Cardiovascular, Pulmonary and Renal Pathology

Inhibition of NF- κ B Signaling Reduces Virus Load and Gammaherpesvirus-Induced Pulmonary Fibrosis

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Idiopathic pulmonary fibrosis (IPF) is a chronic progressive lung disorder of unknown etiology. Several studies have demonstrated an association between pulmonary infection with a herpesvirus and IPF. Based on those observations, we have developed a mouse model in which interferon (IFN) γ R^{-/-} mice infected intranasally with murine gammaherpesvirus 68 (MHV68) develop lung fibrosis. We hypothesize that viral load was a critical factor for the development of fibrosis. Because nuclear factor (NF)- κ B signaling is required to efficiently establish gammaherpesvirus, latency we infected IFN γ R^{-/-} mice with a MHV68 virus that expresses a mutant dominant inhibitor of the NF- κ B signaling pathway, called I κ B α M. Striking differences were observed at the onset of the chronic infection, which correlated with a decreased virus load in mice infected with MHV68-I κ B α M compared with mice infected with control MHV68 (MHV68-MR). IFN γ R^{-/-} mice infected with MHV68-I κ B α M lacked vasculitis and fibrosis 15 to 120 days post infection. Inhibition of NF- κ B in MHV68-infected cells of the lungs diminished the expression of the fibrocyte recruiting chemokines monocyte chemoattractant protein 1 (MCP-1) and CXCL12, ameliorated macrophage expression of markers of alternative activation, and failed to increase expression of the integrin α v β 6, which is implicated in the activation of the profibrotic factor TGF- β . Thus, the inhibition of NF- κ B signaling in the infected lung cells of IFN γ R^{-/-} mice reduces virus persistence and ameliorates profibrotic events. Host determinants of latency might

therefore represent new therapeutic targets for gammaherpesvirus-associated pulmonary fibrosis. (Am J Pathol 2010; 177:608–621; DOI: 10.2353/ajpath.2010.091122)

Idiopathic pulmonary fibrosis (IPF) is a destructive lung disease of unknown cause, with no proven effective therapy other than lung transplantation.¹ Although the cellular and molecular pathways that drive the pathogenesis of IPF are complex and not fully delineated, increasing evidence suggests that a key event in its pathogenesis is ongoing alveolar epithelial injury in association with an abnormal host repair response. Several studies have implicated viral infections as an important factor in IPF pathogenesis. Specifically, Epstein-Barr virus (EBV) DNA and protein have been detected in 40 to 70% of lung tissue of IPF patients, compared with 10 to 17% of lung controls.^{2–7} Our group has detected viral DNA for EBV, Kaposi's sarcoma-associated herpesvirus (KSHV), and Cytomegalovirus herpesvirus in >95% of lung samples of IPF patients, with statically higher frequency compared with patients with non-IPF lung diseases.⁵

We have developed a model of chronic herpesvirus-induced pulmonary fibrosis infection using the herpesvirus murine gammaherpesvirus 68 (MHV68), a natural pathogen of wild murid rodents that has strong genetic and biological similarities with the human gammaherpesviruses EBV and KSHV.^{8–10} In immunocompetent animals, intranasal infection with MHV68 leads to an acute phase of lytic replication in the lung. Within several weeks, infectious virus is undetectable, and latent virus

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persists in B cells, macrophages, dendritic cells, and lung epithelial cells during the chronic life-long infection. This latency period is characterized by the maintenance of a nonintegrated viral episome and the limited transcription of viral gene expression in the absence of infectious virion production. Thus, in the immunocompetent mouse, lung histology during chronic infection is normal and intermittent reactivation to lytic infection occurs at low, barely detectable levels.^{11–14} In contrast, MHV68 infection in a mouse with genetic alterations of the interferon (IFN) γ pathway shows persistent replication of virus during the chronic phase of infection. These infected animals develop progressive lung fibrosis that shares common features observed in IPF lungs, including patchy and subpleural fibrosis, high levels of transforming growth factor (TGF)- β production, the presence of myofibroblast transformation, hyperplasia and epithelial mesenchymal transition of alveolar epithelial cells, and the activation of alveolar macrophages by the alternative pathway.^{15–18}

Our previous studies demonstrated that lytic replication in the chronic phase of infection is critical for fibrotic progression in IFN γ R^{−/−} mice infected with MHV68. The administration of the antiviral drug cidofovir starting at 45 days post infection (dpi) reduced virus replication and halted fibrotic progression.¹⁶ Infection with a recombinant MHV68 that lacks v-cyclin, a gene that functions to control reactivation, developed vasculitis and fibrosis at the onset of the chronic phase (day 15) but failed to drive fibrosis in the late chronic phase (day 150).^{16,19} In C57BL/6 mice, lytic MHV68 infection has been used as a cofactor to exacerbate established pulmonary fibrosis.^{20,21} Additionally, latent MHV68 infection has been recently reported to augment the response to a subsequent fibrotic stimulus with bleomycin or fluorescein isothiocyanate.²² However, the role for a host determinant of latency establishment in setting the levels of persistence that trigger the fibrogenic response in a susceptible host like IFN γ R^{−/−} mice has not been investigated.

Several studies indicate that the gammaherpesvirus life cycle can be regulated through the cellular nuclear factor (NF)- κ B signaling pathway.^{23,24} Expression of the NF- κ B subunit p65 inhibits lytic replication of MHV68, suggesting that high levels of NF- κ B promote the establishment of latency.²⁴ In a resting nonactivated state NF- κ B dimers are sequestered in the cytoplasm as a result of their association with inhibitory proteins including I κ B α .^{25–27} On stimulation, phosphorylation of I κ B α at serines 32 and 36 by I κ B kinases induces ubiquitination and degradation by proteasomes. Removal of the I κ B protein exposes a nuclear localization sequence on the NF- κ B complex resulting in translocation of the complex into the nucleus.^{26,28,29} We previously generated a recombinant MHV68 that expresses a mutant form of I κ B α (MHV68-I κ B α M) that functions as a dominant inhibitor of NF- κ B signaling.²³ *In vivo* infection of C57BL/6 mice with this recombinant MHV68 demonstrated that inhibition of NF- κ B in the host cell during virus infection does not impact virus replication. However, interference with NF- κ B signaling in infected cells results in deficient establishment of latency.²³

To determine the role of NF- κ B signaling in virus latency and virus-induced profibrotic response, we in-

fecting IFN γ R^{−/−} mice with MHV68-I κ B α M. We found that expression of the super repressor of NF- κ B in infected cells impaired latency and persistence in the lung and protected mice from fibrosis as evidenced by low collagen deposition and decreased expression of smooth muscle specific- α actin (α SMA) and PAI-1 and the chemokines MCP-1, CXCL12, as well as the profibrotic factor TGF- β . Taken together, the levels of viral load that directly relate to the profibrotic events that occur in the IFN γ R^{−/−} mice during the chronic phase of infection can be controlled by a host determinant of latency establishment.

Materials and Methods

Animals and Animal Treatment

Mice were bred, maintained, and used at Emory University, Atlanta, Georgia, in accordance with all university and federal guidelines after institutional approval. Mice expressing the GFP-luciferase transgene under the control of a NF- κ B-responsive promoter (NF- κ B-Luc) on C57BL/6 (H-2b) background were kindly provided by Dr. Blackwell.³⁰ IFN γ R^{−/−} mice on C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were 8 to 12 weeks of age and of mixed sex. Mice of the same sex were housed together with two to six mice per cage.

