Role of Endoplasmic Reticulum Stress in Age-Related Susceptibility to Lung Fibrosis

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Abstract

The incidence of Idiopathic pulmonary fibrosis (IPF) increases with age. The mechanisms that underlie the age-dependent risk for IPF are unknown. Based on studies that suggest an association of IPF and gammaherpesvirus infection, we infected young (2-3 mo) and old (≥18 mo) C57BL/6 mice with the murine gammaherpesvirus 68 (MHV68). Acute MHV68 infection in aging mice resulted in severe pneumonitis and fibrosis compared with young animals. Progressive clinical deterioration and lung fibrosis in the late chronic phase of infection was observed exclusively in old mice with diminution of tidal volume. Infected aging mice showed higher expression of TGF-β during the acute phase of infection. In addition, aging infected mice showed elevation of pro-inflammatory cytokines and the fibrocyte recruitment chemokine CXCL12 in bronchoalveolar lavage. Analyses of lytic virus infection and virus reactivation indicate that old mice were able to control chronic infection and elicit anti-virus immune responses. However, old infected mice showed a significant increase in apoptotic responses determined by in situ TUNEL assay, levels of caspase-3 and expression of the pro-apoptotic molecule BIM. Apoptosis of type II lung epithelial cells in aging lungs was accompanied by upregulation of ER stress marker BiP and splicing of XBP1. These results indicate that the aging lung is more susceptible to injury and fibrosis associated with ER stress, apoptosis of type II lung epithelial cells and activation of pro-fibrotic pathways.

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Keywords

Idiopathic Pulmonary Fibrosis, lung, aging, gammaherpesvirus, type II lung epithelial cell, apoptosis, ER stress

Abbreviations

Murine gammaherpesvirus 68 (MHV68)
Epstein Barr Virus (EBV)
Idiopathic Pulmonary Fibrosis (IPF)
Inositol-Requires Enzyme 1 (IRE 1)
Endoplasmic Reticulum (ER)
Unfolded Protein Response (UPR)
Heavy-chain-binding protein (BiP)
X-box binding protein 1 (XBP1)
Spliced X-box binding protein 1 (XBP1s)
Tidal Volume (TV)
Day post-infection (dpi)
Interferon gamma receptor (IFNγR)
Introduction

Idiopathic Pulmonary Fibrosis (IPF) is characterized by progressive destruction of the normal architecture of the lung. Its cause is unknown and there is not a proven effective therapy other than lung transplantation. The prevalence of IPF increases with age, with most patients aged more than 60 years (1, 2). IPF remains a progressive, irreversible, and lethal disease with a median survival from 3 to 5 years after the diagnosis. The cellular and molecular pathways that drive the pathogenesis of IPF are not fully delineated. However, several clinical studies have found DNA or protein from the human gammaherpesvirus, Epstein Barr Virus (EBV) (3-6) in 40 to 70% of the IPF cases (3-6). We have established a mouse model of progressive pulmonary fibrosis using the murine gammaherpesvirus 68 (MHV-68), a virus closely related to EBV (7). We have shown that gammaherpesvirus infection causes persistent virus infection, injury of lung epithelial cells and pulmonary fibrosis in IFNγR-/- mice with similar features to the human disease. Progressive fibrosis was not found in virus infected wild type mice (8-13).

Aging is an important risk factor for the development of IPF. The process of aging is complex, multifactorial and is associated with alterations in the physiological responses to injury and repair. There are structural and functional age-related changes in the lung even in the absence of disease. In addition, there is a propensity for oxidative stress, pro-inflammatory responses and apoptosis in stress conditions (14, 15). One of the potential mechanisms involves alterations in the homeostasis of the endoplasmic reticulum (ER), predisposing to ER stress. ER stress results from misfolding of proteins
and leads to up-regulation of a signaling pathway called the ER stress response or the unfolded protein response (UPR). The most evolutionary conserved UPR signaling pathways existing in mammals is the activation of the inositol-requiring enzyme 1α and β (IRE1α and β). This activation results in the excision of a 26 bp fragment from the mRNA encoding the transcription factor X-box-binding protein 1 (XBP1) by an unconventional splicing event that generates XBP1s, a potent inducer of a subset of UPR target genes (16). UPR is characterized by the induction of chaperones like BiP (heavy-chain-binding protein), degradation of misfolded proteins and attenuation of protein translation. Notably, prolonged and severe ER stress triggers apoptosis (17). ER stress and UPR activation are common features of the alveolar epithelium in familial and sporadic IPF (18, 19).

