CBA/J lung (G and H); Reovirus 1/L-infected CBA/J lung on day 14 postinfection (I and J); Reovirus 1/L-infected CBA/J mice treated daily with MPS (beginning on day 14 postinfection) on day 21 postinfection (K and L). Representative of four independent experiments containing 2 mice per timepoint (A to D, I, J). Representative of one independent experiment containing 2 mice per timepoint (E to H). Representative of two independent experiments containing 4 mice per timepoint (K and L). Objective magnification, $\times 20$.

either day 14 or day 21 post-reovirus 1/L infection (Figure 2). The significant decrease in HP content in the lungs of MPS treated, reovirus 1/L-infected mice is consistent with the observation of limited areas of fibrotic polyps observed in Figure 1, C and D. Therefore, our results demonstrate both histologically and biochemically that the

Figure 2. Modulation of total hydroxyproline content in the lungs of reovirus 1/L-infected mice. CBA/J mice were i.n. infected with 1×10^6 PFU reovirus 1/L and HP content as a measurement of total collagen content in the lungs was determined. Results are expressed as a percentage of HP as compared to saline-inoculated control mice (open bar) on either day 14 (solid bar) or day 21 (striped bar) post-reovirus 1/L infection. Each data-point represents the mean $\pm$ SD of four mice. * $P < 0.05$ as compared to saline-inoculated control mice. ** $P < 0.05$ as compared to reovirus 1/L-infected mice.

administration of MPS beginning on day 5 postinfection to mice receiving 1×10^6 PFU reovirus 1/L was effective at inhibiting fibrosis associated with reovirus 1/L-induced BOOP. While fibrotic lesion development was significantly inhibited by MPS treatment beginning on day 5 postinfection, prominent FB lesions (condensing lymphoid follicles) were still present (Table 1).

To evaluate the effect of MPS administration on the resolution of BOOP lesions, mice were i.n. infected with 1×10^6 PFU of reovirus 1/L, and MPS treatment was begun 10 or 14 days postinfection when fibrotic lesion development had already begun. Treatment with MPS at 10 mg/kg daily beginning on days 10 or 14 post-reovirus 1/L infection also demonstrated 20% mortality (Table 1). At day 21 postinfection, although fibrotic lesions are still observed in mice treated with MPS beginning on day 14 postinfection (Figure 1, K and L), these areas of fibrosis are smaller, more discrete, and less severe than the fibrotic lesions observed in untreated, reovirus 1/L-infected mice on day 14 (Figure 1, I and J) or day 21 (Table 1; Figure 1, A and B). In both untreated and MPS-treated, reovirus 1/L-infected mice, both fibrotic lesions and FB resolved by day 28 to 35 postinfection (data not shown)²³.

Depletion of Either CD4⁺ or CD8⁺ T Cells Before Reovirus 1/L Infection Inhibits the Development of Fibrotic Lesions

To determine whether the infiltration of T cells plays a significant role in reovirus 1/L-induced BOOP fibrotic lesions, CBA/J mice were depleted of either CD4⁺ or CD8⁺ T cells before infection with 1×10^6 PFU reovirus 1/L. Verification of depletion of either CD4⁺ or CD8⁺ T cells was determined by flow cytometry before infection with reovirus 1/L and maintenance of the depleted state was verified at both days 7 (data not shown) and 14 postin-

Figure 3. Modulation of cellular infiltration and phenotype post reovirus 1/L infection. Untreated (◆) or MPS-treated (■) CBA/J mice were infected i.n. with 1×10^6 PFU reovirus 1/L for a time-course of 21 days. Cells were harvested and counted from the BAL fluid (A). Each data-point represents the average $\pm$ SD from two experiments with three mice per timepoint. * $P < 0.05$ reovirus 1/L-infected mice as compared to reovirus 1/L-infected, MPS-treated CBA/J mice. Cells were pooled and stained for surface phenotype expression using antibodies to B220 (B), CD3 (C), Mac-1 (D). Each data point represents the percent positive cells from the average of two independent experiments with pooled cells from three mice per timepoint.