The development of recombinant MHV68-I κ B α M and the marker rescue MHV68-MR (MR) have been described before.^{23,31} Mice were inoculated intranasally as we have described before with 1×10^5 plaque forming units (pfu) of wild-type MHV68, recombinant MHV68-I κ B α M (α M), or a marker rescue of recombinant MHV68-I κ B α M (I κ B α M-MR) mixed in Dulbecco modified Eagle medium (DMEM)–serum free in a total volume of 20 to 40 μ l.¹⁷ Supernatant of homogenated uninfected NIH3T12 cells was used to inoculate mock treated mice or naïve mice were used as controls. At the indicated times post infection, mice were sacrificed and bronchoalveolar lavage (BAL) was performed through a tracheal canula by twice instilling then withdrawing 0.6 ml of serum free complete medium (Cellgro, Herndon, VA). BAL fluid was centrifuged and the supernatant collected and filtered through 0.22- μ m membranes. Samples were stored at -80°C for later determination of cytokine concentrations. White blood cells in the BAL pellet were counted on a hemocytometer, while cytologic examination was performed on cytopsin preparations fixed and stained using Diff Quick (American Scientific Products, Stone Mountain, GA). Differential counts were based on counts of 100 cells using standard morphological criteria to classify the cells as eosinophils, lymphocytes, or other mononuclear leukocytes (alveolar macrophages and monocytes). Counts were performed by a single observer who was blinded to the study group. Lungs lysates were prepared from naïve and virus-infected NGL mice at different time points post infection. Aliquots of lung lysate (10 μ g) were analyzed for luciferase activity in a luminometer. All ani-

mal studies were performed according to National Institutes of Health guidelines.

Cell Culture Infections

Cells were plated at 2×10^5 cells per well onto six-well plates 1 day before infection. For synchronous infections, LA-4 cells were infected as a high multiplicity of infection of five MHV68 infectious particles per cell. For virus growth curves, RLE-6TN cells were infected at a low multiplicity of infection of 0.01 of wild-type or recombinant MHV68 per cell. Infections were performed for 1 hour at 37°C with rocking every 15 minutes. Immediately after infection; plates were overlaid with cMEM (DMEM) supplemented with 100 U of penicillin per ml, 100 mg of streptomycin per ml, 10% fetal calf serum (FCS), and 2 mmol/L L-glutamine. The cells and conditioned medium were harvested together and titered by plaque assay as described below.

For plaque assays, NIH3T12 cells were plated at 2×10^5 cells per well onto six-well plates 1 day before infection. Organs were subjected to four rounds of mechanical disruption of 1 minute each using 1.0-mm zirconia/silica beads (Biospec Products, Bartlesville, OK) in a Mini-Beadbeater-8 (Biospec Products). Serial 10-fold dilutions of organ homogenate were plated onto NIH 3T12 monolayers in a 200- μ l volume. Infections were performed for 1 hour at 37°C with rocking every 15 minutes. Immediately after infection, plates were overlaid with 2% methylcellulose in cMEM. After 6 to 8 days, the monolayer was fixed with methanol and stained with 0.1% crystal violet in 20% methanol, and plaques were scored. The limit of detection for this assay was 50 pfu per organ.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Analysis

Cell lines and lung cell suspension were washed once in PBS and resuspended in hypotonic lysis buffer (10 mmol/L HEPES, pH 7.9, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.1 mmol/L EDTA, 1 mmol/L DTT, 0.5 mmol/L PMSF, protease inhibitor cocktail; Roche, Indianapolis, IN) for 15 minutes before the addition of 1/20 volume of 10% Nonidet P-40. Nuclei were spun down, washed in hypotonic lysis buffer, and then resuspended in high-salt buffer (25% glycerol, 20 mmol/L HEPES, pH 7.9, 420 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, 0.5 mmol/L PMSF, protease inhibitor cocktail; Roche) with vigorous shaking at 4°C. The supernatant collected after $10,000 \times g$ centrifugation for 10 minutes at 4°C was nuclear extract.

Nuclear extracts were assayed for NF- κ B activation by electrophoretic mobility shift assay. 2.5 to 7.5 μ g of nuclear extract were incubated with a 32 P-labeled oligonucleotide containing the NF- κ B consensus site, 5'-AGTTGAGGGGACTTTCCAGGC-3', in a binding reaction containing 2 mmol/L HEPES (pH 7.9), 1 mmol/L EDTA, 5 mmol/L dithiothreitol, 0.05% Triton-X 100, 5% glycerol, 2 μ g poly d(I-C) (Roche) for 30 minutes at room temperature. Supershift experiments were performed by over-

night incubation of nuclear extracts with 0.2 to 4.0 μ g of antibodies against NF- κ B subunits (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C. Nucleoprotein complexes were subjected to electrophoresis in 5% native polyacrylamide gels at 180 V, dried under vacuum, and analyzed by phosphorimager analysis (Typhoon 9410, Amersham Biosciences, Piscataway, NJ).

Histopathology, Immunofluorescence, and Immunohistochemistry

As we have described before, after sacrifice, lungs were perfused with 4% paraformaldehyde¹⁷ or OCT media. Sections from paraffin blocks were stained with H&E and Masson trichrome to determine histopathological changes and fibrosis. The nuclear localization of NF- κ B p65 (Santa Cruz) was visualized in MLE cells that were fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO), and permeabilized in 0.02% Triton X-100 (Sigma-Aldrich). We performed indirect immunofluorescence on frozen lung sections, using an anti-MHV68 rabbit polyclonal antibody produced by Dr. Sam Speck. For IHC staining, we used anti-TGF- β antibody (BD Pharmingen, San Diego, CA) overnight at 4°C followed by horseradish peroxidase (HRP)-conjugated secondary antibodies.

Morphometric Analysis

As we have described before, quantification of collagen deposition was made from slides of mock and infected lungs after Masson trichrome staining.¹⁶ A 0 to 4 point scale was used as follows: 0 = normal lung architecture; 1 = lymphocytic infiltrates in perivascular, peribronchial, and subpleural areas but not fibrosis; 2 = lymphocytic infiltrates and perivascular and peribronchial fibrosis; 3 = lymphocytic infiltrates and fibrotic thickening of the inter-alveolar septa; 4 = lymphocytic infiltrates, presence of foamy macrophages, formation of multiple fibrotic foci, and fibrotic thickening of the pleura. Due to the patchy pathology of the infected lungs, the score for 10 random fields was recorded and the highest score found was assigned for each individual specimen.¹⁶

Limiting Dilution Analysis of Persistent Viral Replication in the Lungs

Preformed infectious virus in the lungs was measured by a modified form of the limiting-dilution *ex vivo* reactivation assay.³¹ Briefly, the lower left lobe of the lung from each animal were subjected to four rounds of mechanical disruption of 1 minute each using 1.0-mm zirconia/silica beads followed by an additional four rounds of disruption using 0.5-mm zirconia/silica beads (BioSpec Products). Disruption of cells was verified by trypan blue exclusion (Sigma). The homogenate was brought up to 3.4 ml with cMEM and plated in twofold serial dilutions onto Murine Embryonic Fibroblasts (MEFs) in 96-well tissue culture plates. Twelve dilutions of 16 replicate wells were plated.

Cytopathic effect was monitored microscopically at 14 to 21 days postplating.

Quantitative PCR

Quantitative PCR for MHV68 ORF50 and the cellular GAPDH normalization control was performed using a Bio-Rad iCycler. Each reaction mixture contained 12.5 μ l iQ SYBR green supermix (Bio-Rad, Hercules, CA), 10 pmol each primer, 200 ng DNA, and water to a final volume of 25 μ l, and each reaction was performed in triplicate in optical 96-well plates. Primers for the reactions were as follows: *orf50*, 5'-GGCCGCAGACATTTA-ATGAC-3' and 5'-GCCTCAACTTCTCTGGATATGCC-3'; *GAPDH*, 5'-CCTGCACCACCAACTGCTTAG-3' and 5'-GTGGATGCAGGGATGATGTTC-3'. Cycling conditions were as follows: 95°C for 5 minutes and then 40 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The number of copies of ORF50 and GAPDH in each sample was determined by comparison with a series of standard curve reactions using a plasmid control containing appropriate sequences. The standard curve dilutions used represented a range from 10^8 to 10^1 , in serial 10-fold dilutions.

