

Latent cytomegalovirus infection exacerbates experimental pulmonary fibrosis by activating TGF- β 1

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Abstract. The aim of the present study was to investigate the hypotheses that cytomegalovirus (CMV) may trigger idiopathic pulmonary fibrosis (IPF) in a susceptible host and/or that the presence of CMV may alter IPF in response to a well-defined trigger of pulmonary fibrosis. A mouse model of murine CMV (MCMV) infection was established, and the mice were divided into a control group, bleomycin group and an MCMV+bleomycin group. Changes in the weights of the mice were determined in the three groups. Pulmonary fibrosis was detected using a histopathological method. The activity of transforming growth factor (TGF)- β 1 was measured, and the levels of E-cadherin, Vimentin and phosphorylated (phospho)-small mothers against decapentaplegic (SMAD)2 were determined using western blot analysis. MCMV was found to invade the lungs, however, it did not cause pulmonary fibrosis. The progression of fibrosis in the mice treated with MCMV+bleomycin was more rapid, compared with that in the control mice. The protein levels of Vimentin and phospho-SMAD2 were upregulated, whereas the level of E-cadherin was downregulated in the MCMV+bleomycin group. The results suggested that latent MCMV infection aggravated pulmonary fibrosis in the mouse model, possibly through the activation of TGF- β 1.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a common and life-threatening disease, with a median survival rate of ~3-5 years following diagnosis. However, its pathogenesis is complex and remains to be fully elucidated (1). Microarray

analysis has shown that the instillation of bleomycin, a profibrotic drug, alters the gene transcription pattern in the mouse lung by increasing the expression of proinflammatory mediators, certain components of the pulmonary extracellular matrix and genes that are induced by transforming growth factor- β 1 (TGF- β 1) (2). TGF- β 1 contributes to pulmonary fibrosis in numerous animal models and in human pulmonary fibrotic disease by inducing the epithelial-to-mesenchymal transition (EMT) of alveolar epithelial cells (3,4).

Human cytomegalovirus (HCMV) is a member of the herpes family of viruses. CMV infection is relatively common, with ~40-70% of adults being infected in the latent state. Latent CMV infection, defined by being a carrier of the CMV genome without active replication, is commonly asymptomatic in immunocompetent individuals, whereas active CMV infection is associated with clinical signs and symptoms, which include fever, sore throat and leukopenia. HCMV infects the respiratory tract, and it has been evaluated with regards to its association with IPF. Patients with IPF show significantly higher DNA copy numbers in their blood, compared with healthy controls (5). There is evidence that suggests an association between the incidence of HCMV infection and the incidence of IPF, and that CMV induces the secretion of TGF- β 1 from infected fibroblasts, astrocytes and osteosarcoma cells (6). However, the mechanism by which HCMV may affect fibrosis remains to be elucidated.

In the present study, a mouse model of murine CMV (MCMV) infection was used to investigate the hypotheses that CMV may trigger IPF in a susceptible host, and/or that the presence of CMV may alter IPF in response to a well-defined trigger of pulmonary fibrosis, namely the chemical bleomycin. In addition, the precedent exists for the possibility that CMV-infected alveolar epithelial cells may induce the production or activation of TGF- β 1 in pathological settings. TGF- β 1 has been associated with EMT and promotion of pulmonary fibrosis.

Materials and methods

Animals and materials. BALB/c mice (age, 4-6 weeks; weight, 15.9 \pm 1.5 g; n=27) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained at 18-22°C with a 10-14 h light/dark cycle and access to food and water

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ad libitum. The mice were divided into 3 groups of 9 mice. MCMV was cultured, as described previously (7). In brief, the salivary gland-passed MCMV (Smith strain) was prepared by homogenizing the salivary glands of 10 BALB/c mice, sacrificed by cervical dislocation, which had been infected with 10^4 plaque forming units (PFU) 3 weeks previously. Aliquots (1 ml) of the homogenized supernatants of the salivary glands were stored in liquid nitrogen in RPMI 1640 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 5% fetal bovine serum (Thermo Fisher Scientific, Inc.), and titrated onto 2.5×10^5 /ml NIH-3T3 cells (American Type Culture Collection, Manassas, VA, USA) and cultured at 37°C in 5% CO₂ for 7 days. Polymerase chain reaction (PCR) reagents were purchased from Applied Biosystems; Thermo Fisher Scientific, Inc. The PCR primers were purchased from Invitrogen; Thermo Fisher Scientific, Inc. Bleomycin was purchased from Sigma-Aldrich (St. Louis, MO, USA). The present study was approved by the ethics committee of Anhui Medical University (Hefei, China).