fection (Figure 4E). Both CD4- and CD8-depleted mice inoculated with saline did not develop any abnormalities and 100% of the animals survived through day 21 (data not shown). In addition, no mortality was observed in either CD4- or CD8-depleted reovirus 1/L-infected mice (Table 1). As determined by H&E (Figure 1, E and G) and Mason's trichrome (Figure 1, F and H) staining, a significant inhibition of fibrotic lesion development on day 21 was observed in both reovirus 1/L-infected CD4- (Figure 1, E and F) and CD8-depleted (Figure 1, G and H) mice, as compared to untreated, reovirus 1/L-infected mice (Figure 1, A and B). However, both FB and foamy macrophages are still observed in the lungs of CD4- and CD8-depleted reovirus 1/L-infected mice at day 21 postinfection (Figure 1, E and G), although these lesions were not prominent. In addition, the measurement of total lung collagen on either day 14 or 21 from CD4- or CD8-depleted reovirus 1/L-infected mice was significantly lower than that observed in reovirus 1/L-infected mice (Figure 2). This significant decrease in HP content in the lungs of CD4- or CD8-depleted reovirus 1/L-infected mice is consistent with the observation of limited areas of fibrotic polyps observed in Figure 1, E and G.

The Administration of MPS or Depletion of Either CD4⁺ or CD8⁺ T Cells Modifies the Inflammatory Response to Reovirus 1/L

To determine the percentage over time of different leukocyte subsets present in the inflammatory infiltrate after MPS treatment beginning 5 days postinfection with 1×10^6 PFU reovirus 1/L cells obtained from the BAL were

Figure 4. Phenotype of cells in the BAL fluid of reovirus 1-L-infected CBA/J mice. CBA/J mice were i.n. infected with 1×10^6 PFU reovirus 1/L and two-color flow cytometric analysis of the infiltrating cell populations in the BAL were analyzed on day 14 postinfection. Reovirus 1/L-infected CBA/J mice (A); MPS-treated, reovirus 1/L-infected CBA/J mice (B); CD4-depleted, reovirus 1/L-infected CBA/J mice (C); CD8-depleted, reovirus 1/L-infected CBA/J mice (D); spleen cells from normal, CD8- or CD4-depleted reovirus 1/L-infected animals (E). Representative of two independent experiments containing pooled cells from three mice per timepoint (A and B). Representative of one independent experiment containing two mice per time point (C to E).

analyzed by flow cytometry using monoclonal antibodies specific for T-cell subsets (CD3, CD4, CD8), B cells (B220), macrophages (CD11b), and NK cells (pan-NK). BAL cells were stained with the leukocyte common antigen (Ly-5) anti-CD45 mAb and only anti-CD45-positive cells were acquired for analysis. Isotype-matched controls were run for each sample (data not shown).

Over the course of the infection, the total cell number recovered from the BAL fluid in MPS-treated mice was significantly decreased only on day 14 post infection (Figure 3A) (12×10^5 in untreated reovirus 1/L-infected mice versus 6×10^5 in MPS-treated, reovirus-infected mice). In addition, over the time course of 21 days in either MPS-treated or untreated mice the predominant cell types found in the BAL were T cells, B cells, and macrophages (Figure 3, B to D). However, at day 14, MPS-treated reovirus 1/L infected mice demonstrated a decrease in both CD4 (6% versus 12%) and CD8 (10% versus 15%) populations. No significant infiltration of PMNs was observed in either MPS-treated or -untreated reovirus 1/L-infected animals (data not shown). Similar to the cellular response observed from cells obtained by

BAL, few differences in the percentages of T or B lymphocytes and macrophages were observed in the interstitial areas of untreated reovirus 1/L-infected mice *versus* MPS-treated, reovirus 1/L-infected mice (data not shown).

