Lung infection with γ-herpesvirus induces progressive pulmonary fibrosis in Th2-biased mice

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IDiOPATHIC PULMONARY FIBROSIS (IPF) is a progressive, fibrotic interstitial lung disease of unknown etiology. No proven effective treatment is available other than lung transplantation. Spontaneous remissions do not occur, and death due to respiratory failure usually ensues within 3–5 years of diagnosis. The histological hallmark of IPF is heterogeneous fibrosis with areas of dense acellular fibrosis alternating with normal alveolar structures and the presence of clusters of activated fibroblasts termed fibroblastic foci (1). Although cellular and molecular pathways that drive the pathogenesis of IPF are complex, increasing evidence suggests that IPF results from ongoing alveolar epithelial injury and abnormal wound repair (15, 28). Alveolar epithelial injury induces the proliferation of fibroblasts and their differentiation into myofibroblasts. Activated fibroblasts produce extracellular matrix proteins like collagen and a variety of cytokines, including the profibrotic cytokine transforming growth factor (TGF)-β. Increased deposition and turnover of extracellular matrixes result in destruction of alveolar capillary units, fibrosis, and loss of lung function. An imbalance between anti- and profibrotic activities in the lungs of IPF patients is also evidenced by the predominance of T helper type 2 (Th2) cytokines over Th1 cytokines (39, 40). Th2 cytokines (IL-4, IL-5, IL-10, IL-13) activate fibroblasts and induce their production of extracellular matrix, whereas Th1 cytokines (IFN-γ) suppress fibroblast proliferation and production of collagen and fibronectin (29). Low levels of IFN-γ have been found in some IPF patients, and in an attempt to restore the balance between Th1 and Th2 cytokines, administration of IFN-γ has been evaluated as a potential therapeutic agent (25, 45).

Several studies have implicated viral infection as the cause of the ongoing epithelial injury in IPF and therefore an important factor in the pathogenesis of IPF (35). In several series, Epstein-Barr virus (EBV) protein and DNA are detected in lung tissue of almost half the cases of IPF (12, 30, 37). Recently, we expanded the search for an association between herpesvirus infection and IPF by testing for all eight known human herpesviruses in lung tissue of IPF patients and of lung disease controls. Using PCR to detect viral DNA in lung specimens, we found that 97% of 33 IPF patients (7 with familial IPF and the rest sporadic) tested positive for one or more herpesviruses (35). Furthermore, we found that antiviral treatment of an IPF subject whose lung tissue was positive for EBV by PCR and by immunohistochemistry with an oral antiviral agent stopped any further decline in pulmonary function (35).

To determine the pathogenetic role of chronic herpesvirus infection in pulmonary fibrosis, we explored the effects of herpesvirus infection in mice. We have chosen instillation of murine γ-herpesvirus 68 (MHV68) into the respiratory tract of Th2-biased mice (IFN-γ receptor-deficient mice) for the following reasons. MHV68 is a natural pathogen of rodents that is closely related to the human γ-herpesviruses (HHV)-8 and EBV (38). In immunocompetent mice, experimental MHV68 infection results in acute pneumonitis, which is cleared after 7–10 days, followed by transient splenomegaly and an infectious mononucleosis-like syndrome (32). Latent virus persists in B cells, macrophages, dendritic cells, and lung epithelial cells (13, 31, 32, 42). However, in immunocompetent mice,
persistent replication of virus during the chronic phase of infection is very low, and there are not associated long-term pathological effects (14). In contrast, when mice deficient in IFN-γ or lacking the IFN-γ receptor are injected intraperitoneally with MHV68, there is persistent replication of virus during the chronic phase of infection, and animals develop severe disease characterized by splenic fibrosis and vasculitis of the great elastic arteries (8, 11, 41). However, it is unknown whether these animals develop pulmonary fibrosis. Therefore, we hypothesize that instillation of MHV68 into the respiratory tract in mice defective in IFN-γ signaling will result in chronic herpesvirus lung infection with epithelial injury and progressive pulmonary fibrosis.

MATERIALS AND METHODS

Animals and animal treatment. The animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee before we conducted the experiments. IFN-γR−/− mice on a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). IFN-γR−/− mice are reported to have no apparent phenotypic anomalies and a normal lifespan of 12 mo or more (9, 18). Mice were then bred and maintained at Emory University in accordance with university and federal guidelines. Mice were inoculated intranasally with 10^5 plaque-forming units (pfu) of MHV68 mixed in DMEM-10% fetal calf serum to a total volume of 40 μl. Virus was propagated in NIH/3T12 cells. Cell homogenate from infected cells was centrifuged for 15 min at 4,220 g, and supernatants were titrated.