Mouse treatments. The salivary gland-passaged MCMV was suspended in 200 μ l of Hanks' balanced salt solution (Thermo Fisher Scientific, Inc.) with 3% fetal bovine serum. The mice were infected via intraperitoneal injection with 10^5 PFU or, as a control, mice were injected with Hanks' balanced salt solution/3% fetal bovine serum. The mice (n=9; 3/group) were sacrificed by cervical dislocation 3, 14 and 28 days following infection and viral loads were analyzed using PCR. After 4 weeks, subsets of the MCMV-infected and uninfected mice, respectively, were intratracheally instilled with bleomycin or phosphate-buffered saline (PBS) to induce fibrotic changes. Briefly, the mice were anesthetized with sevoflurane inhalation (Abbott Laboratories, Abbott Park, IL, USA) and placed in dorsal recumbency. Transtracheal insertion of a 24-G animal feeding needle was used to instillate bleomycin (0.75 U/ml) or vehicle (PBS), in a volume of 80 μ l. The body weights of the mice were measured at least twice each week thereafter. The mice were sacrificed 7 and 14 days following instillation, and the lungs were removed for further analysis, as described below.

PCR assessment of viral loads. Extraction of the MCMV DNA from the tissues was performed using a QIAamp DNA Mini kit (Qiagen, Hilden, Germany). In brief, 25 mg samples of tissues (10 mg of spleen; salivary gland; lung; liver) were sonicated following the addition of 80 μ l PBS. Qiagen lysing buffer (100 μ l; Qiagen) and 20 μ l proteinase K (Thermo Fisher Scientific, Inc.) were added, and the sample was incubated at 56°C for ~3 h until it was completely lysed. RNase A (200 μ l) was then added, along with Qiagen buffer AL (Qiagen). The sample was incubated at 70°C for 10 min and 200 μ l ethanol (96-100%) was added, followed by centrifugation at 6,000 x g for 1 min at 4°C in a spin column several times with 500 μ l Qiagen buffers AW1 and AW2 (Qiagen). The sample was then incubated for 5 min at room temperature and centrifuged at 6,000 x g for 1 min at 4°C using 150 μ l distilled water, following which the filtrate containing the MCMV DNA was collected. The DNA quality was confirmed in an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). The viral loads of MCMV were determined using standard and real-time quantitative (q)

PCR using an ABI 7500 (Applied Biosystems; Thermo Fisher Scientific, Inc.). A quantity of 50 ng total DNA (5 μ l) was used in each reaction with 26.5 μ l PCR Master mix (Thermo Fisher Scientific, Inc.), 15.75 μ l distilled water, 1 μ l forward and reverse primers (10 μ M), and the primer sequences were as follows: forward 5'-ATCTG-GTGCTCCTCAGATCAGCTAA-3' and reverse 5'-ATTGTTTCATTG-CCTGGGGAGTTT-3'. The thermocycling conditions were as follows: 95°C for 10 min; and 40 cycles of 95°C for 15 sec and 60°C for 1 min. The experiments were performed three times independently and results were compared using the 2^{- $\Delta\Delta$ C_q} method (8).

Histopathology. Lungs from each mouse were fixed overnight with neutral-buffered formalin (Sigma-Aldrich) and embedded in paraffin (Sigma-Aldrich). Sections of tissue (5 μ M thick) were mounted and stained with hematoxylin and eosin (Beyotime Institute of Biotechnology, Haimen, China) to assess the degree of fibrosis. The histological sections were reviewed by an experienced pathologist using a fluorescence microscope (BX41; Olympus Corporation, Tokyo, Japan).