Quantification of mouse α -SMA, plasminogen activator inhibitor-1 (PAI-1), and collagen 1A1 and 18S rRNA was performed by amplification of cDNA using the LightCycler real-time thermocycler as described previously.^{32,33} Optimized amplification conditions were 100 nmol/L primers for murine PAI-1, Collagen 1A1 and α -SMA, 4 mmol/L MgCl₂, annealing at 68°C; for 18S, 50 nmol/L universal 18S rRNA primers, 4 mmol/L MgCl₂ and annealing at 62°C; extension at 72°C. Copy numbers were calculated by the instrument software from standard curves generated from human PAI-1, Collagen 1A1, α -SMA and 18S templates. Primers used were α -SMA Forward 5'-GCAGCCCAGCCAAGCACTGTCAGGAAT-3', α -SMA Reverse 5'-AGCCCAGAGCCATTGTACACACCAAGG-3'; Col1A Forward 5'-CCTAAGGGTCCCCAATGGTGAGAC-3'; Col1A Reverse 5'-GGGGGTTGGGACAGTCCAGTCTTC-3'; PAI-1 Forward 5'-GTTCATCGCTGCACCTTTGAGAAA-3', PAI-1 Reverse 5'-GAGGATTGTCTGTGCGGGTTGTGC-3'.

Flow Cytometry

Cells were resuspended at 1×10^6 cells per ml in PBS containing 2% FCS for 30 minutes on ice in the dark and stained with 1:100 to 1:300 dilution of a combination of the following antibodies: R-phycoerythrin (PE)-conjugated antibody to CD62L; fluorescein isothiocyanate (FITC)-conjugated antibody V β 4; and Pacific Blue-conjugated antibody to CD8. The p79 tetramer to an ORF61 peptide (H-2K^b) were synthesized at the NIH Tetramer Core Facility at Emory University and conjugated to streptavidin-allophycocyanin (Molecular Probes, Carlsbad, CA) according to the Core protocol. Rat anti-mouse CD16/CD32 (Fc block) was used to block Fc receptors before staining. All reagents were obtained from BD Biosciences (San Jose, CA) and EBioscience (San Diego, CA). Data were collected on a LSRII (BD Bio-

sciences) and analyzed using FlowJo software (Tree-star, Ashland, OR).

Cytokine and Chemokine Levels in BAL and Lung Tissue

Mouse TNF- α , MCP-1, and IFN- γ levels were measured in BAL fluid using a multiplex bead immunoassay (Linco, Millipore, Billerica, MA) according to manufacturer's recommendations.¹⁶ Concentration of CXCL12 was measured from lung lysates in PBS and a cocktail of protease inhibitors (Sigma) using an ELISA kit (R&D Systems, Minneapolis, MN) according to manufacturer recommendations.

Isolation of Alveolar Macrophages

As we have described before, BAL was performed by instilling $5 \times 600 \mu$ l of serum free DMEM media.¹⁵ BAL fluid was incubated in 24-well plates for 2 hours at 37°C 5% CO₂. Attached cells were analyzed by flow cytometry, and more than 80% of the cells were positive for CD11b (BD Biosciences, San Jose, CA) indicating mononuclear cell type. Isolated mononuclear cells were used for preparation of whole cell extracts.

Western Blot

As we have described before, lung and alveolar macrophage whole cell extracts were prepared using ELB lysis buffer (0.15 NP-40, 50 mmol/L Hepes, pH 7.0, 250 mmol/L NaCl, 5 mmol/L EDTA, pH 8.0). Aliquots of lung lysates (10 μ g) and BAL fluid (12 μ l) were resolved in 4 to 20% SDS-PAGE, and transferred onto nitrocellulose membranes.¹⁵⁻¹⁷ Western blotting for Ym1/2 (kindly provided by Dr. Toshihiko Iwanaga, Hokkaido University Japan), Arginase 1 (Santa Cruz Biotech), Collagen 1 (Santa Cruz biotech), MMP9 (Sigma), and, TGF- β (Pharmingen BD), were performed and filters stripped and re-probed with an antiserum against β -actin (Santa Cruz Biotech) or with an antibody against Surfactant A (Chemicon International) as a loading control for lung homogenates and BAL fluid, respectively. Films were scanned using a Fluor-Chem Multiimage apparatus and analyzed using AlphaEase-FC software (α Innotech Corporation, San Leandro, CA).

Gelatin Zymography

Gelatin zymography was performed by using a 10% SDS-PAGE gel saturated with 1 mg/ml gelatin (Sigma Chemical, 300 bloom) as described previously.^{16,34}

Statistical Analyses

Data were plotted and statistically analyzed using InStat 3 and Graph-Pad Prism 4 (Graph Pad Software, San Diego, CA). Luciferase assays, cytokine concentrations, viral genome copies, and differential cell counts were analyzed

Figure 1. NF- κ B activation in response to MHV68 infection can be suppressed by MHV68-I κ B α M *in vivo*. **A:** Nuclear extracts were prepared from LA-4 cells, murine airway epithelial cells, treated 15 minutes with TNF- α , or infected at an MOI of 5.0 for 24 hours and analyzed by electrophoretic mobility shift assay (EMSA). NF- κ B subunits present in the nucleoprotein complexes were identified by supershift analysis using antibodies against the indicated NF- κ B subunits. **Lines** point to specific complexes; **bracket** indicates supershifted complexes. **B:** Immunofluorescence of MLE15 cells infected at an MOI of 0.01 48 hpi demonstrates nuclear localization of NF- κ B p65 subunit in infected but not uninfected cells. **C:** Nuclear extracts were prepared from the lungs of a naïve IFN γ R^{-/-} mouse (N) or infected (1 and 2) IFN γ R^{-/-} mice 7 dpi and analyzed by EMSA. The composition of the complexes was determined by the addition of antibodies against the indicated NF- κ B subunits. **D:** Lung lysates were prepared from NF- κ B luc reporter mice mock-infected or infected with MHV68-MR or MHV68-I κ B α M at the indicated dpi, normalized by protein content, and subjected to luciferase assay. The data shown represent four to six mice per experimental group. RLU, relative luciferase units. **P* < 0.05.

with unpaired *t*-tests using Mann-Whitney test. Two-tailed *P* values <0.05 were considered significant.

Results

NF- κ B Activation in Response to MHV68 Infection Can Be Reduced by Infecting with a Recombinant MHV68 Expressing an NF- κ B Transdominant Inhibitory Transgene

The NF- κ B signaling pathway is activated during the productive infection of fibroblasts and latent infection of B cells with MHV68,^{23,31} but the activation profile in lung epithelial cells in culture or lung tissue *in vivo* has not been investigated. To examine the activation profile on *de novo* infection of murine airway epithelial cells, nuclear extracts were prepared from LA-4 cells infected at a multiplicity of infection (MOI) of 5 pfu per cell and analyzed for binding to an oligonucleotide containing a NF- κ B binding sites by EMSA. NF- κ B activation was evident 24 hours post infection (hpi) in MEF cultures (Figure 1A), and this complex was competed by a wild-type but not by a single base-pair mutant competitor oligonucleotide (data not shown). Supershift analyses revealed that the NF- κ B complexes from the nuclear extracts of infected epithelial cells contained the p65/RelA and p50 and subunits, as seen on treatment with TNF α (Figure 1A). Activation of p65 was also indicated by nuclear localization of p65 in the murine lung epithelial MLE15 cell line, 48 hpi with MHV68 (Figure 1B). Higher levels of p65/p50 heterodimers and p50 homodimers were present in the lung extracts of infected IFN γ R^{-/-} mice 7 dpi compared with a naïve control mouse (Figure 1C).