Our data support that virus induced lung injury in aging mice increases ER stress responses in type II lung epithelial cells resulting in severe apoptosis and activation of pro-fibrotic pathways.
**Materials and Methods**

A detailed description of methodologies is provided in the online supplement.

*Animals and animal treatment*

Young (2-3 mo) and old (≥18 mo) C57BL/6 mice were inoculated intranasally with $1 \times 10^5$ plaque forming units (pfu) of MHV68 as we described before (10). Infected mice were maintained in BSL2 facility.

*Histopathology, Immunofluorescence and Immunohistochemistry*

After sacrifice, lungs were perfused with 4% paraformaldehyde (10) or Optimal Cutting Temperature Tissue-Tek Compound (OCT). Sections from paraffin blocks were stained with H&E and Masson trichrome to determine histopathological changes and fibrosis. Morphometric analyses were performed as described before (9). Immunohistochemistry analyses were performed using anti-XBP1 antibody (Santa Cruz Biotech, Santa Cruz, CA, USA). Dual immunofluorescence staining was performed using methods outlined previously (18, 20).

*Histopathological 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
thickening of the pleura. Because of 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*

Hydroxyproline content in whole mouse lung was used to quantify lung collagen content
and was measured colorimetrically by a method described previously (21).

*Western blot Analyses*

Extracts from lung tissue samples and cell cultures were prepared as previously
described (9). Western blotting for BiP (Millipore) was performed, and then the blot was
re-probed with an antiserum against β-actin (Santa Cruz Biotech) as a loading control.
Western blots were quantified using NIH ImageJ software (v.1.43).

*Quantitative Real-Time PCR analysis*

Total DNA and RNA were extracted from lung tissue using DNeasy and RNeasy kits,
respectively (Qiagen, Valencia, CA, USA), according to manufacturer’s
recommendations. Real-time RT-PCR was performed using SYBR Green and primers
specific for the genes of interest and normalized using 18S RNA and RPL19 genes.
Quantitative PCR for MHV68 DNA was performed as described before (13) using
ORF50 primers and GAPDH as housekeeping gene.

*Cytokine and chemokine expression*
Mouse IL-6, IFN-γ, IL-10, and MCP-1 levels were measured in bronchoalveolar (BAL) fluid using a multiplex bead immunoassay (Linco, St. Charles, MI) according to manufacturer’s recommendations, while TGFβ was determined with a single analyte ELISA (Qiagen; Valencia, CA, USA). CXCL12 was measured in lung whole cell extracts using an ELISA kit (R&D Systems).

**Statistical Analyses**

Data were plotted and statistically analyzed using InStat 3 and Prism 5 (GraphPad Software, San Diego, CA). Differences among groups were assessed using one-way analysis of variance (ANOVA) and between pairs using Student’s t-test. Results are presented as mean +/- standard error of the mean. Significant differences have p<0.05.
Results

Virus-Induced Lung Fibrosis in Aging C57BL/6 Mice

Our previous studies showed that young (2 months old) C57BL/6 mice infected with gammaherpesvirus control rapid lytic virus replication during the acute phase of infection (day 7-15) and developed mild pneumonitis that completely reverses by day 25 post-infection (10). To determine whether aging results in a differential response to herpesvirus infection, we infected old (≥ 18 months old) C57BL/6 mice. In an examination of weight loss with time, infection of old mice resulted in a steep decline in weight that peaked around 15 dpi (days post-infection), recovered to the starting weights by 90 dpi and then declined again at 120 dpi without recovering over the remaining 130 days time course (Figure 1A). In sharp contrast, young virus-infected mice never experienced weight loss during the course of the acute or chronic phase of infection and remained nearly indistinguishable from their non-infected counterparts (Figure 1A). At day 250 post-infection, lung function was measured using a whole body plethysmograph. Old C57BL/6 animals showed a significant reduction in tidal volume, corresponding to a restrictive pulmonary defect in lung function (Figure 1B).

Lungs were analyzed for collagen deposition by Masson trichrome staining at 15 dpi (immediately following the acute phase of infection) and at 250 dpi (late in the chronic period of infection). Compared to naïve and young mice, old (≥18 months old) C57BL/6 mice infected with gammaherpesvirus developed more severe pneumonitis with collagen deposition indicated by blue staining at 15 dpi that persisted as a patchy interstitial fibrosis during the late chronic phase in 30% of the old infected mice (Figure
1C). Overall, there was only a mild degree of lung pathology in young mice infected with MHV68 at 15 and 250 dpi as compared with the pathology scores of old infected mice (Figure 1D). Lung fibrosis was also assessed by collagen content by measuring levels of OH-proline (Figure 1E). We found significant higher levels of OH-proline in aging mice at 15 and 250 dpi. Taken together, these data provided evidence for increased virus-induced lung fibrosis in aging mice.