Preparation of tissue extracts. The isolated lung tissues were rinsed in sterile normal saline to remove blood and were briefly placed on a sterile gauze to dry. To obtain tissue extracts, the collected tissues were cut into small sections, and ~30-50 mg of the tissues were minced and sonicated in 500 μ l lysis buffer (Qiagen) with 50 mM Tris-HCl (pH 7.5), containing 100 mM sodium fluoride, 30 mM sodium pyrophosphate, 2 mM sodium molybdate, 1 mM sodium orthovanadate, 1 mM glycerophosphate and 1X protease inhibitor cocktail, on ice. The samples were centrifuged at 12,000 x g for 20 min at 4°C. The clear supernatant was collected and stored in aliquots at -80°C. Protein quantification of the lysate was performed using the bicinchoninic acid method.

Bioassay of TGF- β 1 activity. TGF- β 1 activity was measured by the inhibition of (3H) thymidine incorporation into lung epithelial cells (CCL64), as described by Jennings *et al* (9). Acid activation was performed, with minor modification, to isolate free TGF- β molecules from the latent complex. Briefly, 30 μ l of tissue extracts (equivalent to 100-300 μ g protein) were added to 200 μ l of minimal essential medium/bovine serum albumin, followed by the addition of 10 μ l 4 N HCl, and agitated for 1 h at 4°C. Acid activation was terminated by neutralization with 10 μ l 4 N NaOH. The tissue extracts were assessed prior to and following activation. The activated tissue extracts were also examined following the neutralization of TGF- β 1 activity with rabbit anti-pan-TGF- β serum (British Biotechnology, Oxon, UK; 1:100) at 37°C for 30 min. The controls consisted of (3H) thymidine incorporation into cells treated with a range of concentrations of purified TGF- β 1 with and without anti-TGF- β antibodies, cells incubated with 4 mM HCl, TGF- β -neutralizing serum alone (1:100), medium alone, or non-immune rabbit serum.

Reverse-transcription-qPCR. Total RNA was obtained from the lung tissues using an RNeasy kit (Qiagen), according to the manufacturer's protocol. The RNA quality was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.). The RNA was reverse transcribed into cDNA using an

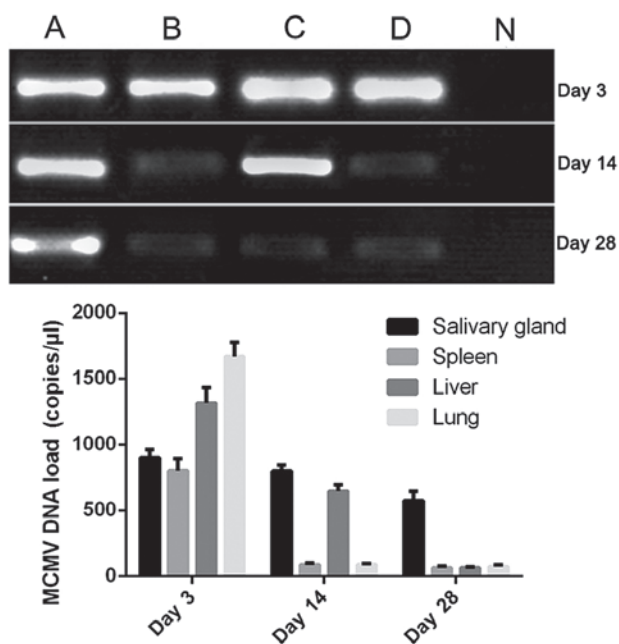


Figure 1. Quantitative polymerase chain reaction analysis of the MCMV DNA load in different organs. (A) Salivary gland; (B) spleen; (C) liver; (D) lung; (N) control. Data are presented as the mean \pm standard error of the mean from experiments repeated in triplicate. MCMV, murine cytomegalovirus.

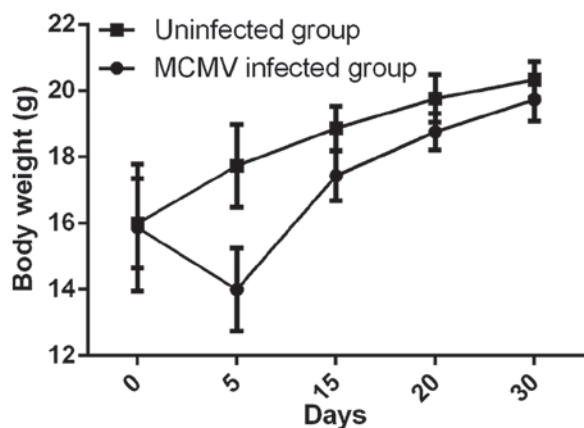


Figure 2. Body weight changes of the mice MCMV, murine cytomegalovirus. Data are presented as the mean \pm standard error of the mean from experiments repeated in triplicate.