At various time points from 15 to 180 days postinoculation, mice were killed. Bronchoalveolar lavage (BAL) was performed through a tracheal cannula with two instillations of 0.6 ml of serum-free complete medium (Cellgro, Herndon, VA) for cytokine determination. For Western blot analysis of surfactant proteins, BAL was performed using PBS and a cocktail of protease inhibitors (Sigma-Aldrich, St. Louis, MO). Cell pellet from BAL was used for DNA extraction, and PCR analysis was used for the viral gene Orf50.

After BAL, lungs were removed and processed for histological and immunohistological examination or utilized to prepare lung cell suspensions as follows. A sample of lung tissue was minced and digested with 150 U/ml collagenase type IV and 10 U/ml DNase (Sigma-Aldrich) for 1 h at 37°C and passed through a 70-μm cell strainer to dissociate cells. These cell suspensions were subjected to fluorescence-activated cell sorting (FACS) analysis and also used for isolation of lung lymphocytes. Additional lung tissue was used for DNA extraction for PCR of viral genes or for preparation of whole cell protein extracts for Western analysis.

A total of four experiments were conducted. In the first set of experiments, mock and infected IFN-γR−/− mice were compared with mock and infected C57BL/6 wild-type mice. The second to fourth sets of experiments only included virus- and mock-infected IFN-γR−/− mice. Twenty-five C57BL/6 wild-type mice and 142 IFN-γR−/− mice were analyzed in the four sets of experiments. Because of the small size of mice lungs, not all tests or analyses were performed on every animal. No mortality was observed in control and MHV68-infected mice (observed up to 180 days after infection) with the virus dose used.

Histology, immunohistochemistry, and immunofluorescence. An average of 3–4 mice was used per group at each experimental point for histopathology and immunohistochemistry analysis. After inflation and fixation with 4% paraformaldehyde for 24 h, lung tissue was paraffin embedded, sectioned, and stained with hematoxylin and eosin (H&E) for routine histological examination and Masson Trichrome or Verhoeff-Van Gieson staining to delineate collagen. Immunohistochemistry was performed for TGF-β1 (Santa Cruz Bio-technology), α-smooth muscle actin (α-SMA; Sigma), B220 (Santa Cruz Biotechnology), surfactant protein C (SP-C; Chemicon International, Temecula, CA), and matrix metalloproteinase-7 (MMP-7; Chemicon International). Diaminobenzidine (Vector) was used as the chromogen. Indirect immunofluorescence was performed in sections from frozen blocks. Slides were fixed with 4% paraformaldehyde for 20 min at room temperature. Anti-MHV68 (41) and anti-Pro-SP-C (Santa Cruz Biotechnology) were used for immunostaining for 6 h at room temperature followed by the respective secondary conjugated antibodies. Nuclei were detected by 4′,6′-diamidino-2-phenylindole staining.

Morphometric analysis. Quantitation of collagen deposition was made using Verhoeff-Van Gieson staining. The intensity of red color staining was quantified using Scion Image software from three images at ×20 magnification per slide and three to seven mice per time point. Measurements were recorded in pixels.

Electron microscopy. Electron microscopy was performed on lung tissue after fixation in 3% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer at pH 7.3. Samples were postfixed in 1% osmium tetroxide and embedded in Eponate 12 resin (Ted Pella, Redding, CA). Ultrathin sections were cut, stained with lead citrate and uranyl acetate, and examined with a Zeiss EM 10C electron microscope.

Lung mechanics. Serial measurements of lung function were made using a whole body plethysmograph (Buxco, Sharon, CT). Unrestrained mice were placed in chambers, and after 10 min of adaptation, measurements were taken every 5 min for an interval of 20 min total. Measurements obtained were tidal volume, respiratory rate, minute ventilation, and enhanced pause (Penh), a parameter related to pulmonary resistance.

Determination of cytokine levels. Mouse IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, and IFN-γ levels were measured in BAL fluid and cell culture supernatants using a multiplex bead immunoassay (Linco, St. Charles, MO) according to the manufacturer’s recommendations. Cell culture supernatants were obtained from lung lymphocytes, which in turn were purified from the cell suspensions using Lympholyte-M gradient (Cedarlane). Cells were cultured (2 × 10^5 per 100 μl per well) and stimulated with plate-bound anti-CD3 (5 μg/ml) and anti-CD28 (2.5 μg/ml). After 72 h, supernatants were collected and stored at −80°C for later determination of cytokine levels.

FACS analysis. Lung cell suspensions were obtained by enzymatic disruption as we described above and cultured for 6 h in the presence of Golgi Plug (PharMingen) starting 1 h after restimulation (10 ng/ml PMA and 1 μM ionomycin; Sigma-Aldrich). After fixation and permeabilization, intracellular immunofluorescence staining was performed using phycoerythrin (PE)-anti-IL-4, allophycocyanin (APC)-anti-IL-5 plus FITC-anti-IFN-γ (PharMingen).