**Control of Lytic and Latent MHV68 Infection with Age**

To determine whether there is an age-associated decline in the ability to control lytic and persistent infection, we monitored virus replication during the acute phase of infection at 7 dpi, a period of peak replication in the lung after intranasal infection. Viral titer of the lung was comparable between young and old mice at this time point (Figure 2A). The level of latent virus was measured at 7 and 15 dpi in the lung using a quantitative PCR assay which allows determination of a latency-associated gene product (Figure 2B). We found that latent virus in the lungs at 7 and 15 dpi, was similar in young and old mice. Additionally, we analyzed virus reactivation and the presence of preformed infectious virus in spleens by a sensitive limiting dilution reactivation assay upon explant at 15 dpi. Virus reactivation was nearly ten-fold lower in aging mice in comparison with their young counterparts (Figure 2C). Preformed infectious virus detected in the disrupted splenocytes was also appreciably lower in the aged mice. We failed to detect lytic virus in the lung or spleen late during chronic infection at 250 dpi.
Because T cell responses are important contributors to control MHV68 infection, we determined the frequency of MHV68-specific CD8 T cells in lungs of naïve and virus infected mice during the acute (11 days) and chronic (30 days) phase of infection using MHC class I tetramers specific for two well-characterized MHV68 epitopes, p56 (ORF6487-495) and p79 (ORF61524-531). The data showed a similar percentage of p56 and p79–specific CD8 T cells in young and old mice (Figure 1A supplement). In addition, we determined the percentage of vβ4-CD8 T cells, which have been shown to be driven by the M1 latency gene. We found similar expansion of these CD8 T cells in spleens at 30 dpi (Figure 1B supplement). These data suggest that aging mice can control chronic MHV68 infection and that the fibrotic response to infection in old mice is not due to impaired anti-viral responses.

High Levels of Pro-inflammatory Cytokines and Chemokines in MHV68 Infected Aging Mice in the Acute Phase of Infection

To characterize the inflammatory response in infected aging mice, we performed differential counts from bronchalveolar lavage (BAL) of young and old mice at 15 dpi. Similar numbers of cells were found between naïve young and naïve old animals (Figure 3A). After infection, higher number of macrophages and lymphocytes were found in aging mice compared with young animals; however, the relative percentage of lymphocytes in the young infected mice was higher than their old infected counterparts (54.7% vs 30.2%).
In BAL, we found upregulation of both IL-6 and IFN-γ in aging and young mice at 7 dpi although significantly higher levels of IL-6 were found in old animals (Figure 3B-C). In contrast, levels of the anti-inflammatory cytokine IL-10 were significantly higher in young animals (Figure 3D). We also compared levels of the chemokines MCP-1 (Figure 3E) and CXCL12 (Figure 3F) in the BAL and lung tissue, respectively. These two chemokines have been associated with the recruitment of fibrocytes. We found high levels of both chemokines in infected lungs of aging mice at day 7 and 15 post-infection. However, determination of fibrocyte recruitment to the infected lungs by FACS using CD45+ CXCR4+ Collagen1+ markers showed similar percentage of fibrocytes in lungs of young and old mice (Figure 2 supplement). These results suggested that increased fibrosis observed in MHV68 infected aging mice is independent of fibrocyte recruitment.

**Age-related changes in TGF-β Expression**

Our previous studies in IFN-γR deficient mice have shown that MHV68 infection induces up-regulation and activation of the pro-fibrotic factor TGF-β1. We found a higher concentration of active TGF-β1 in BAL of naïve and acutely infected aging mice (Figure 4A). In concordance, quantitative RT-PCR analyses of total lung lysates showed that naïve and 7 dpi infected aging mice have higher levels of TGF-β transcripts (Figure 4B). Chronic infected young and old mice show similar high levels of TGF-β transcripts (Figure 4B). Our data suggest that aging lungs upregulate TGF-β during the acute phase of virus infection.