In either CD4- or CD8-depleted reovirus 1/L-infected mice, the predominant cell types found in the BAL were macrophages (30% - 35% in CD4- or CD8-depleted reovirus 1/L-infected mice *versus* 25% reovirus 1/L-infected mice or 26% MPS-treated reovirus 1/L infected mice) (Figure 4). CD4-depleted, reovirus 1/L-infected mice showed an infiltration of CD8⁺ cells (15%) without a significant infiltration of CD4⁺ cells (2%) (Figure 4C) in the BAL fluid. A similar response was observed with CD8-depleted, reovirus 1/L-infected mice (16% CD4⁺ cells *versus* 1% CD8⁺ cells) (Figure 4, C and D). No significant infiltration of PMNs was observed in either CD4- or CD8-depleted reovirus 1/L-infected animals (data not shown). Depletion of either CD4⁺ or CD8⁺ cells was verified on both day 7 (data not shown) and day 14 (Figure 4E) post-reovirus 1/L infection. As shown in Figure 4E, the phenotype of spleen cells from normal mice was 15% CD4⁺ and 10% CD8⁺ cells, while animals depleted of either CD8, or CD4 demonstrated the appropriate phenotype (CD8-depleted animals: 13% CD4⁺ and 2% CD8⁺ cells; CD4-depleted animals: 0.3% CD4⁺ and 18% CD8⁺ cells).

Cytokine and Chemokine Expression are Modulated in the Lungs of MPS-Treated CBA/J Mice Receiving 1×10^6 PFU Reovirus 1/L

Cytokine and chemokine mRNA expression in total lung tissue was evaluated by RPA. Three cytokine RPA template sets (Figure 5, A to C) and one chemokine RPA template set (Figure 5D) were evaluated in MPS-treated or untreated CBA/J mice after i.n. inoculation with 1×10^6 PFU of reovirus 1/L over a time-course of 21 to 28 days. Together, these four template sets allow an analysis of the expression and modulation of mRNAs for cytokines and chemokines which have been implicated to play a role in the pulmonary fibrotic process (such as MCP-1, IL-6, IFN- γ) as well as others contained within the template sets.

Substantial expression of mRNA for a number of cytokines and chemokines were observed in the lungs of CBA/J mice infected i.n. with 1×10^6 PFU reovirus 1/L. (Figure 5). These include IL-9 (Figure 5A), the proinflammatory cytokines IL-1 α , IL-1 β , IL-1 Ra, IL-6 (Figure 5B), IFN- γ , (Figure 5, A to C), MIF (Figure 5, B and C), and the chemokines, MIP-1 β , MIP-1 α , MCP-1, RANTES, and IP-10 (Figure 5D). In comparison to CBA/J mice infected with 1×10^6 PFU reovirus 1/L, MPS-treated reovirus 1/L-infected i.n. CBA/J mice, in general, demonstrated a decreased expression of most of the cytokines and chemokines present on the four template sets. Expression of the following cytokines and chemokines was observed in MPS-treated reovirus 1/L-infected CBA/J mice: IL-9 (Figure 5A) IL-1 α , IL-1 β , IL-1 Ra, IL-6 (Figure 5B), IFN- γ , (Figure 5, A to C), MIF (Figure 5, B and C), MCP-1, RANTES, and IP-10 (Figure 5D). Cytokine mRNA expres-

sion was not induced for a number of cytokine genes in either MPS-treated or untreated reovirus 1/L-infected mice. These included IL-2, IL-3, and IL-13. In addition, mRNA expression for the chemokines eotaxin, MIP-2, and TCA-3 were not significantly induced.