Cells infected with a recombinant MHV68 expressing the I κ B α M-transgene are inhibited for NF- κ B activation in EMSAs and NF- κ B-dependent reporter assays in cell culture.²³ We next sought to determine whether the level of NF- κ B activation could be altered in the lung tissue of infected mice. NF- κ B luc mice are transgenic mice with a NF- κ B-dependent luciferase construct that are respon-

sive to NF- κ B activating stimuli in multiple tissues and organs including the lungs.^{30,35} MHV68-MR infection of the NF- κ B luc reporter mice led to the detection of luciferase activity at 4, 7, and 12 dpi, with the peak of luciferase activity at 4 dpi to levels nearly 200-fold over the uninfected naïve mice (Figure 1D). Luciferase expression was significantly reduced in NF- κ B luc mice infected with MHV68-I κ B α M by nearly threefold at each time point. Thus, MHV68-I κ B α M can effectively diminish NF- κ B activation in the lungs of infected mice during the known period of acute replication (Figure 1D).

Persistent Virus Infection and Fibrotic Disease Course Is Prevented on Inhibition of NF- κ B Signaling during MHV68 Infection of IFN γ R^{-/-} Mice

NF- κ B is a critical mediator and regulator of immune responses and is required for optimal establishment of latency during chronic infection by MHV68.^{23,31} To examine the role of NF- κ B signaling in the mouse model of gammaherpesvirus-driven pulmonary fibrosis, we examined for overt signs of illness and lung pathology in IFN γ R^{-/-} mice on intranasal infection with a recombinant MHV68 expressing I κ B α M, the transgenic super-repressor of NF- κ B signaling (MHV68-I κ B α M), and the marker rescue repair virus (MHV68-MR). The infection of IFN γ R^{-/-} mice with wild-type MHV68 results in a rapid period of weight loss that can reach nearly 20% of initial body mass. In an examination of weight loss with time, infections with wild-type, MHV68-MR, and MHV68-I κ B α M resulted in a steep decline in weight that peaked around 9 dpi (Figure 2A). Mice infected with MR or wild-type MHV68 recovered to their starting weights by 25 dpi yet failed to thrive as evidenced by a very slight weight gain over the remaining six-month time course in surviving mice. In sharp contrast, the I κ B α M virus-infected mice rebounded dramatically from the initial drop in body mass, reaching the weights of naïve mice by 25 dpi and remaining nearly indistinguishable from their uninfected

Figure 2. Defective establishment of latency by inhibition of NF- κ B in gammaherpesvirus infection protects IFN- γ R-deficient mice from virus-induced fibrosis. **A:** Data are represented as the percentage of the initial body weight (dotted line). More severe illness was observed in MHV68-wild-type and MHV68-MR-infected mice. Data were compiled from two independent experiments ($n = 4$ –18 mice per data point). **B** and **C:** Masson trichrome staining of lung slides from two different mice infected with MHV68-MR (marker rescue) at day 120 post infection. Collagen deposition is demonstrated by blue staining. Notice the thickening of the pleura and alveolar walls. **Inset** in **C** is shown at higher power view in **D**. **E** and **F:** Masson trichrome staining of lung slides from two different mice infected with MHV68-I κ B α M at day 120 post infection. Lymphocytic infiltrates are observed in perivascular areas, but there is no collagen deposition in pleura or alveolar walls. **Inset** in **F** is shown at higher power view in **G**. **H:** Semiquantitative morphometric analysis of lung histopathology in virus-infected mice with MHV68-MR and MHV68-I κ B α M at day 120 of infection. MHV68-MR-infected mice had higher pathology scores corresponding to thickening of the intraalveolar septa and the pleura. In contrast, mice infected with MHV68-I κ B α M had lymphocytic infiltrates ($n = 13$ for MHV68-MR and $n = 9$ for MHV68-I κ B α M). **I:** Masson trichrome staining of naive IFN γ R $^{-/-}$ mouse. **Inset** in **I** is shown at higher power view in **J**. **K:** Viral load in the lung was quantified by real-time PCR detection of the virus-genome specific orf50-DNA as compared with cellular GAPDH DNA in 200 ng total DNA prepared from lung tissue of four individual mice infected with MHV68-wild-type (closed squares), MHV68-MR (inverted triangles), and MHV68-I κ B α M (open triangles) at 198 dpi. Bars represent geometric mean ($*P < 0.01$). Dotted line represents detection limit of the assay. **L:** Persistence in the lungs was analyzed by limiting dilution analysis of disrupted lung tissue isolated from IFN γ R $^{-/-}$ mice infected with the indicated viruses 198 dpi. Symbols for each sample represent the mean percentage of 12 wells, positive for cytopathic effect $\pm$ the SEM on plating six fourfold dilutions of mechanically disrupted lung tissue from an individual mouse on an indicator MEF monolayer ($n = 3$ –4 mice). Curve fit lines were derived from nonlinear regression analysis. Magnification, $\times 4$ (**B**, **C**, **E**, **F**, and **D**).

counterparts throughout the remainder of the experiment (Figure 2A).

Lungs were analyzed for collagen deposition by Masson trichrome staining at 120 dpi, late during the chronic period of infection. There was a dramatic alteration in the histopathology of the lungs of MHV68-MR-infected mice as compared with the MHV68-I κ B α M-infected mice and naïve mice (Figure 2, B–J). MHV68-MR-infected mice had extensive thickening of the pleura, accompanied by areas of abnormal alveolar septation and enlargement of alveolar spaces. Collagen deposition indicated by blue staining demonstrated interstitial fibrosis and widespread regions of collagen deposition in both the pleural and subpleural regions in the MHV68-MR-infected mice (Figure 2, B–D). In sharp contrast to the multifocal fibrosis of MHV68-MR-infected mice, there were low levels of collagen deposition in the MHV68-I κ B α M-infected mice,

largely localized around the large airways (Figure 2, E–G). Overall, there was only a low degree of lung pathology in mice infected with MHV68-I κ B α M as compared with the pathology scores that ranged from mild to quite severe in mice infected with the MHV68-MR virus (Figure 2H).

Ongoing virus replication has been demonstrated to mediate the lung fibrosis in MHV68-infected IFN γ R $^{-/-}$ mice.¹⁶ We now hypothesized that the pathology and weight loss data were also directly related to virus load in the lungs. Indeed, there was a more than 20-fold reduction in viral load in the lungs of MHV68-I κ B α M-infected mice compared with wild-type and MHV68-MR control virus as determined by quantitative PCR at 198 dpi (Figure 2K). The presence of preformed infectious virus has been reported in the lungs of IFN γ R $^{-/-}$ mice at 90 dpi.¹¹ Consistent with the inability of IFN γ R $^{-/-}$ mice to control

Figure 3. Inhibition of NF- κ B in MHV68 infection does not impair virus replication or inflammation during the acute period of infection in IFN γ R^{-/-} mice. **A:** Acute replication in the lungs of IFN γ R^{-/-} mice. On the indicated days post infection, lungs were harvested, disrupted, and titered on NIH 3T12 cells. The data were compiled from one or two experiments with five to ten mice analyzed per experiment. Data are shown as log₁₀ titer, and the bar indicates the geometric mean titer. The dashed line indicates the limit of detection of this assay as log₁₀1.7 or 50 pfu/ml of sample homogenate. **B:** Multistep growth curve in RLE-6TN cells infected with an MOI of 0.01 pfu per cell. Samples were harvested at the indicated time points and titers were determined on NIH3T12 cells. **C–F:** H&E staining of lung sections from mice infected with MHV68-MR, at 4, 9, and 12 dpi, respectively. Severe inflammation is noted in subpleural areas, around small and medium blood vessels, and surrounding airways by 12 dpi. **Inset** in **E** is shown at higher power view in **F**. **G–J:** H&E staining of lung sections from mice infected with MHV68-IκBαM, at 4, 9, and 12 dpi, respectively. **Inset** in **I** is shown at higher magnification in **J**. Magnification, $\times 10$ (**C–E**, **G–I**).