RT2 First Strand kit (Qiagen), and the resulting cDNAs were used according to the manufacturer's protocol of the commercial primer/probe sets (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR was conducted as described above. The results were normalized to 18S RNA and expressed as the fold change from baseline (control group). The experiments were performed three times independently and the results were compared using a standard curve.

Immunoblotting assays. The protein in the lung tissue extracts was quantified using the BCA Protein assay kit (Beyotime Institute of Biotechnology) 50 μ g samples were separated on 10% SDS-PAGE gels (Thermo Fisher Scientific, Inc.), which were then transferred onto polyvinylidene fluoride membranes (Merck Millipore, Darmstadt, Germany).

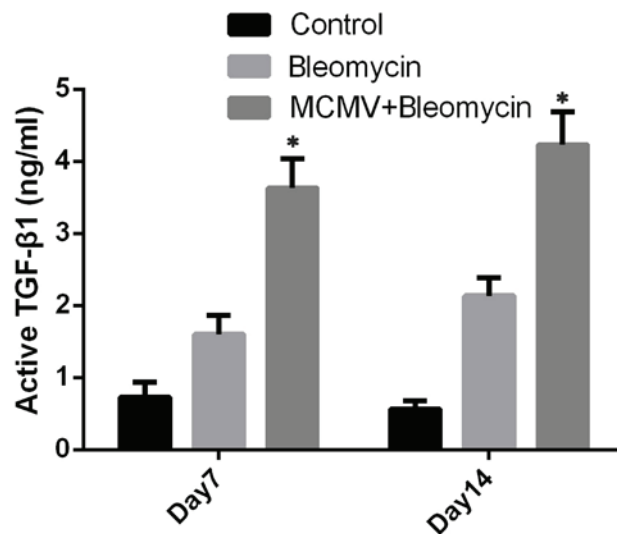


Figure 3. Activity of TGF- β 1 in the treatment groups. Data are presented as the mean \pm standard error of the mean from experiments repeated in triplicate. * P <0.05 vs. the bleomycin group. MCMV, murine cytomegalovirus; TGF- β 1 transforming growth factor- β 1.

Subsequently, the membranes were blocked with 50 ml 3% fat free milk for 1 h and incubated with primary antibodies as follows: Mouse monoclonal anti-E-cadherin (1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA; cat. no. 14472), mouse monoclonal anti-Vimentin (1:1,000; Cell Signaling Technology, Inc.; cat. no. 3390), rabbit monoclonal phospho-SMAD2 (1:1,000; Cell Signaling Technology, Inc.; cat. no. 3108) and β -actin (1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; sc-8432) at 4°C overnight. Following washing with PBS three times, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse (1:2,500; Santa Cruz Biotechnology, Inc.; cat. no. 2005) and goat anti-rabbit (1:2,500; Santa Cruz Biotechnology, Inc.; cat. no. sc-2004) secondary antibodies. at room temperature for 1 h. The membranes were washed again, and the antigen-antibody reaction was visualized and analyzed using an Amersham ECL detection system (GE Healthcare Life Sciences, Amersham, UK).

Statistical analysis. Statistical analysis was performed with GraphPad Prism 6.01 software (GraphPad Software, Inc., La Jolla, CA, USA) using Student's two-tailed t-test. P <0.05 was considered to indicate a statistically significant difference.

Results

RT-qPCR results. The results of the RT-qPCR analyses are shown in Fig. 1. The results revealed that MCMV invaded the lung tissues, however it did not cause pulmonary fibrosis. The viral DNA loads in the salivary glands, spleen, liver and lungs were highest following inoculation for 3 days. Subsequent to this, the numbers gradually reduced and were maintained at a low level, which is similar the latent infection in humans.