**Apoptosis of Lung Epithelial Cells in Infected Aging Mice**
Several lines of evidence imply that apoptosis may play a role in the aging process and the age-related functional declines of multicellular organisms. To determine the apoptotic response in young and old mice infected with MHV68 we performed *in situ* TUNEL assay in lung slides from naïve and infected mice at 15 and 250 dpi. Naïve young and aging mice showed low number of apoptotic cells but the frequency of apoptotic cells increased dramatically after infection, especially in aging mice (Figure 5A). Semi-quantitative analyses of the signal from the *in situ* TUNEL assay showed significant elevation of the apoptotic responses after infection in lung samples from aging mice (Figure 5B). Apoptosis can be triggered by members of the Bcl-2 protein family, such as BIM. By quantitative RT-PCR, we found higher expression of BIM in naïve and infected aging lungs compared to samples from young animals (Figure 5C). Injury of lung epithelial cells is believed to be critical for the initiation of the fibrotic process in the lung. Additionally, a potential role for apoptotic macrophages in pulmonary inflammation and fibrosis has been reported (22). To determine the type of cells undergoing apoptosis, we performed TUNEL assay and staining using anti-Mac3 and anti-Pro-surfactant C antibodies, as markers of macrophages and type II lung epithelial cells, respectively (Figure 5D). Numerous TUNEL-positive type II lung epithelial cells (Pro-surfactant C positive) were present in infected lungs from aging mice whereas only few apoptotic cells were Mac-3 positive. Less abundant apoptotic type II lung epithelial cells were found in lungs of infected young animals (Figure 5E).

*Activation of ER stress responses in Lung Epithelial Cells from Infected Aging Mice*
Susceptibility to lung injury and apoptosis has been associated with ER stress responses in the lung. The transcription factor XBP1s, is a key component of the ER stress response in lung epithelial cells (23). We analyzed the XBP1 expression in lungs from uninfected and infected young and old mice by immunohistochemistry analysis. No signal was observed in naïve young and old mice. At 15 dpi, airway epithelial cells from infected young mice showed XBP1 positive signal whereas old infected mice had extensive staining in lung epithelial and inflammatory cells (Figure 6A). In conditions of ER stress, XBP1 mRNA undergoes IRE1-dependent splicing that permits translation of the biologically active XBP1 protein. The ratio of the spliced isoform to total XBP1 mRNA can be used as a marker of the IRE1 mediated ER stress response (13). We found predominance of unspliced XBP1 in naïve young and old mice (Figure 6B). After infection, splicing of XBP1 was evident at 15 dpi in young mice; however, old animals showed evidence of ER stress earlier at 7 dpi with a further increase by 15 dpi (Figure 6B). ER stress also induces expression of the chaperone BiP. RT-PCR analyses showed that lungs from old infected mice have higher expression of BiP compared to young animals (Figure 6C). Immunoblot analysis confirmed higher protein levels in the aging lung (Figure 6D). To determine if markers of ER stress were expressed in type II lung epithelial cells from aging mice, we performed co-immunostaining for type II lung epithelial cells (anti-Pro-surfactant C protein, green) and XBP1 or BiP (red). Co-localization of type II lung epithelial cell markers and ER stress markers were observed in infected lungs from aging mice at 15 dpi (Figure 6E) and persisted high at 250 dpi (Figure 3 supplement). These data provided evidence that type II lung epithelial cells in
aging mice have lower resistance to ER stress responses after virus infection and that this process is accompanied by increased apoptosis.
Discussion

The incidence and prevalence of IPF increase noticeably with age. A recent study showed that prevalence of IPF increased 50 to 60-fold when comparing adults less than 35 years with those over 75 years (1, 2). However, the pathogenic mechanisms involved in the higher susceptibility of aging individuals to lung fibrosis are unknown. Based on several studies that show detection of viral proteins and/or viral genome of herpesvirus in the lungs of IPF patients, we have established a murine model of MHV68-induced lung fibrosis. Here we examined whether aging in C57BL/6 mice is sufficient to increase the susceptibility to lung fibrosis after herpesvirus infection. We found that compared to infected young C57BL/6 mice, which develop only a mild reversible pneumonitis, infected old C57BL/6 mice have more severe and progressive inflammation and collagen deposition. The severity of the virus-induced lung pathology in aging mice was associated with a dramatic increase of ER stress markers and apoptosis of type II lung epithelial cells with subsequent fibrosis.

Aging has been associated with impaired immune responses that might have an effect in the control of acute virus infection. Less is known of chronic, persistent and latent virus infections such as MHV68 infection. Previous studies have shown that aging mice can control acute and latent MHV68 infection with optimal maintenance of functional virus specific CD8 T cells (24). Similarly, our studies found that de novo infected old mice do not have deficiencies in virus control. We found no significant differences in virus replication and load in the lung and no evidence for increased viral load in the spleen. We report that aging mice exhibited normal IFN-γ production and induction of virus-specific CD8 T cells. Moreover, aging mice show higher percentage of
effector and central CD8 memory phenotype T cells (Figure 4 supplement) in the lungs before and after infection that might contribute to immune responses and maintenance of low levels of viral persistence in the lung.