MHV68 reactivation, robust levels of productive infection were detected in the MHV68-MR and control wild-type MHV68 infections (Figure 2L). The level of productive infection was reduced approximately tenfold in mice infected with MHV68-IκBαM (Figure 2L), consistent with the lower level of viral DNA detected by quantitative PCR (Figure 2K). Taken together, these results show that the lack of lung fibrosis in MHV68-IκBαM-infected mice is associated with impaired latency and persistent gamma-herpesvirus infection.

NF-κB Signaling during MHV68 Infection Is Not Essential for Acute Virus Replication and Early Inflammatory Responses

Intranasal administration of MHV68 results in acute productive infection of alveolar lung epithelial cells causing injury and potential activation of repair responses. To examine for NF-κB-dependent effects on the lungs of infected IFN γ R^{-/-} mice, we first analyzed the levels of productive infection in the lungs of mice infected with MHV68-IκBαM virus at early times after infection. Consistent with the weight loss data in Figure 2A MHV68-IκBαM underwent robust replication on days 4–12 after infection. The levels of virus production were similar at 4 and 9 dpi, periods of peak replication, with a maximal difference of 6.5-fold in geometric mean titer at 4 and 9 dpi (Figure 3A). At 12 dpi, replication is reduced to nearly undetectable levels and marks the transition from the acute to chronic phase of infection (Figure 3A). To confirm our *in vivo* data, we examined the role of NF-κB signaling in the replication of MHV68 in rat lung epithelial cells, RLE-6TN. Similar to growth curves performed in permissive primary or transformed fibroblast cells,^{23,31}

the inhibition of NF-κB signaling had no impact on virus replication in lung epithelial cells (Figure 3B).

Histological analysis of H&E-stained lung sections at 4, 9, and 12 dpi revealed little differences in the inflammatory infiltrate between MHV68-IκBαM- and MHV68-MR-infected mice (Figure 3, C–J). Overall, there was a moderate and increasing degree of inflammatory infiltration between 9 and 12 dpi, concomitant with decreasing levels of virus production (Figure 3A). This lymphocytic infiltrate was localized in the interstitium and around airways and blood vessels in both MHV68-IκBαM- and MHV68-MR-infected mice. Taken together, the levels of virus replication and inflammatory infiltration during the acute phase seem largely NF-κB-independent in IFN γ R^{-/-} mice.

Early Reduction of Viral Load at an Early Chronic Phase of Infection Diminishes Profibrotic Responses in the Lung

We next examined the histopathology of the lungs during the initial period of chronic infection at 15 dpi. Inflammation was striking in the MHV68-MR-infected mice at 15 dpi as evidenced by the large numbers of lymphocytic infiltrates surrounding the airways and in the small and large blood vessels of subpleural areas (Figure 4A). This infiltration was accompanied by extensive interstitial collagen deposition visualized by Masson trichrome staining (Figure 4, B and C). This is in sharp contrast to the lower levels of infiltration in the MHV68-IκBαM-infected mice that appeared to resolve slightly from the degree of infiltration observed at 12 dpi (Figure 4D). Furthermore, collagen deposition was greatly diminished in the MHV68-IκBαM-infected mice (Figure 4, E and F). In concordance

Figure 4. NF- κ B inhibition reduces viral load and collagen deposition in the lung tissue of IFN γ R $^{-/-}$ mice at the transition from acute to chronic infection. **A:** H&E staining of lung section from IFN γ R $^{-/-}$ mouse infected with MHV68-MR at day 15 pi. **B:** Masson trichrome staining for collagen deposition of lung section from IFN γ R $^{-/-}$ mouse infected with MHV68-MR at day 15 pi. **Inset in B** is shown in **C**. **D:** H&E staining of lung section from IFN γ R $^{-/-}$ mouse infected with MHV68- α M at day 15 pi. **E:** Masson trichrome staining for collagen deposition of lung section from IFN γ R $^{-/-}$ mouse infected with MHV68- α M at day 15 pi. **Inset in E** is shown in **F**. Notice the increased degree of pleural and alveolar thickening in mice infected with MHV68-MR, as compared with MHV68- α M. **G:** Semiquantitative morphometric analysis of lung histopathology in virus-infected mice with MHV68-MR and MHV68- α M at 15 dpi. MHV68-MR-infected mice had higher pathology scores corresponding to thickening of the intraalveolar septa. **H–J:** Quantitative RT-PCR analysis for plasminogen activator 1 (PAI 1), collagen type 1 (Col1), and smooth muscle actin (α -SMA) transcripts in the lungs of IFN γ R $^{-/-}$ mice infected with the indicated viruses at 15 dpi (* P < 0.05, n = 4). **K and L:** Viral antigen in immunostained frozen lung sections from MHV68-MR versus MHV68- α M-infected IFN γ R $^{-/-}$ mice. Insets in **K** and **L** are shown in **M** and **N**, respectively. **O:** Quantitative PCR analysis of virus load in the lungs of individual IFN γ R $^{-/-}$ mice infected with MHV68-MR (inverted triangles) or MHV68- α M (open triangles) at 15 dpi. Bars represent geometric mean (* P < 0.01). Magnification, $\times 10$ (**A–D**); $\times 20$ (**K** and **L**).

with the fibrotic differences observed in the lungs of infected mice (Figure 4G), we found higher expression of collagen 1 (Figure 4H), α SMA (Figure 4I), and the antifibronolytic factor PAI-1 (Figure 4J) in tissue obtained from MHV68-MR-infected lungs than MHV68- α M-infected mice. Up-regulation of these transcripts is known to be mediated by the profibrotic factor TGF- β , a cytokine associated with fibrosis and the presence of myofibroblasts and activated type II alveolar epithelial cells.^{36,37}

Lung fibrosis in MHV68-infected IFN γ R $^{-/-}$ mice is associated with persistent virus replication in the lungs.¹⁶ Since virus replication in the lungs of mice at 15 dpi was near the limit of detection by plaque assay (data not shown), the distribution and abundance of viral antigen was analyzed by immunofluorescence with polyclonal antibody against MHV68 lytic cycle-associated proteins. There was an abundance of viral antigens detected in frozen lung sections from mice infected with MHV68-MR at 15 dpi (Figure 4, K and M). Positive staining was detected in cells localized in the alveolar walls and in perivascular inflammatory infiltrates. Lungs of mice infected with MHV68- α M showed diminished positive staining for viral antigen as compared with their MHV68-MR counterparts (Figure 4, L and N). We further examined viral load in the lung at 15 dpi by quantitative PCR analysis of viral genomes. Viral DNA copy number was reduced 10-fold in the lungs of MHV68- α M-in-

fecting mice as compared with MHV68-MR-infected mice (Figure 4O). Additionally, we performed *in situ* hybridization for MHV68 tRNA-like transcripts in lungs from MHV68-MR and MHV68- α M-infected mice to detect latent infected cells at day 25 post infection and observed extensive positive signal in the lung tissue of mice infected with MHV68-MR-infected lungs, compared with lungs infected with MHV68- α M or naïve mice (data not shown). Taken together, these data indicate that interference with NF- κ B signaling in the infected cells has a critical role in the persistence of infection that was directly associated with early collagen deposition. This suggested that the ensuing fibrotic pathology likely initiates at a very early stage of chronic infection and led us to further examine events during the transition from the acute to chronic phase of infection with MHV68- α M or control MHV68-MR.