IL-4 and MMP-7 expression in the lung. The determination of mRNA levels was done by a semiquantitative bioluminescence-based RT-PCR assay as previously described (33). RNA from lung tissue was prepared using Quigen kit according to the manufacturer’s recommendations (Valencia, CA). cDNA was generated from 5 μg of total RNA using random hexamers and superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Biotinylated PCR primers and digoxigenin-labeled probes were synthesized by Genosys Biotechnologies (The Woodlands, TX). They were as follows: MMP-7 F-primer 5′-TGAGGACGGCAGGAGGTGA-3′; MMP-7 R-primer 5′-TCCA- TCCACAGCAAAAG-3′; MMP-7 probe 5′-TGAATGCTGCAATGTCG-3′; IL-4 F-primer 5′-TGGTGTGCTCTGCTTCTTT-3′; IL-4 R-primer 5′-CTAGCAGTAATGACATTTG-3′; IL-4 probe 5′- CTGGTGTTGCTCTGTT-3′; β-actin F-primer 5′-ATGGATGAC-GATATCGCT-3′; β-actin R-primer 5′-ATGGAGTACCCGATCTGCT-3′; β-actin probe 5′-GAGATGCTAGCATGACCT-3′. Comparison and standardization was made to β-actin mRNA.

Western blot. Whole cell extracts from lung tissue samples were prepared using cell lysis buffer (0.15 Nonidet P-40, 50 mM HEPES, pH 7.0, 250 mM NaCl, 5 mM EDTA, pH 8.0). Aliquots of lung lysates (10 μg) and BAL fluid (12 μl) were resolved in 12% SDS-PAGE gels and transferred onto nylon or polyvinylidene difluoride.
membranes. For hydrophobic surfactant proteins (SP-B and SP-C), BAL fluid was centrifuged at 16,000 g for 90 min. The pellets were resuspended in Laemmli sample buffer and electrophoresed using Bio-Rad 4–20% SDS-PAGE gels. Western blotting for Pro-SP-C, SP-A, SP-B, SP-D (Chemicon International), and TGF-β1 (PharMin- gen) was performed according to manufacturer’s recommendations. Filters were stripped and reprobed with an antiserum against β-actin (Santa Cruz Biotechnology) as a loading control for lung homogenates.

DNA extraction. Extraction of DNA for PCR analysis was performed on BAL cell fractions and whole lung cell suspensions. Lung cell suspension and BAL were obtained as previously described, with formed on BAL cell fractions and whole lung cell suspensions. Lung homogenates.

DNA extraction. Extraction of DNA for PCR analysis was performed on BAL cell fractions and whole lung cell suspensions. Lung cell suspension and BAL were obtained as previously described, with the exception of using 5 × 1 ml washes for BAL. The cell fraction was collected by centrifugation for 1 min at 14,000 g. DNA was extracted from 5 × 10⁶ lung cells and all available BAL cells using the DNeasy Tissue Kit (Quiagen) following the protocol for animal cells.

MHV68 Orf50 PCR. DNA amplification was performed on 100 ng of DNA extracted from lung cells, BAL cells, and S11E cell DNA (cell line transfected with virus plasmid, positive control). Single-round PCR amplified a 586-bp region of the viral gene Orf50, using primers KM86 (5'-AACTGGAACTCTTCTGTGGC-3') and KM89 (5'-GGCCGCAGACATTTAATGAC-3'). A standard PCR reaction was carried out in a 50-μl reaction volume [0.5 units Taq DNA polymerase, 1× PCR buffer, 200 μM (each) dNTPs, and 200 nM each primer]. The reaction was carried out for 45 cycles of 15 s at 94°C, 30 s at 60°C, and 30 s at 72°C. Twenty microliters of PCR products and 10 μl of positive control product were run on a 1.5% agarose gel with ethidium bromide. For control amplification, one round of PCR was performed using primers C4 117 (5'-TCCCTATGC-AGGTGTGCATG-3') and C4 118 (5'-CCCACCTCATGCATG-AAG-3').

Statistical analyses. Data were plotted and analyzed using Instat 3 and GraphPad Prism 4 (Graph Pad Software, San Diego, CA). Nonparametric analysis of intensity of collagen staining, levels of cytokines in BAL fluid, and determination of relative expression of IL-4 and MMP-7 by RT-PCR were carried out using a two-tailed Mann-Whitney test. Tidal volume results were analyzed with unpaired t-tests.