Weight changes in the mice. Changes in the body weights of the mice are shown in Fig. 2. The results suggested that the weights of the mice decreased sharply following infection

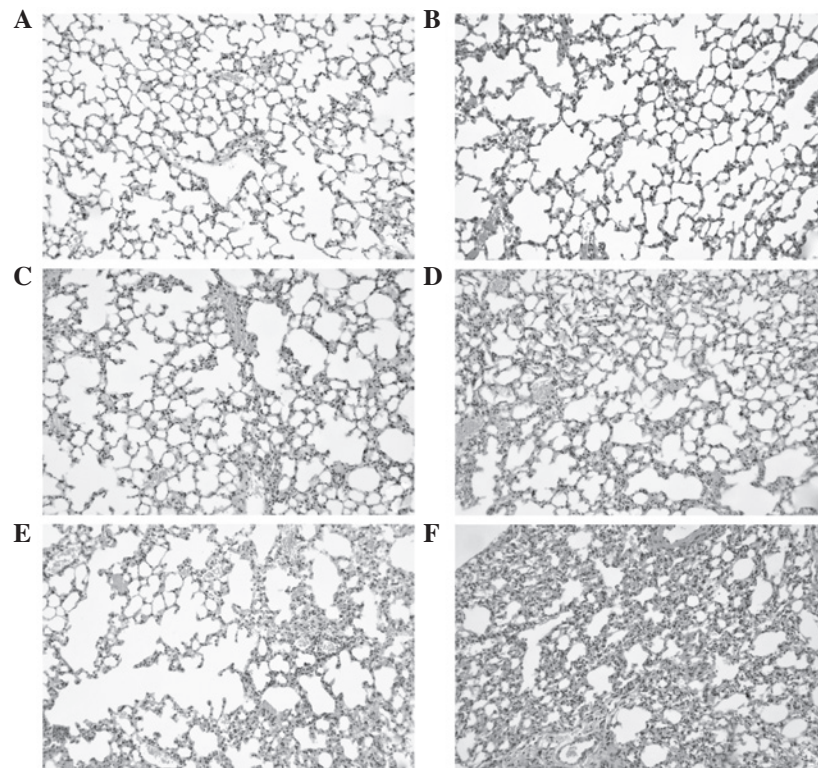


Figure 4. Results of pathological evaluation. Histopathological assessment using fluorescence microscopy was performed to evaluate tissues from the (A) control group, (B) MCMV infection group and (C) bleomycin group following treatment for 7 days; (D) MCMV+bleomycin group following treatment for 7 days. (E) Bleomycin group following treatment for 14 days. (F) MCMV+bleomycin group following treatment for 14 days. Magnification, x40. MCMV, murine cytomegalovirus.

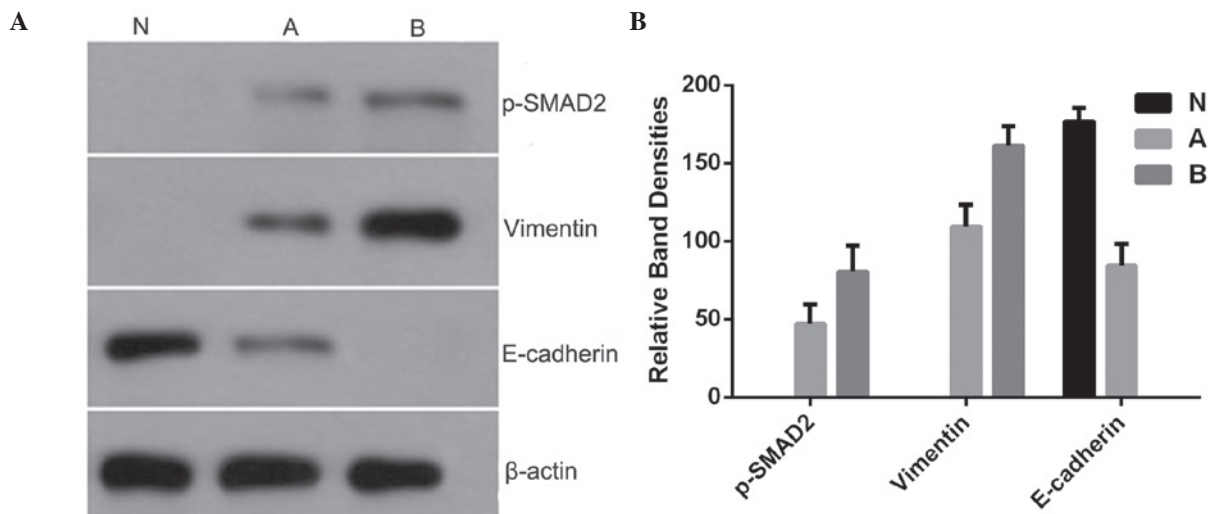


Figure 5. (A) Western blot analysis of the protein levels of Vimentin, p-SMAD2 and E-cadherin. (B) The intensity of the protein products was quantified. Data are presented as the mean \pm standard error of the mean from three experiments. Results are expressed as relative expression following normalization to β -actin. N, control; A, bleomycin group; B, MCMV+bleomycin. MCMV, p-SMAD2, phosphorylated small mothers against decapentaplegic 2; MCMV, murine cytomegalovirus.