Since both IFN- γ and MCP-1 have been implicated in the fibrotic process induced by reovirus 1/L,^{26,27} we quantitated the autoradiographs and found a significant up-regulation of both IFN- γ and MCP-1 (Figure 6A) mRNA in CBA/J mice infected with 1×10^6 PFU reovirus 1/L. However, in CBA/J mice infected with 1×10^6 PFU reovirus 1/L and treated with MPS beginning on day 5 postinfection, a significant decrease in the mRNA expression of IFN- γ (Figure 6A) was observed. Although the expression of MCP-1 was decreased after MPS treatment, this decrease as compared to control mice was not statistically significant (Figure 6A). In a similar manner, while a significant increase in IFN- γ protein in the BAL fluid was observed in both MPS treated and untreated reovirus 1/L-infected mice as compared to uninfected controls (Figure 6B), a decrease in IFN- γ protein expression was observed between MPS-treated and untreated, reovirus 1/L-infected mice (Figure 6B). Similar to the expression of mRNA for MCP-1, a decrease (although not statistically significant) in the protein expression of MCP-1 in the BAL fluid of MPS-treated reovirus 1/L-infected was observed (Figure 6B). In contrast, in both CD4- and CD8-depleted mice, a reduction in the protein expression of both MCP-1 and IFN- γ was observed (Figure 6C). In all cases IFN- γ and MCP-1 was not detected in control, saline-inoculated mice (data not shown).

To demonstrate a role for IFN- γ in the development of fibrotic lesions in reovirus 1/L induced BOOP, reovirus 1/L-infected (10^6 PFU BOOP) CBA/J mice were treated i.p. every 3 days beginning on day 3 postinfection with either 100 μ g anti-IFN- γ antibody (R4-6A2) in PBS (Figure 7, C and D) or 100 μ g normal rat IgG (Figure 7, A and B). Mice were evaluated on days 14 (Figure 7, A and C) and 21 (Figure 7, B and D) postinfection for the development of fibrotic lesions by H&E staining. As can be observed with H&E staining, significant fibrotic lesion development was observed in reovirus 1/L-infected mice who were treated with normal rat IgG on both days 14 and 21 (Figure 7, A and B). However, in reovirus 1/L-infected, anti-IFN- γ treated mice, although follicular bronchiolitis (FB) lesions were observed on day 14 and to a lesser extent on day 21 (Figure 7D) postinfection, this was accompanied by little to no fibrotic lesion development (Figure 7, C and D).

Discussion

In this study, we have used a reovirus 1/L-induced model of BOOP to study cellular and molecular events important in the development of this type of pulmonary fibrosis. Using reovirus 1/L-infected mice that were either treated with corticosteroids or depleted of either CD4⁺ or CD8⁺ T cells, our results demonstrate that T cells play a major role in the development of intraluminal fibrosis associated with BOOP. Corticosteroid treatment of reovirus 1/L-in-

Figure 5. Differential cytokine and chemokine mRNA expression from the lungs of untreated and MPS-treated reovirus 1/L-infected CBA/J mice. CBA/J mice were i.n. infected with 1×10^6 PFU reovirus 1/L and treated with MPS i.p. daily beginning on day 5 postinfection. RNA was harvested from the whole lungs at the indicated timepoints, and probed by RPA analysis using the following multiprobe template sets: mCK-1b (T-cell-derived cytokine panel) (A); mCK-2b (inflammatory cytokine panel) (B); mCK-3b (cytokines associated with fibrosis) (C); and mCK-5 (chemokine panel) (D). The position of individual cytokine or chemokine mRNA expression is indicated on the **left side** of each panel. Representative of three independent experiments containing two mice per timepoint.