RESULTS

MHV68 infection causes lung inflammation in wild-type and IFN-γR−/− mice, but lung fibrosis is only seen in IFN-γR−/− mice. To evaluate morphological changes in the lung, IFN-γR−/− and C57BL/6 wild-type mice were infected intranasally with MHV68 and killed at several time points during the acute (1–15 days) and the chronic (>15 days) phases of infection. Wild-type mice demonstrated a moderate lymphocytic infiltrate at the peak of the acute phase mainly around airways and blood vessels (Fig. 1, A and B). By day 45,
wild-type mice had complete resolution of the interstitial pneumonia and vasculitis (Fig. 1C). As a control, IFN-γR−/− and wild-type mice were mock infected using a lysate of uninfected NIH/3T12 cells. Lung structure of mock-infected wild-type and IFN-γR−/− mice were normal and comparable to uninfected mice (Fig. 1D). In contrast, acutely infected IFN-γR−/− mice had severe inflammation in subpleural areas, around small and medium blood vessels and surrounding airways (Fig. 1E). The infiltrate consisted of lymphocytes, plasma cells, neutrophils, and eosinophils. Contrary to wild-type mice, lymphocytic infiltrates in IFN-γR−/− mice persisted in the chronic phase of the infection. Mice analyzed at day 45 (Fig. 1F) and day 100 (Fig. 1, G and H) showed abundant lymphocytes in perivascular, peribronchiolar, and subpleural areas. The subpleural infiltrates were triangular or wedge shaped, which is similar to the pathological lesions found in lungs of IPF patients by high-resolution computed tomography (Fig. 1H) (1). Immunostaining analysis demonstrated that B cells were the main component of the lymphocytic infiltrated as they stained positively with anti-B220 antibody (Fig. 1F).

Lungs of infected mice were evaluated for interstitial fibrosis at 15- to 30-day intervals. In IFN-γR−/− mice, by day 45, the lungs began to show evidence of subpleural fibrosis, which became interstitial fibrosis by day 150. At 180 days of infection in IFN-γR−/− mice, the thickening of the alveolar walls and pleura was much more evident. To confirm collagen deposition, we performed Verhoeff-Van Gieson staining. Collagen deposition in mock-infected mice was found only around large airways, and there was minimal deposition of collagen in the interstitium and pleura (Fig. 2, A and B). In contrast, virus-infected IFN-γR−/− mice demonstrated a heterogeneous appearance on low-power magnification, with areas of normal collagen deposition alternating with areas of moderate-to-severe fibrosis, particularly in the peripheral regions of the lung (Fig. 2C). Interstitial fibrosis was most intense in subpleural areas and in areas where the alveolar spaces were filled with large macrophages (Fig. 2, D–H). A generalized thickening of the pleura was noted, and areas of abnormal alveolar septation consisting of absent or shortened alveolar septa and enlargement of alveolar spaces were seen. The intensity of collagen staining was quantified using Scion Image software. The deposit of collagen increased fivefold (P = 0.03) in IFN-γR−/− mice at day 180 postinfection compared with uninfected IFN-γR−/− mice and fourfold compared with mock-infected mice (P = 0.04) (Fig. 3A). Although some perivascular lymphocytic infiltrates were observed in chronically infected wild-type mice, the progressive fibrosis was an exclusive finding in IFN-γR−/− mice and did not revert spontaneously (Fig. 3, B and C). The progressive and multifocal pulmonary fibrosis after MHV68 infection in IFN-γR−/− mice was also visible by electron microscopy that showed regions of collagen fibrils inside the alveolar walls and surrounding type II epithelial cells (Fig. 3, D and E). The increased interstitial inflammation and the presence of interstitial fibrosis were evident in H&E-stained sections at day 180 postinfection (Fig. 3F).

![Figure 2](http://ajplung.physiology.org/)

Fig. 2. IFN-γR−/− mice infected chronically with MHV68 develop lung fibrosis. A and B: deposition of collagen was analyzed by Verhoeff-Van Gieson staining of lung tissue from mock-infected IFN-γR−/− mice at day 180. Normal presence of collagen around large airways is denoted by red staining; black staining denotes elastin in blood vessel walls. Box 1 in A is shown at higher power view in B. C–H: lung section of MHV68-infected IFN-γR−/− mice Verhoeff-Van Gieson stained. Boxes 2–6 in C are fibrotic areas shown in higher power view in D through H, respectively. Extensive thickening of pleura was detected exclusively in virus-infected animals (arrow in C). A and C, ×2 magnification; B, D–H, ×20 magnification.

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The presence of fibroblasts was associated with myofibroblast transformation as evidenced by positive staining with anti-α-SMA. Myofibroblasts were absent in mock-infected animals or in areas with normal lung architecture in virus-infected IFN-γR−/− mice (Fig. 3G), but they were often found in areas of interstitial thickening in infected IFN-γR−/− mice and in some cases forming fibroblastic foci (Fig. 3, H and I).