Alteration of innate immune responses have also been described during aging that include the propensity for increased production of pro-inflammatory mediators (24). We found that aged MHV68 infected mice expressed higher levels of pro-inflammatory cytokines during the acute phase of infection associated with elevated CXCL12, a recruiting chemokine for fibrocytes. Using the bleomycin-induced lung injury model and accelerated senescence mice, we have observed similarly high levels of CXCL12 after injury in senescence mice (14, 25, 26). Those studies showed a senescence related change in the bone marrow cell population characterized by increased proportion of fibrocytes in the bone marrow and a diminution of mesenchymal stem cells. In addition to this pro-fibrotic mechanism, the susceptibility to fibrosis in aging mice can be related to high levels of TGF-β. Our data indicate that there are age-related changes in the levels of TGF-β transcripts and protein in naïve and acutely infected mice. However, differences between young and old infected mice are not longer obvious when young mice reach 250 dpi, a time point when young mice have reached almost a year old of age.

Previous studies have demonstrated the importance of the cross-talk between fibroblasts and epithelial cells in the control of the pro-fibrotic responses in the lung (27). Our data suggest that aging lungs are more susceptible to ER stress and injury of lung
epithelial cells. Abnormal function of the ER might lead to an evolutionarily conserved cell stress response, the UPR, which is aimed to compensate the damage but can eventually trigger cell death if the ER dysfunction is persistent or severe. Three distinct UPR signaling pathways exist in mammalian cells that include IRE1α and β, pancreatic ER kinase (PERK), and activating transcription factor 6 (ATF6) (28). Activation of IRE1 results in an unconventional splicing event that generates XBP1s (29). We have found that aging mice exposed to MHV68 infection have persistent and progressive increasing levels of XBPs in lung epithelial cells and inflammatory infiltrates, as well as high levels of BiP. Markers of ER stress have been found in alveolar type II cells from lungs of IPF patients. Moreover, epithelial cells from both IPF and COPD lungs undergo apoptosis as evidenced by caspase-3 activation and Bax dimerization, but only IPF specimens show activation of ER stress responses (19). ER stress in IPF lungs has been also associated with altered surfactant protein processing and herpesvirus infection. The association of herpesvirus infection with ER stress and IPF was evaluated in patients with familial IPF including family members with the surfactant protein C mutation L188Q, individuals with familial interstitial pneumonia without surfactant C protein mutations, and individuals with sporadic IPF. In these studies herpesvirus protein expression was found in alveolar epithelial cells from 15/23 IPF patients and co-localized with UPR markers in alveolar epithelial cells from these patients (18). More recently, a mouse model with transgenic expression of the surfactant C protein mutation L188Q in alveolar epithelial cells demonstrated induction of ER stress but lung fibrosis was only identified in the presence of a profibrotic stimulus (23). These data suggest that susceptible individuals to ER
stress, such patients with alterations in the processing of surfactant C protein, have predisposition to fibrosis after a second hit like herpesvirus infection.

Our studies support the concept that the susceptibility to ER stress increases during aging. ER stress initially stimulates an adaptive UPR to promote cellular survival, whereas in the case of persistent, chronic stress, UPR can trigger apoptotic cell death program (30, 31). The aging process contains abundant characteristics that might affect the ER stress response, e.g. increased oxidative stress, disturbance in calcium homeostasis, misfolding and aggregation of proteins, and impairment in global protein synthesis (32-34). All these age-related changes imply that aged cells might be more vulnerable to ER stressors, leading to apoptosis. Thus, similarly to patients with alterations in the processing of surfactant C protein, aging individuals exposed to injury may have vulnerable epithelial cells that are prone to ER stress, resulting in increased pro-apoptotic and pro-fibrotic signals. Moreover, ER stress might induce inflammatory responses by production of reactive oxygen species (ROS), release of calcium from the ER, activation of the transcription factor NF-κB and mitogen-activated protein kinase (MAPK) pathway. Our studies show that type II lung epithelial cells are particularly sensitive to ER stress in the aging lung. Recently, ER stress has been associated with epithelial mesenchymal transition (EMT) of type II lung epithelial cells (35, 36). Further studies will be required to determine if increased ER stress in the aging lungs has a role in EMT and fibroblast accumulation by this pathway.
In summary, our studies show the increased susceptibility during aging to lung injury by herpesvirus infection, resulting in pulmonary fibrosis. We show the propensity to disrepair in old animals is unrelated to control of chronic virus infection. Our studies suggest that aging individuals develop persistent and more severe ER stress responses than younger counterparts causing exacerbated apoptosis and fibrosis. These studies confirm that there are age-related changes in the physiological responses to injury, and that the understanding of these mechanisms might result in better therapeutic approaches for IPF.
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References


Figure 1. Severe clinical disease and fibrosis in MHV68 infected aging mice.