infected mice both inhibited the development of fibrotic lesions when administered early in the time-course and promoted the resolution of fibrotic lesions when corticosteroid administration was delayed. Daily treatment with corticosteroids inhibited the total cellular infiltration into the lung post reovirus 1/L infection, and also resulted in an inhibition of the proinflammatory and profibrotic cytokines, IFN- γ and MCP-1. Further, the depletion of either CD4⁺ or CD8⁺ T cells before reovirus 1/L infection also inhibited fibrotic lesion development as well as IFN- γ and MCP-1 protein expression. Consistent with these results, T-cell-deficient neonatally thymectomized (nTx) CBA/J mice do not develop intraluminal fibrosis after infection with 1×10^6 PFU reovirus 1/L and both the phenotype of

the infiltrating cells and the expression of both IFN- γ and MCP-1 were significantly altered in these nTx CBA/J mice as compared to normal CBA/J mice.²⁷ Taken together, this study combined with our previous studies suggest a significant role for T cells in the development of reovirus 1/L-induced BOOP fibrotic lesions and suggests that TH1 derived cytokines, especially IFN- γ , may play a key role in fibrotic lesion development.

Currently few small animal models of BOOP exist. However, several studies have shown that the infiltration of T lymphocytes may be important in the development of other forms of pulmonary fibrosis although the data from both animal models and patients has been equivocal. Intratracheal administration of bleomycin in rodents, a

Figure 6. Expression of IFN- γ and MCP-1 in corticosteroid-treated or CD4- or CD8-depleted CBA/J mice infected with 1×10^6 PFU reovirus 1/L. Relative differences in mRNA expression of either IFN- γ or MCP-1 (**A**) over time were determined by comparing the ratio of cytokine/chemokine mRNA with the housekeeping gene, L32 from RPA autoradiograms. IFN- γ expression was evaluated between days 5 and 10 postinfection. MCP-1 expression was evaluated between days 3 and 10 postinfection. Control, uninfected mice (**open bar**); reovirus 1/L-infected CBA/J mice (**solid bar**); MPS-treated, reovirus 1/L-infected CBA/J mice (**striped bar**). The mean $\pm$ SD of the densitometric measurements from two independent experiments with two mice per timepoint (four independent autoradiographs) over the indicated timepoints are presented. Detection of IFN- γ or MCP-1 protein by ELISA in the BAL fluid over time from reovirus 1/L-infected CBA/J mice ($\blacklozenge$) and MPS-treated reovirus 1/L-infected CBA/J mice ($\blacksquare$) (**B**). The mean $\pm$ SD of two independent experiments, evaluated in duplicate, is shown. Detection of MCP-1 ($\blacksquare$) or IFN- γ ($\square$) proteins by ELISA in the BAL fluid evaluated on day 14 post-reovirus 1/L infection from reovirus 1/L-infected normal, CD4-depleted, or CD8-depleted CBA/J mice (**C**). Representative of one experiment with two animals per timepoint, performed in duplicate. * $P < 0.05$ as compared to saline-inoculated control mice. ** $P < 0.05$, as compared to reovirus 1/L-infected mice.

model for idiopathic pulmonary fibrosis (IPF), results in interstitial fibrosis accompanied by a significant infiltration of both T and B lymphocytes.^{17,18} In this model, some reports have demonstrated that the inhibition or depletion of lymphocytes by anti-lymphocyte antibody, mAb to T-cell subsets, or treatment with steroids inhibited the development of bleomycin-induced fibrosis,^{34–36} while other studies found no effect of T-cell depletion on fibrotic lesions induced by bleomycin instillation.^{37–39} Similarly, in nude mice lacking T cells or SCID mice deficient in both T and B cells, conflicting evidence for a role of T cells in the fibrotic process has been reported.^{40–42} A role for T cells has also been proposed for Bronchiolitis Obliterans Syndrome (BOS) which is the major limitation to survival postlung transplantation and is characterized by persistent peribronchiolar inflammation that leads to airway fibrosis/obliteration.^{3,16} Models of BOS demonstrate increases in CD4⁺ and CD8⁺ T cells, B cells, and macrophages post allograft transplantation.^{15,16,43,44} Further, in SCID allograft models of BOS no influx of lymphocytes or fibrosis was observed, suggesting that lymphocytes may play an important role in the development of fibrosis during chronic graft rejection.¹⁶