Pulmonary function was measured weekly during the acute phase of infection and then monthly thereafter. Consistent with a restrictive pulmonary defect due to interstitial fibrosis, studies of pulmonary physiology showed significant reduction in tidal volume in infected IFN-γR−/− animals at 180 days postinfection compared with mock-infected IFN-γR−/− controls (P = 0.0173) (Fig. 4A) but not at any earlier time points. In contrast, airway obstruction, as measured by Penh, showed a significant increase at the peak of the acute phase and at day 180 postinfection (data not shown).

One of the most potent regulators of connective tissue synthesis is TGF-β. To determine whether MHV68 infection in IFN-γR−/− mice induced TGF-β expression, we performed immunostaining analysis. Areas of the lung with mild fibrosis showed increased TGF-β expression that was limited to epithelial cells and alveolar macrophages (Fig. 4B). However, in areas of the lung where there was extensive fibrosis, TGF-β expression was observed that extended to the interstitial connective tissue (Fig. 4C). Long-term infected wild-type mice had limited positive staining for TGF-β, which was primarily associated with lymphocytic infiltrates around blood vessels (Fig. 4D). Protein levels of TGF-β were measured in BAL fluid by Western blot analysis. Increased levels of the TGF-β1 latency-associated peptide complex (55 kDa) and active TGF-β1 homodimer (12.5 kDa) were found in acute and chronic MHV68-infected IFN-γR−/− mice compared with mock-treated animals (Fig. 4E).

MHV68 infects lung epithelial cells and alveolar macrophages. To assess the location of the virus in the lungs of infected IFN-γR−/− mice, we performed immunofluorescence microscopy using the polyclonal antibody anti-MHV68,
which was generated using viral lysate of NIH/3T12 infected cells injected into rabbits, therefore detecting productive lytic infection. Lung sections of mock-infected animals at day 180 were negative for viral antigen (Fig. 5A), whereas infected mice during the acute phase of the infection showed numerous positive cells for the MHV68 antibody (Fig. 5B). At day 180, lung tissue from infected IFN-γR−/− mice showed viral antigen in type II alveolar epithelial cells (Fig. 5, C–E). Type

Fig. 4. Pulmonary fibrosis in MHV68-infected IFN-γR−/− mice is associated with physiological dysfunction and increased expression of TGF-β. A: at day 180 postinfection (n = 6 mock group and n = 5 virus infected), there was a significant decrease in tidal volume. B: TGF-β expression as determined by immunostaining of lungs of MHV68-infected IFN-γR−/− mice at day 180 postinfection. Areas of the lung with mild fibrosis or normal lung parenchyma showed TGF-β expression in lung epithelial cells and alveolar macrophages. C: in the same animal shown in B, TGF-β expression in lung areas with advanced fibrosis and remodeling showed marked TGF-β expression in extracellular matrix, fibroconnective tissue, and macrophages. D: immunostaining for TGF-β on a lung section from a wild-type mouse at day 180 postvirus infection. B and D, ×40 magnification; C, ×20 magnification. E: Western blot analysis for the relative levels of latent vs. active TGF-β protein in bronchoalveolar lavage (BAL) fluid of mock (M) and MHV68-infected IFN-γR−/− mice collected at the indicated days postinfection (pi). Increased levels of the latent and active forms of TGF-β1 were found in BAL samples from virus-infected animals.

Fig. 5. Lung epithelial cells and macrophages are targets of MHV68 infection. A: no virus protein was detected by immunofluorescence analysis of lung frozen sections of mock-treated IFN-γR−/− mice at day 180. Virus antigen detection was performed using an anti-MHV68 antibody (green). Type II cells were detected using an anti-Pro-surfactant protein C (SP-C) antibody (red). Slides were counterstained with 4',6-diamidino-2-phenylindole, which stains nuclei blue. B: frozen sections from lungs of IFN-γR−/− mice at day 7 postinfection were stained with anti-MHV68 (green) and anti-Pro-SP-C (red) antibodies. Merged image shows scattered yellow cells, indicating double-positive cells. C–E: immunostaining of lung frozen section of IFN-γR−/− mice at day 180 postinfection using anti-MHV68 (green) and anti-Pro-SP-C (red) antibodies. Yellow cells indicate type II cells supporting lytic infection of the virus. Boxes 1 and 2 are shown in a higher power magnification in D and E, respectively. F and G: viral antigen is detected in alveolar macrophages using the anti-MHV68 (green) and anti-Mac3 antibodies (red). Yellow cells indicate positive double staining. A–C, ×20 magnification; D–G, ×100 magnification. H: viral DNA was detected by single-round PCR using specific primers for Orf50 in 100 ng of DNA isolated from BAL cellular pellet and lung cells of IFN-γR−/− animals at day 180. PCR reactions were performed in 2 MHV68 and 2 mock (M)-infected IFN-γR−/− mice. H2O, PCR without DNA; S11, PCR using DNA from cell line S11E (positive control); C4, control PCR for genomic DNA.
II epithelial cells positive for MHV68 were in clusters associated with the presence of alveolar macrophages and alternated with areas of virus-negative type II cells. Virus antigen was also present in alveolar macrophages, although in fewer numbers than epithelial cells (Fig. 5, F and G). We also investigated for the presence of viral genome-positive cells in the lung of infected IFN-γR−/− mice at day 180, probing for Orf50. Positive PCR reactions were obtained in DNA preparations from lung and BAL cells of infected mice. Mock-infected mice were negative for MHV68 DNA (Fig. 5H).