(A) Weight loss data are presented as total body weight. More severe illness was observed in aging infected mice. Number of mice: 5-13 per group and time point. Data are representative of 3 different experiments. (B) Lung function determined by the measurement of Tidal Volume using a whole body plethysmograph. Measurements were performed at day 120 post-infection. Number of animals: 3-5 per group. (*, p<0.05) (C) Masson trichrome staining of lung sections from 3 mo (young), and >18 mo (old) C57BL/6 mice infected with MHV68 at indicated times post-infection. Collagen deposition is shown in blue. Notice loss of normal lung architecture of the lung in old infected mice associated with higher interstitial collagen deposition. Young mice only show collagen deposition around blood vessels at day 250 post-infection. (D) Semiquantitative morphometric analysis of lung histopathology in virus infected young and old mice at the indicated time post-infection. Old infected mice showed higher pathology scores corresponding to pneumonitis and thickening of the interalveolar septa compared to young animals (n=5-8). (E) Normalized lung collagen content based on hydroxyproline microplate assay at 7, 15 and 250 dpi (*, p < 0.05, n=3).

Figure 2. Control of chronic virus infection in young and aging mice.

(A) Acute replication in the lungs of young and old C57BL/6 mice at day 7 post-infection. Lungs were harvested, disrupted, and titered on NIH 3T12 cells by plaque assay. Data are shown as log10 titer, and the bar indicates the geometric mean titer. (B) Quantitative PCR analysis of virus load of individual lungs from MHV68 infected
young and old C57BL/6 mice at 7 and 15 dpi. Viral load was determined as the levels of viral DNA (targeting the ORF50 region) normalized by cellular DNA (targeting GAPDH). Bars represent geometric mean. (C) Reactivation of MHV68 in spleens from infected young and old mice was assessed using standard limiting dilution analyses at 15 dpi ($n = 3–4$ mice). Serial dilutions of bulk intact or disrupted splenocytes were plated on monolayers of mouse embryonic fibroblast (MEFs). The presence of reactivating virus was determined by the presence of cytopathic effect (CPE). CPE observed in the disrupted splenocytes indicate preformed infectious virus. Symbols represent the mean percentage of wells positive for CPE ± SEM. Curve fit lines were derived from nonlinear regression analysis.

Figure 3. Higher levels of pro-inflammatory cytokines and chemokines in MHV68 infected aging mice.

(A) Differential cell counts in the bronchoalveolar lavage (BAL) of naïve and infected young and old mice at 15 dpi. (**, $p<0.01$, $n=8$). (B-F) IL-6, IFN-$\gamma$, IL-10 and MCP-1 levels were measured in bronchoalveolar lavage (BAL) fluid from naïve and MHV68 infected young and old mice at the indicated time points post-infection, while CXCL12 was measured in lung tissue. Bars represent mean ($n= 5$ per group) ± SEM. (*, $p<0.05$).

Figure 4. High expression of TGF-β in naïve and virus-infected aging mice

(A) Active TGF-β levels were measured in BAL fluid from naïve and MHV68 infected young and old mice at 7 and 15 dpi ($n=3$). (B) Quantitative RT-PCR analysis for TGF-β
transcripts in the lungs of naïve and infected young and old mice at different time point post-infection (*, p < 0.05, n=4).

**Figure 5. Apoptosis in type II epithelial cells from infected old C57BL/6 mice.**

(A) Representative images from *in situ* TUNEL assay in lung sections from young and old mice at 15 and 250 dpi. Magnification: 40x. (B) Semi-quantitative analyses in high-power field show a greater percentage of TUNEL positive cells in old infected mice (n=3). (C) Quantitative RT-PCR analysis for BIM transcripts in the lungs of naïve and infected young and old mice at different time point post-infection (n=4). (D) Macrophage and type II lung epithelial cell death was evaluated in paraffin-embedded tissue sections from 15 dpi using TUNEL assay (brown). Macrophages and type II lung epithelial cells were identified using Mac-3 and Pro-surfactant-C antibodies (red). Higher number of apoptotic type II lung epithelial cells (arrows) was found in old infected mice. (E) Semi-quantitative analyses in high-power field show a greater percentage of double stained cells (TUNEL positive nucleus + cell type marker) in old infected mice (n=4).