for 3 days, following which the weights recovered rapidly in the infected group. No significant differences were observed between the control group and infected group from 15 days post-infection.

Detection of TGF- β 1 activity. The results of the analysis of TGF- β 1 activity are shown in Fig. 3. It was found that the

activity of TGF- β 1 was higher in the MCMV+bleomycin group, compared with the activity of TGF- β 1 in the bleomycin group and control group following treatment for 7 days. The activity reached the highest level following treatment for 14 days. These results suggested that the progression of fibrosis in the mice treated with MCMV+bleomycin was more rapid, compared with that in the control mice.

Detection of histopathology. The results of the pathological detection are shown in Fig. 4. The results showed that the degree of pulmonary fibrosis was more marked in the MCMV+bleomycin group, compared with the bleomycin group following treatment for 7 and 14 days.

Western blot analysis of protein expression levels. The results of the western blot analysis are shown in Fig. 5. The results showed that the protein levels of Vimentin and phospho-SMAD2 increased following treatment with bleomycin and MCMV+bleomycin, whereas the protein levels of E-cadherin decreased following treatment with bleomycin and MCMV+bleomycin.

Discussion

The etiology of IPF remains to be elucidated. However, previous studies based on animal models of pulmonary fibrosis and lung tissues from patients with IPF have suggested a dynamic pathobiological process, involving excessive wound healing with chronic inflammation, apoptosis of epithelial and endothelial cells, mesenchymal cell proliferation and activation with the formation of fibroblasts/myofibroblasts foci, and excessive deposition of extracellular matrix resulting in destruction of the lung architecture and loss of lung functions (10). High throughput genomic profiling studies have characterized certain gene changes, which occur during the development of pulmonary fibrosis (9). During EMT, alveolar epithelial cells demonstrate loss of epithelial characteristics and cellular adhesions, develop changes in the actin cytoskeleton, induce the expression of fibrogenic molecules and acquire a migratory phenotype (11). These fibroblastoid alveolar epithelial cells are key contributors to pulmonary fibrosis, as the inhibition of TGF- β 1-mediated EMT prevents and reverses experimentally induced pulmonary fibrosis in animal models (12,13).

Infection with HCMV is bimodal, occurring through vertical and horizontal transmission in early childhood and through sexual transmission in young adults. The viral loads in the broncho-alveolar lavage cells of patients with IPF and healthy volunteers are elevated, relative to the respective viral load in the blood leukocytes, suggesting that the lungs are important in the pathobiology of HCMV (14,15). The production of TGF- β 1 can be induced by transient transfection with expression plasmids containing the HCMV immediate early 1 and 2 genes into fibroblasts and astrocytoma cells. In previous studies, increases in TGF- β 1 were associated with the induction of TGF- β 1 mRNA. However, the local effects of TGF- β 1 are often controlled *in vivo* by activation of the extracellular latent form (16,17). In the present study, it was found that MCMV invaded the lungs, however it did not cause pulmonary fibrosis. The progression of fibrosis in the mice treated with MCMV+bleomycin was more rapid, compared with that in the control mice, and the degree of pulmonary fibrosis was more severe in the MCMV+bleomycin group, compared with the bleomycin group following treatment. The protein levels of EMT-associated genes, including Vimentin and phospho-SMAD2 were upregulated following treatment

with MCMV+bleomycin. These results suggested that latent MCMV infection aggravated pulmonary fibrosis in the mouse model, possibly through the activation of TGF- β 1.

In conclusion, the present study found that the presence of CMV may alter IPF in response to a well-defined trigger of pulmonary fibrosis, namely the chemical bleomycin. Latent MCMV infection may have aggravated pulmonary fibrosis in the mouse model through the activation of TGF- β 1.

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