MHV68 infection induces Th2 responses in IFN-γR−/− mice. Because the cytokine pattern of the inflammatory response in the lungs of IPF patients is predominantly a Th2 response, we found increased levels of IL-5 and IL-10 (both Th2 cytokines) in samples of BAL fluid from IFN-γR−/− mice at 10 days postinfection (Fig. 6A). In addition, we determined expression of IL-4 by semiquantitative RT-PCR using RNA obtained from lung tissue. We found a 2.5-fold increase on IL-4 levels in infected IFN-γR−/− mice at day 8 postinfection compared with mock-infected IFN-γR−/− mice (P = 0.007). Levels of IL-4 persisted at later time points of the infection (15, 45, 155 days) but to a lesser extent (1.5-fold compared with mock-treated mice; Fig. 6B). To determine the frequency of Th2 cytokines producing cells in total lung cell suspension during the chronic phase of the infection, we stained suspensions of lung cells for IFN-γ-, IL-5-, and IL-4-producing cells. Shown are representative data from 1 of 2 experiments with similar results. (Fig. 6C). An expected enlargement in the pool of IFN-γ-producing cells was also noted after viral infection and presumably due to the incapacity of this cytokine to signal a response in the absence of IFN-γR.

Morphological analysis of pulmonary type II cells and abnormal surfactant protein homeostasis after γ-herpesvirus infection. IFN-γR−/− mice infected with MHV68 had multipolar or hypertrophic type II alveolar epithelial cells that formed cuboidal epithelial monolayers (Fig. 7A). These areas with hyperplastic epithelium were detected during the acute phase of the infection but also were seen during the chronic phase and overlay areas with extensive deposition of collagen (Fig. 7B and C). The identity of the cuboidal cells was confirmed by immunostaining analysis using an anti-Pro-SP-C antibody (Fig. 7D). Hyperplasia of type II cells is frequently associated with a reparative process after epithelial damage. In concordance with this hypothesis, we found increased apoptosis in lung areas with ongoing extensive alveolar remodeling during the chronic phase and upregulation of matrilysin expression, an MMP involved in lung epithelial repair (Fig. 7, E and F) (5, 7). Expression of matrilysin was also measured by a semiquantitative RT-PCR. A significant diminution of matrilysin expression was observed at the peak of lung virus replication after intranasal infection (day 8), followed by a significant increase of the levels at the end of the acute phase (day 15) and in long-term infected mice (day 155) (Fig. 7G).

Pronounced changes in type II cell morphology were also observed in IFN-γR−/− mice by electron microscopy. Early after infection, type II cells from infected mice were severely enlarged with lamellar bodies (Fig. 8, A and B). During the chronic phase, type II cells were either hyperplastic with cuboidal shape and containing multiple dense inclusions as we showed in Fig. 7C or hypertrophic with the presence of abundant lamellar body inclusions (Fig. 8C). These abnormal cells were observed most often in areas with extensive collagen deposition within the alveolar wall. In some instances, hypertrophic type II cells showed apoptotic nuclei, and there was...
SP-D levels were increased during acute and chronic MHV68 infection (Fig. 8E). A potential impairment in the processing of hydrophobic surfactant proteins at later time points of infection was evidenced by the accumulation of intermediate forms of SP-C protein in lung tissue (Fig. 8E).