**Figure 6. ER stress in lung epithelial cells from infected old C57BL/6 mice.**

(A) Immunohistochemistry analyses of XBP1 expression in lung sections of young and old mice at day 15 of mock or MHV68 infection. Notice abundant positive staining in old MHV68 infected mice. (B) Determination of mRNA levels of unspliced and spliced XBP1 in lung samples of naïve and MHV68 infected mice at day 7 and 15 post-infection. Ratio of spliced versus total XBP1 mRNA of samples is shown on the right. (C) Quantitative RT-PCR analysis for BiP transcripts in the lungs of naïve and infected
young and old mice at different time point post-infection (n=3-4; *, p<0.001). (D) Immunoblot assay of lung lysates from naïve (N) and infected young and old C57BL/6 mice at the indicated time point post-infection using anti BiP antibody. Blot was stripped and reprobed with an anti-β-actin antibody as a loading control. (E) Dual immunofluorescent staining in lung sections of aging mice infected at 15 dpi for Pro-surfactant-C (green) and the markers of ER stress BiP (red) and XBP1 (red). Yellow cells in the merge column indicate type II cells supporting ER stress. Nuclei were visualized by DAPI staining (blue).
Figure 1

A

B

C

D

E

190x254mm (96 x 96 DPI)
Figure 3
Figure 4

![Figure 4](image-url)
Figure 5

A

15 dpi

250 dpi

B

C

D

E

190x254mm (96 x 96 DPI)
Role of Endoplasmic Reticulum Stress in Age-Related Susceptibility to Lung Fibrosis


Online Data Supplement
Lung Mechanics

Using a whole body plethysmograph (Buxco, Sharon, Connecticut, USA) lung function was measured in naïve and MHV68 infected mice at 250 dpi (days post-infection). The unrestrained animal was placed in a chamber and, after 10 minutes of adaptation, measurements were taken every 5 minutes for an interval of 60 minutes total. Measurements obtained were tidal volume (TV), respiratory rate and minute ventilation.

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 0.5 ml of PBS, after which 0.5 ml of 10 N HCl was added. The samples were hydrolyzed at 110°C for 24 hours and then neutralized with 0.4ml of 10N NaOH. One hundred microliters of each sample was mixed with 400μl oxidizing solution (300mg of chloramine-T in 50ml of citrate-acetate buffer: 5% citric acid, 1.2% glacial acetic acid, 7.25% sodium acetate, and 3.4% sodium hydroxide) and the mixture was incubated for 30 minutes at room temperature. Ehrlich’s solution (400μl) was added and the samples were incubated at 65°C for 30 minutes. Absorbance was measured at 570 nm. A standard curve was generated for each experiment using reagent hydroxyproline as a standard. Results were expressed as micrograms of hydroxyproline per milligrams of lung.

TUNEL Assay
In situ TUNEL assay were performed in paraffin lung tissue sections using dUTP HRP labeled kit (Chemicon) accordingly to manufacturer recommendations. Macrophages and type II lung epithelial cells were identified with Mac-3 (BD Biosciences) and Pro-surfactant C antibodies (Chemicon), respectively. Semi-quantitative analysis of TUNEL positive cells was performed in three independent lung sections from old and young mice, five fields were viewed per section.

Immunofluorescence Assay

Briefly, after blocking with 1% BSA, sections were incubated with a rabbit polyclonal antibody against pro-surfactant protein C (pro-SP-C) (Chemicon, Temecula, CA) and either a rat polyclonal antibody against BiP, also known as glucose regulated protein-78 (GRP78) (Santa Cruz Biotechnology, Santa Cruz, CA) or a goat polyclonal anti-XBP-1 (Santa Cruz Biotechnology). After the sections were washed with phosphate buffered saline, they were stained with fluorescent secondary antibodies (FITC conjugated donkey anti-rabbit and Cy3 conjugated donkey anti-goat or anti-rat) (Jackson Immunoresearch, West Grove, PA). Nuclear staining was done with DAPI and then mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Confocal fluorescent microscopy was performed using an Olympus IX81 Inverted Research Microscope configured with an Olympus IX2 Biological Disk Scanning Unit (DSU) (Olympus, Tokyo, Japan) with Z-stack images obtained.

RT-PCR Primers
Primers have been designed using Beacon Designer Software.