While these models have been used to evaluate potential mechanisms related to fibrosis, they may not fully recapitulate what occurs in similar human pathologies. As an example, while bleomycin-induced fibrosis in rodents is used as a model for human IPF, many features of bleomycin-induced fibrosis are not shared with the human condition.⁴⁵ However, in this regard, the reovirus 1/L-induced model of BOOP recapitulates the histological (intraluminal fibrosis) and phenotypic characteristics of human BOOP and thus, is an excellent small animal model of human BOOP. Furthermore, unlike other animal models for pulmonary fibrosis, reovirus-induced pulmonary fibrosis offers the advantage in that it also provides a model that recapitulates the response of human disease to clinical treatments currently in use. Since these lesions occur in a well-defined temporal sequence that proceeds from initial peribronchiolar inflammatory lesions to characteristic, fibrotic cellular BOOP lesions, this model can be used to evaluate the cellular and molecular signals that may lead to fibrotic lesion development in human BOOP.

The results described in this manuscript as well as in our previous publications all consistently demonstrate a

Figure 7. Inhibition of fibrosis associated with BOOP after treatment with a neutralizing monoclonal antibody to interferon- γ . CBA/J mice were i.n. infected with reovirus 1/L and treated i.p. every 3 days beginning on day 3 postinfection with either 100 μ g normal rat IgG (**A** and **B**) or 100 μ g anti-IFN- γ antibody (**C** and **D**) in PBS. Mice were evaluated both on days 14 (**A** and **C**) and 21 (**B** and **D**) postinfection for the development of fibrotic lesions by H&E stain. **A** and **B**: Reovirus 1/L-infected CBA/J mice treated with normal Rat IgG. **C** and **D**: Reovirus 1/L-infected CBA/J mice treated with an anti-IFN- γ antibody. Representative of one independent experiment containing four mice per timepoint (magnification $\times 20$).

clear dependence of T cells on the fibrotic process associated with reovirus 1/L-induced BOOP.²⁶ Thus, we analyzed cytokines and chemokines that have previously been associated with fibrotic lesion development. We believe that our data supports a role for IFN- γ in the fibrotic process and that it acts as a profibrotic agent in the spectrum of fibrosis induced in reovirus 1/L-infected mice.^{22,25–27} Our data demonstrate that when the inflammatory and fibrotic process in BOOP is inhibited either by corticosteroid treatment or CD4 or CD8 depletion, the concentration of IFN- γ in the BAL fluid decreases. Both a decrease in IFN- γ expression and limited fibrotic lesion development was also demonstrated in T-cell-deficient nTx mice that were infected with reovirus 1/L-induced BOOP.²⁷ However, in reovirus 1/L-induced ARDS, the fibrotic component was not inhibited and IFN- γ expression levels remained high in either corticosteroid-treated or nTx animals.^{25–27} Further, treatment of reovirus 1/L-induced BOOP with an anti-IFN- γ neutralizing monoclonal antibody inhibited fibrotic lesion development on both days 14 and 21 postinfection, further supporting our hypothesis that IFN- γ plays a significant role in fibrotic lesion development. Our data also demonstrate an increased expression of MCP-1 in reovirus-1/L induced BOOP that was modified after corticosteroid treatment or within CD4- or CD8-depleted mice. This data are also in agreement with our previous studies that demonstrated an increase in MCP-1 expression in reovirus 1/L-induced ARDS whose fibrosis is not modified either in nTx mice or by corticosteroid treatment.^{26,27} However, in reovirus 1/L-induced BOOP a decrease in MCP-1 expression as well as fibrosis was observed in nTx mice.²⁷ Taken together,

these data and our previously published results demonstrate a direct correlation of proinflammatory cytokine expression such as IFN- γ and MCP-1 and the development of fibrosis.