DISCUSSION

Our finding that >95% of patients with IPF have evidence of chronic pulmonary infection with one or more of three herpes-viruses, EBV, cytomegalovirus (CMV), and/or HHV-8, suggested that these viruses can be a trigger element for the pathogenesis of IPF (35). In this study, we show that, in mice biased to develop a Th2-type response, chronic pulmonary infection with a MHV induces epithelial damage and inflammatory responses that evolve into progressive interstitial fibrosis. In contrast, fibrosis is not seen in wild-type mice. These animals only develop an acute, mild pneumonitis. Also, as shown in Table 1, the histopathological features of the pulmonary disease after chronic γ-herpesvirus infection in the Th2-biased animals recapitulates most of the patterns that are described in the lungs of IPF patients such as increased TGF-β expression, myofibroblast foci, hyperplasia of type II alveolar epithelial cells, imbalance of Th1 and Th2 cytokines, altered surfactant proteins, and vascular changes. However, honeycomb changes seen in end stage of IPF were absent in our experimental model. The absence in MHV68-infected mice of more severe fibrotic lesions and honeycomb characteristics of human IPF might be the reflection of differences in species, time following initial infection, or age-related dissimilarities.

The pathological changes in lungs of IPF patients are accompanied by enhanced expression of TGF-β1 in epithelial cells, macrophages, and associated extracellular matrix. Although high levels of TGF-β1 are not specific for IPF, overexpression of TGF-β1 in animals by alveolar epithelial cells results in pulmonary fibrosis that resembles the alveolar remodeling observed in IPF (20, 44). We speculate that chronic MHV68 infection causes injury to the epithelial cells inducing TGF-β secretion in a similar pattern to that occurring in IPF lungs. The absence of IFN-γ signaling in IFN-γR−/− mice may contribute to the increase in TGF-β or perpetuate the high levels since IFN-γ has been reported to mediate the expression of Smad7, a natural inhibitor of the TGF-β signaling pathway (23).

In addition to the extensive alveolar remodeling after MHV68 infection in IFN-γR−/− mice, MHV68 has been reported to cause vascular damage consisting of an elastic arteritis. Viral antigen has been reported in lung capillary endothelial cells during the acute phase of the infection and in smooth muscle cells of the media weeks to months after intraperitoneal inoculation of the virus (8). In our studies, we detected vasculitis during the acute phase and around day 100 postinfection. We also observed red blood cell extravasation with concomitant deposition of hemosiderin around vessels and alveoli compatible with previous episodes of hemorrhage. Recent reports indicate that microvascular injury has a potential role in the induction of fibrosis in IPF patients in concert with attachment of the cell to the basement membrane. This type II cell phenotype seen after MHV68 infection has a striking resemblance to the morphology of type II cells found in a kindred of patients with familial IPF and an associated mutation in the SP-C gene (36). Type II cells from these patients contain many abnormal lamellar bodies. To establish whether the changes in the phenotype of type II cells after MHV68 infection is associated with alterations in the expression of surfactant proteins, we performed Western blot analysis in homogenates of lung tissue and BAL fluid in IFN-γR−/− infected and mock-infected mice. We observed that levels of SP-D were enhanced in BAL fluid at 7 days postinfection compared with mock-infected animals. SP-D levels persisted at high levels during the chronic stage of the disease, whereas SP-A and SP-B levels were normal (data not shown). Contrary to this pattern, SP-C in BAL fluid decreased at 7 days postinfection and normalized at 15 days to diminish again during the chronic phase of the infection (Fig. 8D). In lung tissue samples, SP-D levels were increased during acute and chronic MHV68 infection (Fig. 8E). This result is in line with the findings in the IFN-γR−/− mice.
with chronic persistent CMV infections and the presence of anti-endothelial cell antibodies (22). Heterogeneous vascular remodeling seems to be present in lungs of IPF patients so that vascular density is higher than controls in areas with low-grade fibrosis and lower in areas with extensive fibrosis. These findings are associated with enhanced expression of angiogenic factors such as IL-8 and VEGF in endothelial and epithelial cells of lungs from IPF patients (10). The MHV68 homologous HHV-8 is vasculotropic as demonstrated by the endothelial abnormalities of cutaneous Kaposi’s sarcoma and the plexiform lesions from patients with primary pulmonary hypertension (6).

Lung pathology associated with viral infections can range from mild to severe pneumonitis depending on the status of the host immune system and the virulence of the virus. The absence of IPF-γR1 has been shown to increase susceptibility to several different viruses, but virus-induced pulmonary pathology is specific to the infectious agent (4). For example, IFN-γR−/− mice infected with respiratory syncytial virus develop predominantly airway disease characterized by bronchiolitis by 8 days postinfection that resolves by day 14 (2). Another example is IFN-γR−/− mice infected with X31 influenza virus. These animals have peribronchial and perivascular inflammation of mononuclear cells and lymphoblasts for up to 9 days postinfection that resolves rapidly after the virus is cleared (24). Together, these data suggest to us that the histopathological changes observed after chronic pulmonary MHV68 infection in IFN-γR−/− mice are particular to this virus.

