Control of Gammaherpesvirus Reactivation Ameliorates Pulmonary Fibrosis in IFNγR Deficient Mice

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Animals and animal treatment

Previous reports showed complete control of MHV68 virus lytic replication using 25 mg/kg (17). Because we observed a 50% mortality with the 25 mg/kg dose after the first week of treatment, we decided to use 7.5 mg/kg starting on day 45 post-infection, a similar dose to the dose recommended in human patients (5 mg/kg). A dose of 15 mg/kg of body weight was used in symptomatic animals starting on day 60 post-infection. .At 120-150 days post viral inoculation, IFNγR-/- mice were sacrificed by cervical dislocation after isofluorane anesthesia and the following tests performed in subsets of animals. A group of 9 mice was infected intranasally with 10^5 pfu of v-cyclin-stop MHV68 mutant virus and sacrificed at day 150 post infection. Bronchoalveolar lavage (BAL) was performed through a tracheal canula by twice instilling then withdrawing 0.6 ml of serum free complete medium (Cellgro, Herndon, Virginia, USA). BAL fluid was centrifuged and supernatants collected and filtered through 0.22μm membranes. Samples were stored at -80^{o}C for later determination of cytokine concentrations. For western blot analysis for TGFβ, BAL was performed using PBS and a cocktail of protease inhibitors (Sigma-Aldrich); BAL fluid was stored at -80^{o}C for later western blot analysis. Following BAL, lungs were removed and processed for the following analyses: for histology lungs were inflated with 4% paraformaldehyde; for immunofluorescence, lungs were inflated with OCT media for the preparation of frozen sections. Additional lung tissue was used for preparation of whole cell protein lysates. A total of 50 IFNγR-/- mice were

analyzed in the mock infected and 85 IFNγR-/- mice were infected with virus in four sets of experiments. Experiment 1 : 7 mock (M), 10 mock antiviral (M AV), 6 virus saline solution (Virus SS) and 10 virus antiviral begun at day 45 (Virus AV 45); Experiment 2: 6 M, 8 M AV, 15 Virus SS and 13 virus AV 45, Experiment 3: 9 M, 7 Virus SS, 12 Virus AV 45 , 2 Virus AV begun day 60 (Virus AV 60), Experiment 4: 5 M, 5 M AV, 13 Virus SS and 7 Virus AV 60. Because the small size of mouse lungs, not all tests or analyses were performed on every animal.

Morphometric Analysis – Pathology Score

A 0 to 4 point scale was used as follow:

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 interalveolar septa;.

4 =lymphocytic infiltrates, presence of foamy macrophages, formation of multiple fibrotic foci and fibrotic thicknening of the pleura.

Because 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.

Hydroxyproline Assay

At the time of sacrifice, all lobes of lung were removed and the extrapulmonary airways and blood vessels excised and discarded. The lung parenchyma was homogenized in 1.0 ml of PBS, after which 1.0 ml of 12 N HCl was added, and the samples were hydrolyzed at 110°C for 24 hours. Five microliters of each sample was mixed with 5 µl of citrate-acetate buffer (5% citric acid, 1.2% glacial acetic acid, 7.25% sodium acetate, and 3.4% sodium hydroxide). One hundred microliters of chloramine-T solution (1.4% chloramine-T, 10% N-propanol, and 80% citrate-acetate buffer) was added, and the mixture was incubated for 20 minutes at room temperature. Ehrlich's solution was added and the samples were incubated at 65°C for 18 minutes. Absorbance was measured at 550 nm. A standard curve was generated for each experiment using reagent hydroxyproline as a standard. Results were expressed as micrograms of hydroxyproline per lung.

Determination of cytokine levels

Samples were obtained at the time of sacrifice on day 120-150 post-infection. The minimum detectable concentrations for the assay were: TNFα, 0.9 pg/ml; IL-5, 0.5 pg/ml; IL-6, 0.7 pg/ml; IL-13, 4.7 pg/ml; IFN-γ, 0.7 pg/ml; MIP-1α, 2.8 pg/ml; MCP-1, 6.3 pg/ml.

Gelatin Zymography

Equal volume of BAL samples (20μl) were loaded onto the gel and electrophoresed at a constant 150 V for 1.5 h. The gels were incubated for 1 h at

room temperature in 2.5% Triton X-100, followed by an overnight incubation at 37°C in gelatinase substrate buffer (50 mM Tris, 10 mM $CaCl_2$, and 0.02% NaN_2, pH 8.0). The gels were stained with 0.5% Coomassie blue followed by subsequent destaining with 50% methanol. Purified MMP-2 and MMP-9 were used to identify zymography bands.

Arginase Activity

Briefly, cells were lysed with 100 µl of a buffer containing 0·1% Triton X-100 and 25 mM Tris-HCl, MnCl2 5 mM pH 7,4. Arginine hydrolysis was performed by incubating the lysate with 100 µl of 0·5 M L-arginine (pH 9·7) at 37°C for 60 min. The reaction was stopped with 800 µl of H2SO4 (96%)/H3PO4 (85%)/H2O (1:3:7, vol/vol/vol). The urea concentration was measured at 540 nm after addition of 50 µl of α-isonitrosopropiophenone (dissolved in 100% ethanol) followed by heating at 95°C for 45 min .

Fibronectin Gene Transcription

To evaluate fibronectin gene transcription, a plasmid expressing luciferase under the control of a fibronectin promoter sequence was introduced into murine NIH/3T3 fibroblasts from the American Type Culture Collection (CRL Rockville, MD) via electroporation to create stable transfectants. The transfected NIH/3T3 fibroblasts were treated with BAL fluid and tested for luciferase activity.

MHV68 glycoprotein B, Ym1/2 and FIZZ1 expression in the lung

Total RNA was extracted from lung tissue using an RNeasy mini kit according to manufacturer's recommendations (Quiagen, Valencia CA). cDNA was generated from 0.5-5 μg of total RNA using random hexamers and Thermoscript reverse transcriptase (Invitrogen, Carlsbad CA). Real time RT PCR was performed using SYBR Green and primers specific for the genes of interest in an iCycler iQ (BIO-RAD).

Primers were designed using Beacon Designer IV (Premier Biosoft International, Palo Alto, CA). The primers used were:

gB: 5' CCTATAACTACATCGCTACCG3', and 5'ATTCTTGACCTCCCTGACC3',

Ym1/2: 5'TTATCCTGAGTGACCCTTCTAAG3' and

5'TCATTACCCAGATAGGCATAGG3'.

Fizz1: 5'GAACTTCTTGCCAATCCAG3' and 5'TCCAGTCAACGAGTAAGC3'.

Bleomycin instillation and cidofovir treatment

Bleomycin was instilled intra-tracheally under xylazine-ketamine anesthesia. Four U/kg of bleomycin, in 0.1 ml sterile phosphate-buffered saline, was injected into the tracheal lumen. After inoculation, the incision was closed and the animals were allowed to recover.