Diminished Proinflammatory Cytokines and Chemokines in MHV68- α M-Infected Mice at an Early Phase of Chronic Infection

To characterize the inflammatory response at this early period of chronic infection we performed differential count of cell pellets harvested from the BAL of infected animals at 15 dpi. A significant larger number of mono-

Figure 5. Alternatively activated macrophages as well as chemokine MCP-1 and CXCL12 expression levels are diminished on the infection of $IFN\gamma R^{-/-}$ mice with MHV68-IkBaM. **A:** Differential cell counts in the bronchoalveolar lavage (BAL) of infected mice at 15 dpi. M indicates mononuclear cells; L, lymphocytes; N, neutrophils; E, eosinophils ($*P < 0.01$, $n = 8$). **B:** Flow cytometry was used to analyze the CD8⁺ T cell profile of $IFN\gamma R^{-/-}$ mice uninfected or infected with MHV68-MR or MHV68-IkBaM at 15 dpi. Bars represent the mean percentage of effector (CD44^{hi}, CD62^{lo}) cells; the mean percentage of activated, MHV68-specific (CD44^{lo}, MHV68-p79-peptide specific (tetramer+)) cells; the mean percentage of CD44^{hi}, Vβ4⁺ populations in gated CD8⁺ T cells $\pm$ SD ($n = 3-4$ mice per group) at 15 dpi. **C-E:** TNF-α, IFN-γ, and MCP-1 levels were measured in bronchoalveolar lavage (BAL) fluid from uninfected and MHV68-MR and IkBaM-infected $IFN\gamma R^{-/-}$ mice at the indicated time points post infection. Bars represent mean ($n = 5$ per group) $\pm$ SEM ($*P < 0.05$). **F:** Levels of CXCL12 in lung lysates of $IFN\gamma R^{-/-}$ mice after infection with MHV68 determined by ELISA in whole lung lysate. Values represent mean $\pm$ SEM ($n = 3-8$, $*P < 0.05$). **G:** Immunoblot analyses for markers of alternatively activated macrophages, Arginase 1 and Ym1/2, in lung lysates of naïve mice (N) and mice infected with MHV68-MR and IkBaM at the indicated time points post infection. Each lane represents a different mouse. Blots were stripped and reprobed with an anti-β-actin antibody as loading control. **H:** Immunoblot assay for Arginase 1 in whole cell extracts from alveolar macrophages isolated from infected MHV68-MR and MHV68-IkBaM mice at day 10 pi.

cytes and lymphocytes were found in the BAL of MR-infected mice compared with their mock and MHV68-IkBaM-infected counterparts (Figure 5A). However, the relative percentage of cell subsets in BAL samples were similar between animals infected with MHV68-MR compared with MHV68-IkBaM, including mononuclear cells (70.8 versus 65.7), lymphocytes (25.6 versus 32.9), and neutrophils (3.1 versus 1.5). Thus, while the intensity of the inflammatory response is much diminished in the MHV68-IkBaM-infected animals, there was no evidence for a change in the cellular profile of the infiltrates at 15 dpi.

We further examined immune responses in MHV68-MR and MHV68-IkBaM-infected mice. CD8⁺ T cells have a critical role in clearing acute replication in the lungs and controlling virus reactivation during chronic infection. We examined for differences in the phenotype of CD8⁺ T cell infiltrates in the lungs of mice infected with MHV68-IkBaM compared with MR at 15 dpi by flow cytometry (Figure 5B). More than 50% of CD8⁺ T cells in mice infected with MHV68-MR or MHV68-IkBaM exhibited a CD44^{hi}/CD62^{lo} effector T cell phenotype (Figure 5B). In addition, there was no difference in the abundance of activated CD44^{hi} CD8⁺ T cells that bound the MHV68

p79 tetramer (Figure 5B). MHV68 infection also elicits persistent activation of CD8⁺ T cells bearing the Vβ4 T cell receptor. The expansion of this T cell population associated with the expression of the latent protein M1 has been implicated in the pathogenesis of the fibrotic disease in the $IFN\gamma R^{-/-}$ mice.³⁸ The persistent activation of CD8⁺ T cells bearing a Vβ4⁺ T cell receptor (CD44^{hi}/Vβ4⁺/CD8⁺ T cells) was detected at similar levels in mice infected with MHV68-MR or MHV68-IkBaM (Figure 5B). This indicates that effector CD8⁺ T cells are recruited at similar levels during the initial stage of chronic infection regardless of NF-κB inhibition and are not a major contributor to the gross differences in lung pathology seen at 15 dpi in MHV68-MR-infected mice.

TNF-α is a NF-κB-regulated immune mediator produced by the alveolar epithelial and inflammatory cells.³⁹ The levels of TNF-α in the BAL fluid peaked at 9 dpi and were detected at slightly higher levels in the IkBaM-infected mice (Figure 5C). TNF-α production was sustained in the MR-infected mice from 12 dpi through 15 dpi, although without significant differences (Figure 5C). The proinflammatory cytokine IFN-γ is critical to control MHV68 reactivation from latency.⁴⁰ Although, IFN-γ is not active in the $IFN\gamma R^{-/-}$ mice, its increased presence in

the MHV68-MR-infected mice indicates greater CD8⁺ T cell effector function in response to the higher level of virus persistence in the MHV68-MR-infected versus MHV68-I κ B α M-infected mice at early and late times after infection (Figure 5D). Lastly, we determined levels of the chemokines MCP-1 and CXCL12 in BAL samples of infected mice during the chronic phase of infection. In mice, MCP-1 and CXCL12 have been associated with the recruitment of fibrocytes, a subgroup of hematopoietic progenitor cells that produce collagen and that contribute to the pathogenesis of lung fibrosis.^{41,42} MCP-1 was found at significantly higher levels in the BAL of mice infected with MHV68-MR compared with MHV68-I κ B α M at 12 and 90 dpi (Figure 5E). We also analyzed lung levels of CXCL12 in MHV68-MR and MHV68-I κ B α M-infected mice at several time points post infection. CXCL12 significantly increased in IFN γ R^{-/-} mice infected with either virus at 9 dpi. However, at 15 dpi there was a significantly higher level of CXCL12 exclusively in MHV68-MR-infected animals that remained present at 90 dpi (Figure 5F).

Th2 environments induce the activation of macrophages by the alternative pathway⁴³. Our previous studies have shown that lung fibrosis in IFN γ R^{-/-} mice infected with wild-type MHV68 is accompanied by the presence of M2 macrophages activated by the alternative pathway.¹⁵ We analyzed the expression of chitinase-like proteins Ym1/2 and Arginase 1, markers of M2 macrophages, in lungs of MHV68-MR and MHV68-I κ B α M-infected mice at several time points after infection. Consistent with our previous results we found that MHV68-MR-infected mice express both markers during the acute and chronic phase of infection.¹⁵ In sharp contrast, expression levels of Ym1/2 and Arginase 1 were reduced in lungs of MHV68-I κ B α M-infected mice (Figure 5G). Because Arginase 1 is also produced by lung fibroblasts, we determined Arginase 1 expression in alveolar macrophages. Macrophages were isolated from BAL of mock and virus infected mice at 10 dpi and whole cell extracts were prepared. We found a dramatic reduction in Arginase 1 expression in infected macrophages on the inhibition of NF- κ B (Figure 5H). These changes in the phenotype of alveolar macrophages were not associated with differences in the expression levels of the Th2 cytokine IL-13 (data not shown) in lungs of MHV68-MR- and I κ B α M-infected mice.

Taken together, these data provide evidence for a dramatic diminution in the inflammatory and profibrotic profile of the MHV68 infected IFN γ R^{-/-} mice associated with low viral load at the onset of the chronic phase of infection.