Our results, demonstrating a role for IFN- γ and MCP-1 in reovirus 1/L-induced BOOP are in agreement with other studies in which both IFN- γ and MCP-1 have been implicated in the fibrotic process in both patients and animal models.^{46–48} A role for IFN- γ in bleomycin-induced interstitial fibrosis is supported by the observations that susceptible versus non-susceptible mouse strains produce high amounts of IFN- γ ,⁴² depletion of T cells down-regulates both IFN- γ expression and fibrosis,^{35,49} and high levels of IFN- γ expression and fibrosis are observed in SCID mice, which are susceptible to bleomycin-induced fibrosis.⁴² Bleomycin treatment of IFN- γ knockout mice (IFN- γ $-/-$) also resulted in both a significant inhibition of pulmonary inflammation and fibrosis.⁵⁰ IFN- γ has also been implicated in the fibrotic process associated with the tracheal transplant model of BOS.^{11,16,51,52} Expression of TH1 cytokines including IFN- γ and IL-10 were up-regulated to a greater extent than TH2 cytokines (IL-4), suggesting that although cytokine production by all T-lymphocyte subsets (TH1, TH2) may be involved in the development of BOS, the TH1 cytokine products may be more important in the development of fibrosis.⁵² INF- γ has also been implicated as a profibrotic factor in lung fibrosis that occurs in patients or animal models with fibrosing alveolitis, IPF, sarcoidosis, chronic beryllium disease, silicosis, and lung allograft fibrosis.^{53–58} MCP-1 is also expressed in a number of inflammatory conditions in patients that demonstrate a

fibrotic component including ARDS, IPF, systemic sclerosis, and BOOP^{59–63} as well as in a number of mouse models of fibrosis.^{63–65} In addition, depletion of MCP-1 by treatment with anti-MCP-1 antibodies or by loss of CCR2 signaling results in a significant reduction in fibroblast proliferation.^{63,64} Taken together these data also suggest a potential role for MCP-1 in the fibrotic process.

Although the process and underlying mechanisms of fibrosis have not been clearly elucidated, the release of cytokines and chemokines from inflammatory cells has been implicated in the development and regulation of fibrosis. Our data clearly support a role for T cells in the development of intraluminal fibrosis associated with BOOP since we observed the lack of fibrotic lesion development in reovirus 1/L-infected nTx CBA/J,²⁷ in CD4- or CD8-depleted CBA/J mice as well as in corticosteroid-treated mice. In contrast to BOOP, a definitive role for T cells in the development of fibrosis associated with reovirus 1/L-induced ARDS has not been established. In reovirus 1/L-induced ARDS, both infection of nTx mice and corticosteroid treatment have shown little effect in both the early inflammatory response as well as the later fibrotic response.^{26,27} We have previously proposed that a positive feedback loop exists between the expression of MCP-1 and IFN- γ in the fibrotic response associated with reovirus 1/L-induced BOOP and ARDS.²⁷ We suggest that while the initial infection of resident epithelial cells by reovirus 1/L leads to MCP-1 expression and cellular infiltration, the sustained production of MCP-1 is the result of an autocrine or paracrine mechanism driven by the secretion of IFN- γ . This sustained production of MCP-1 leads to the continued recruitment of inflammatory cells, eventually leading to fibrotic development. In support of this model, IFN- γ has been shown to induce the expression of MCP-1 from a number of cell types including macrophages, epithelial cells, vascular endothelial cells, and fibroblasts.^{65–69} Although histopathologically similar fibrotic lesions are associated with reovirus 1/L-induced BOOP and ARDS, the mechanism of fibrotic lesion development may be distinct due to differential regulation of infiltrating cells. While T cells may be the predominant cell type producing IFN- γ in BOOP that ultimately leads to fibrosis, non-T cells such as NK cells may be responsible for the production of IFN- γ in ARDS that ultimately leads to fibrosis. Therefore, treatment methods focused only on one aspect of fibrosis may be inefficient at modulating the development of fibrosis in both of these diseases since their disease processes are distinct.

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