MHV68 infection has been previously reported to cause interstitial and intra-alveolar fibrosis during the acute phase of infection in 129/Sv/Ev wild-type and IFN-γR−/− mice that resolved spontaneously at day 45 (11). In contrast, using IFN-γR−/− mice in C57BL/6 background, we observed minimal deposition of collagen during this earlier phase of the infection, and pulmonary fibrosis was only evident in long-term infected mice. Mouse strain differences have been reported to have an impact on immune responses and viral titers after MHV68 infection, and clearly this could have an impact on the histopathological changes in the lung after infection. For example, MHV68 infection induces a higher response of chemokines, IFN-γ, and titers of infective virus in the lung of BALB/c infected mice compared with MHV68-infected C57BL/6 mice (43). In addition, MHV68 infection has been reported to be a cofactor in the development of pulmonary fibrosis in bleomycin-resistant mice (21). In this study, BALB/c mice at day 8 postinfection were treated with repetitive doses of bleomycin and killed at day 36. In correlation with our studies, immunocompetent animals treated with virus alone failed to develop fibrosis. Mice treated with virus and bleomycin showed significant increase of fibrosis and inflammation by histopathological analysis, although there were no overall differences in the collagen content determined by hydroxyproline estimation. In this study, mice received bleomycin treatment at the peak of the inflammatory response during acute MHV68 infection. It is possible that high levels of IFN-γ and chemokines induced by viral infection can have an impact in the development of the inflammatory and fibrotic response induced by bleomycin. Higher levels of IFN-γ have been demonstrated in the bleomycin-susceptible C57BL/6 mice compared with fibrosis-resistant BALB/c mice at 3, 6, and 14 days after bleomycin. Consequently, IFN-γ-deficient mice treated with bleomycin
have decreased inflammation and fibrosis compared with wild-type animals (3, 26). These studies suggest that IFN-γ can mediate in part the bleomycin-induced pulmonary inflammation and fibrosis.

We found alterations in surfactant proteins after MHV68 infection. Upregulation of SP-D expression has been found with chronic lung inflammation and increased Th2 cytokines and associated macrophage proliferation and hypertrophy of type II cells (34). However, the early (by 4 days postinfection) accumulation of lamellar bodies in macrophages and type II cells and the diminished levels of SP-C protein during the chronic infection in our studies are unusual. This abnormal accumulation of lamellar bodies is similar to that seen in SP-C-deficient mice with 129/Sv background and also similar to the reported alterations observed in a kindred of patients with familial IPF and mutations of SP-C gene (16, 36). The mechanism for fibrosis in this kindred is thought to be the result of the accumulation of incompletely processed/misfolded SP-C protein that is toxic to the cells. However, deficiency of SP-C per se can cause inflammation and remodeling presumably because of the role of SP-C in surfactant homeostasis.

We have found that type II cells are the target of MHV68 infection. Infected type II cells often appeared abnormal, showing detachment from the basement membrane and apoptotic nuclei. Thus one mechanism for the remodeling that we observe during MHV68 infection may be dysfunction and death of type II cells causing alterations in surfactant production and function that in turn may predispose to reactivation of latent virus, furthering the epithelial injury. The association between sporadic IPF and genetic polymorphic variants of surfactant proteins has recently been examined (27). SP-A and SP-B alleles were found with higher frequency in IPF patients compared with healthy controls. Collectively, these findings indicate that changes in surfactant proteins may increase the risk for or contribute to the pathogenesis of IPF. An additional consideration is that chronic exposure to Th2 cytokines, such as IL-13, has been linked to inflammation, hypertrophy of type II cells, interstitial fibrosis, and/or altered homeostasis of surfactant proteins (17, 19).

In summary, we have demonstrated that pulmonary infection with a murine herpesvirus in a Th2-biased mouse causes progressive interstitial fibrosis that does not resolve. Our studies suggest that MHV68 infection causes injury of alveolar epithelial cells, inducing hyperplasia of type II cells as a mechanism of epithelial repair. However, the damage to type II cells by the viral infection and the altered homeostasis of surfactant proteins affects their reparative capacity. Fibrosis in this model was accompanied by the enhanced appearance of myofibroblasts and TGF-β production by lung epithelial cells and macrophages in fibrotic areas. We believe that a vicious cycle of injury and disrepair is maintained by chronic and progressive lung epithelial injury mediated through reactivation of infectious virus that can cause de novo infection in new areas of the lung (hence the inhomogeneous and progressive nature of the lesions). Reactivation of virus to a productive infection may be mediated through defective IFN-γR signaling and/or abnormal surfactant protein synthesis.

Further studies in IPF patients will be necessary to determine the contribution of herpesvirus infection to the pathogenesis of the disease. Meanwhile, MHV68 infection in susceptible mice is a unique animal model in which to study the physiopathology of pulmonary fibrosis, to determine molecular mechanisms involved in disrepair of the injured lung, and potentially to define future treatments for IPF.

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