Defective MHV68 Latency Establishment and Persistence Blocks TGF- β 1 Responses

Our previous studies have shown that MHV68 infection induces up-regulation of the profibrotic factor TGF- β 1 in IFN γ R mice.¹⁷ To determine whether infection with MHV68-I κ B α M, a virus impaired in latency and persistence, alters virus-induced TGF- β expression, we ana-

lyzed protein levels of TGF- β in BAL fluid samples from IFN γ R^{-/-} mice infected with MHV68-MR and MHV68-I κ B α M. By immunoblot analysis, we determined that levels of latent TGF- β were induced at comparable levels in the lungs of mice infected with MHV68-I κ B α M or MHV68-MR during the acute phase of infection (Figure 6A) as well as active TGF- β (data not shown). However, infection with MHV68-I κ B α M failed to up-regulate the TGF- β 1-latency associated peptide complex and active TGF- β 1 homodimers in the lung during the chronic phase of infection (Figure 6B). Similarly, immunohistochemical analyses demonstrated a substantial loss of TGF- β staining in mice infected with MHV68-I κ B α M as compared with MHV68-MR (Figure 6C). Activation of latent TGF- β in lung epithelial cells occurs through the α v β 6 integrin.^{44,45} We analyzed levels of integrin β 6 in lungs of IFN γ R^{-/-} mice infected with MHV68-MR and MHV68-I κ B α M. Immunoblot analysis show higher levels of integrin β 6 in MHV68-MR-infected lungs compared with MHV68-I κ B α M during the acute phase of infection (day 9), the transition of acute to chronic phase (day 15) and during the chronic phase of infection (day 90) (Figure 6D). Immunohistochemistry analysis identified positive staining for integrin β 6 in MHV68-MR-infected lungs at day 15 post infection in areas with extensive remodeling (Figure 6E).

TGF- β expression leads to the up-regulation of MMP-9, as well as several extracellular matrix components. MMP-9 expression in lung tissue of infected mice was increased at 15 dpi, but the inhibition of NF- κ B signaling led to a decrease in MMP-9 expression levels at 90 dpi (Figure 7A). We also examined MMP-2 and MMP-9 gelatinolytic activity in BAL samples from mice infected with MHV68-MR and I κ B α M during the acute and chronic phase of infection. BAL fluid samples from MHV68-MR-infected animals compared with naive and MHV68-I κ B α M-infected animals exhibited high gelatinolytic activity that corresponds to MMP-9 and MMP-2 activity (Figure 7B). TGF- β is also a potent stimulator of collagen 1 and α SMA expression. Quantitative PCR analyses showed that lungs from chronic infected mice with MHV68-I κ B α M express collagen and α SMA transcripts at levels that are comparable to naive mice, in contrast to the higher transcript levels of the mice infected with MHV68-MR (Figure 7, C and D). The higher levels of collagen-1 in lung of mice infected with MHV68-MR compared with mice infected with MHV68-I κ B α M at day 90 were confirmed by immunoblot assay (Figure 7E).

These data suggest that MHV68 acute infection induces TGF- β expression and activation by integrin β 6 but high levels of TGF- β in the chronic phase of infection requires the establishment of a robust latent virus infection that sets a higher level of persistent replication.

Discussion

Several studies have detected viral proteins and/or viral genome of herpesvirus in the lungs of IPF patients, suggesting that this virus can be a trigger or a cofactor element for the pathogenesis of pulmonary fibro-

Figure 6. Defective expression and activation of TGF- β in mice infected with MHV68-I κ B α M. **A:** Western blot assay for latent form of TGF- β in BAL collected from naive (N) and MHV68-MR and MHV68-I κ B α M-infected mice at 4 and 9 dpi. Each lane represents a different mouse. Blot was stripped and reprobed with anti-surfactant A (SP-A) antibody to normalize expression of latent TGF- β . **B:** Immunoblot analysis for the latent and active forms of TGF- β in BAL collected at 15 and 90 dpi. Each lane represents a different mouse. Blot was stripped and reprobed with an anti-surfactant A antibody to normalize expression of latent (white bars) and active (gray bars) TGF- β . Decreased levels of latent and active TGF- β were found in MHV68-I κ B α M-infected mice. **C:** Immunohistochemical analysis of lungs from MHV68-MR and MHV68-I κ B α M-infected mice at 90 dpi, using anti-TGF- β specific antibody. Magnification, $\times 10$ (upper); $\times 100$ (lower). Strong positive staining was found in alveolar hyperplastic lung epithelial cells in MHV68-MR-infected mice. **D:** Immunoblot assay of lung lysates from MHV68-MR and MHV68-I κ B α M-infected mice at the indicated time point post infection using an anti-integrin $\beta 6$ antibody. Increased levels of integrin $\beta 6$ were found in samples from MHV68-MR-infected mice. Blot was stripped and reprobed with an anti- β -actin antibody as a loading control. **E:** Immunostaining for integrin $\beta 6$ on lung sections from MHV68-MR and -I κ B α M at 15 dpi. High expression for integrin $\beta 6$ was found in lung sections from MHV68-MR-infected mice. Magnification, $\times 40$.

sis.^{4,5,46–49} Furthermore, expression of the EBV latent protein LMP-1 has been associated with poor prognosis in IPF patients and markers of ER stress.^{6,49} Chronic infection with herpesviruses is characterized by periods of long-term latency with intermittent reactivation events. In our virus induced lung fibrosis model, we have shown that MHV68 reactivation from latency is critical for the development of fibrosis in the IFN γ R^{−/−} mice.¹⁶ Here we examined whether a reduction in the establishment of gammaherpesvirus latency and the consequent diminution of viral load were sufficient to control the induction of

profibrotic responses in this susceptible host. Because NF- κ B signaling is a key determinant of gammaherpesvirus latency, we used a recombinant virus expressing the NF- κ B super repressor I κ B α M to inhibit NF- κ B only in the infected cells. We performed a comparative analysis between IFN γ R^{−/−} mice infected with MHV68-I κ B α M and marker rescue virus (MHV68-MR) during both the acute and chronic stages of infection to identify critical events that might provide mechanistic insight in the pathogenesis of virus induced pulmonary fibrosis. These studies confirmed that persistent infection drives profibrotic

Figure 7. Defective latency by NF- κ B inhibition blunts profibrotic events in MHV68-induced fibrosis. **A:** MMP9 expression in lung homogenates from naive and MHV68-MR and MHV68-I κ B α M-infected mice at 90 dpi. Each lane represents a different mouse. **B:** Gelatin zymography of BAL fluid samples from naive or infected mice at 15 and 90 dpi. Each lane represents a different mouse. Recombinant MMP-9 and MMP-2 were used as controls. High gelatinolytic activity was observed in samples obtained from MHV68-MR-infected mice. **C:** Quantitative RT-PCR analysis for smooth muscle actin (α -SMA) (* P < 0.05, n = 4). **D:** Collagen type 1 (Col1) transcripts in the lungs of IFN γ R^{−/−} mice infected with the indicated viruses at 90 dpi. **E:** Immunoblot analysis of collagen 1 in lung lysates of mice infected with MHV68-MR and MHV68-I κ B α M at 90 pi. Each lane represents a different mouse. Blot was stripped and reprobed with an anti- β -actin antibody as loading control.

events involving the expression of TGF- β in the early and late phase of chronic virus infection.

NF- κ B is a critical host determinant of gammaherpesvirus latency. In MHV68-infected mice, ablation of NF- κ B signaling by the expression of the NF- κ B super repressor I κ B α M led to a dramatic reduction in latent infection in the spleen and lung.^{23,31} This finding was confirmed by a complimentary genetic approach wherein B cells lacking p50 in p50^{+/-} bone marrow chimeric mice failed to support wild-type MHV68 latency. IFN γ R^{-/-} mice infected with MHV68-I κ B α M exhibited a reduction of latency in the spleen (data not shown) and lungs as evidenced by a reduction in viral genomes and persistent replication. The dramatic reduction in fibrotic pathology in mice infected with MHV68-I κ B α M that accompanies this reduction in virus load demonstrates that interference with a host determinant of latency could be an effective strategy for disabling herpesvirus-driven pulmonary fibrosis.

IFN γ R^{-/-} mice exhibit rapid and dramatic weight loss often approaching 20% of their initial body mass that peaks around 9 days after infection. This period of weight loss was concomitant with the acute phase of virus replication and was observed in mice infected with wild-type MHV68, the recombinant MHV68-I κ B α M, or the repair virus MHV68-MR. The inflammatory response in the lung during the acute period of IFN γ R^{-/-} mice as evidenced by histopathological analysis was not dependent on NF- κ B activation within the infected cell, suggesting that the innate host response is responding to generalized tissue damage from virus replication. Because virus replication and the degree of inflammation based on histology during the acute period of infection was not impacted by NF- κ B inhibition, we conclude that the profibrotic differences observed between I κ B α M- and MR-infected mice beginning by 15 dpi are not due to acute virus replication. In concordance, IFN γ R^{-/-} mice infected with the mutant v-cyclin stop-MHV68 that is reactivation deficient but with normal latency, develop fibrosis at day 15 pi but have only mild lung pathology at later time points post infection.

Depletion of CD8 T cells in the IFN γ R^{-/-} prevented cellular atrophy and fibrosis in the spleen in addition to reducing the burden of latently infected cells to the levels of wild-type-infected mice.⁵⁰ In addition, the M1 latency gene of MHV68 that has superantigen-like functions in driving V β 4+CD8+T cell expansion is required for inflammation and fibrosis in IFN γ R^{-/-} mice.³⁸ However, we did not observe any appreciable reduction in activation or effector profile of CD8+ T cells or V β 4+CD8T+ expansion at 15 dpi in the I κ B α M-infected mice compared with MR-infected mice. This indicates that during this period of acute to chronic transition, the T cell function is not disrupted by the inhibition of NF- κ B signaling in the infected cells.

Expression of proinflammatory cytokines like TNF- α is mediated by NF- κ B.⁵¹ However, we see robust TNF- α production in I κ B α M-infected mice at the peak of acute replication 9 dpi. Therefore, there is not a direct dependence on NF- κ B in the infected cell population for proinflammatory cytokines production in the lung as a whole. The dramatic diminution of viral load in the lung from MHV68-I κ B α M-infected mice was also associated with a rapid reduction in the levels of chemokines involved in

the recruitment of fibrocytes, CXCL12 and MCP-1. The recruitment of fibrocytes has been shown that can be an indicator for disease activity and disease progression in the IPF lung.^{41,42} CXCL12 has been demonstrated to be important in mediating fibrocyte recruitment during lung injury and to contribute to fibrosis.⁵² Murine fibrocytes have also been shown to traffic to the lungs in response to MCP-1.⁵³ In the injured lung, CXCL12 is expressed by bronchial and alveolar epithelial cells and enhanced expression is found in alveolar epithelial cells from IPF patients. Similarly to our finding, Vannella et al²² recently reported that MHV68 latently infected alveolar epithelial cells up-regulate the expression of these two chemokines and induce fibrocyte accumulation in lungs of latent MHV68 infected mice.

TGF- β 1 has been widely recognized as a key fibrogenic cytokine. TGF- β -induced cellular processes associated with lung fibrosis including enhanced collagen synthesis, proliferation, migration, adhesion, and trans-differentiation into myofibroblasts, characterized by the expression of α -SMA.⁵⁴ Our previous studies show that gammaherpesvirus infection in the IFN γ R^{-/-} mice up-regulates the expression of latent and active TGF- β in the lung during the chronic phase of infection. Control of virus reactivation using an antiviral agent beginning at day 45 pi resulted in reduction of pulmonary fibrosis and low levels of TGF- β at day 120 pi. In the current study, we found that TGF- β expression is up-regulated in MHV68 infected lungs at day 9 pi but latent and active TGF- β levels were significantly reduced in mice with low viral load even at the time of transition from acute to chronic phase of infection (day 15, Figure 6B) and later time points in the chronic phase of infection. These data suggest that lytic replication of the virus induces TGF- β expression but latent virus infection is required to maintain high levels of TGF- β in the infected lung. In concordance with our findings, *in vitro* studies using a viral cell line model show that EBV lytic phase induction in alveolar epithelial cells increased active and total TGF- β .⁵⁵ In addition, *ex vivo* studies in alveolar epithelial cells isolated from latent infected C57BL/6 mice produced high levels of active TGF- β .²²

TGF- β 1 is secreted as an inactive noncovalent complex with a latency-associated peptide and requires activation before it can bind to its receptors. α v β 6 integrin is responsible for the activation of constitutively expressed latent TGF- β in the lung epithelial cell.⁵⁶ Integrin α v β 6 is up regulated during injury. Mice deficient in integrin- β 6 are resistant to bleomycin-induced lung fibrosis although they have enhanced inflammatory responses.^{56,57} Infection with human Cytomegalovirus has been associated with induction of integrin α v β 6 expression in endothelial cells, leading to activation of TGF- β and collagen synthesis.⁵⁸ Analyses of integrin- β 6 expression in MHV68-I κ B α M-infected lungs show reduced levels compared with MHV68-MR from day 9 to 90 pi (Figure 6E). Because we observed normal levels of latent TGF- β at day 9 during the acute phase of replication in the MHV68-I κ B α M-infected lung, the establishment of latency during the early phase of chronic infection in alveolar epithelial cells is likely to contribute to maintain the high levels of

active TGF- β in the infected lung at late times after infection.

Similar to the cellular tropisms of EBV and KSHV, multiple cell types including epithelial cells, endothelial cells, and fibroblasts may harbor MHV68, yet B cells are the predominant reservoir for latency during chronic infection of mice by MHV68.^{11,21,59} Based on immunohistochemistry, MHV68 protein antigens are detected in epithelial and alveolar macrophages in the lungs of infected IFN γ R^{-/-} mice, indicating these cells are a major reservoir for lytic replication during the acute and chronic phase on infection in the lungs.¹⁷ B cells are a latency reservoir and likely play a role in reactivation and persistence as well. In C57Bl/6 mice, the only alteration in the MHV68-I κ B α M-infected mice is a reduction of activated CD69+ B cells.²³ Our studies demonstrated that expression of integrin β 6, an integrin specifically expressed in epithelial cells,⁴⁵ is diminished in the lungs of mice infected with MHV-68-I κ B α M. We also determined that alveolar macrophages from MHV68-I κ B α M-infected lungs express lower levels of markers of alternative activation in the early and chronic phase of infection. This finding is consistent with the direct infection of alveolar macrophages by MHV68-I κ B α M because activation of NF- κ B in macrophages is reported to induce alternative activated macrophage phenotype through negative cross talk with the Stat1 pathway.⁶⁰

Directly attributing profibrotic events to a particular type of infected cell in the lung is challenging given the broad cellular tropism and dynamics of latency and persistence in the IFN γ R^{-/-} mice. However, a characterization of cell type-specific roles for NF- κ B-mediated gene regulation in the infected cell reservoirs of the lung is a current line of investigation. In addition, the role of NF- κ B activation in a particular cell type *in vivo* might be best approached using recombinant MHV68 driving I κ B α M expression under the control of a cell type-specific promoter.

In summary, gammaherpesvirus-induced lung fibrosis in the IFN γ R^{-/-} mice was nearly abolished early in the chronic phase of infection, by the inhibition of NF- κ B signaling, a pathway that is critical for the establishment of a latent infection by MHV68. Furthermore, the level of persistent gammaherpesvirus infection directly influences the degree of continual up-regulation of TGF- β , CXCL12, and MCP-1 that results in progressive lung fibrosis. Our findings suggest that intervention with virus latency is a therapeutic approach that might control or prevent lung fibrosis that is associated with a gammaherpesvirus infection.

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