BIM (+1)  5'-GCCTGCTTTGCTCTCTCCAT-3'
BIM (-1)  5'-CCTACACGTGTGCCGTTTGT-3'
BiP (+1)  5'-CCTGCGTCGGTGTGTTCAAG-3'
BiP (-1)  5'-AAGGGTCATTCCAAGTGC-3'

**Plaque Assay**

Lungs were subjected to four rounds of mechanical disruption of 1 min each using 1.0-mm zirconia/silica beads (Biospec Products) in a Mini-Beadbeater-8 (Biospec Products as we have described before (13). Serial 10-fold dilutions of organ homogenate were plated onto NIH 3T12 monolayers in a 200μl volume. Infections were performed for 1 h at 37 °C with rocking every 15 min immediately after infection; plates were overlaid with 2% methylcellulose in cMEM. After 6 to 7 days, plates were stained with a neutral red overlay, and plaques were scored the next day.

**Limited dilution ex vivo reactivation analyses.**

As we have described before (11), bulk splenocytes and lung suspensions were plated in serial 2-fold dilutions (starting with 10⁵ cells) onto Mouse Embryonic Fibroblasts (MEF) monolayers in 96-well tissue culture plates. Twelve dilutions were plated per sample, and 24 wells were plated per dilution. Wells were scored for cytopathic effect at 21 to 28 days post-plating. To detect preformed infectious virus, parallel samples of mechanically disrupted cells were plated onto MEF monolayers. This process kills more
than 99% of live cells, which allows preformed infectious virus to be discerned from virus reactivating from latently infected cells (37).

**Quantitative PCR for MHV68**

DNA was isolated from approximately 50 mg of lung tissue using a Qiagen DNeasy Tissue Kit (Qiagen, Valencia, CA). DNA A260/280 ratios were between 1.8 and 2.0. Quantitative PCR for MHV68 ORF50 and the cellular GAPDH normalization control was carried out using an Applied Biosystems 7300 Real Time PCR machine (Carlsbad, CA). Each reaction mixture contained 5µl RT² Real Time Sybr Green/Rox PCR master mix (SA Biosciences, Frederick, MD), 10 pmol each primer, 200 ng DNA, and water to a final volume of 10µl, and each reaction was performed in triplicate in 386-well plates. Primers for the reactions were as follows: **orf50**, 5'-GGCCGCAGACATTTAATGAC-3' and 5'-GCCTCAACTTCTCTGGATATGCC-3'; **GAPDH**, 5'-CCTGCACCACCAACTGCTTAG-3' and 5'-GTGGATGCAGGGATGATGTTC-3'. Cycling conditions were as follows: 95°C for 10 min and then 40 cycles at 95°C for 15 s, 60°C for 1 min. The number of copies of viral ORF50 and cellular GAPDH in each sample was determined by comparison to a series of standard curve reactions using a plasmid control containing the ORF50 and GAPDH sequences. The standard curve dilutions used represented a range from $10^8$ to $10^1$, in serial 10-fold dilutions. Melt curve analysis was performed to verify specificity.

**T cell isolation and FACS**

Peripheral blood cells were subjected to red blood lysis and lung suspensions were obtained by treatment with collagenase and DNAse as we have reported.
previously (10). To detect activated T cell subsets, lung cell suspensions were stained using anti-CD8, anti-CD4, anti-CD44, anti-CD62L and anti-CD69 antibodies. For fibrocyte detection, lungs cell suspensions, and bone marrow cells were stained with a mixture of anti-CD45 and anti-CXCR4 antibodies. Then cells were fixed and permeabilized with a cytoperm and cytofix buffers (BD Biosciences). Cells were incubated with rabbit anti-Collagen I followed by anti-rabbit conjugated with FITC. For analyses, CD45 negative cells were excluded and the percentage of Collagen I positive cells was determined in the CD45\(^+\) CXCR4\(^+\) gate as we have described before (26, 38). Tetramers to ORF79 and ORF59 (H-2Db) were synthesized at the NIH Tetramer Core Facility at Emory University and conjugated to streptavidin-allophycocyanin (Molecular Probes) according to the Core protocol.
Figure E1. MHV68-specific T cell responses in young and aging mice.

(A) Flow cytometry was used to analyze CD8\(^+\) T cell profile of young and old C57BL/6 mice uninfected or infected with MHV68 at 0, 11 and 30 dpi. Representative dot blots are shown gated on CD8\(^+\) cells. Percentage of activated MHV68-specific (CD44\(^{hi}\), MHV68-p56-peptide and MHV68-p79-peptide specific (tetramer\(^+\))) cells were similar between the young and aging mice. (B) The mean percentage of CD44\(^{hi}\), V\(\beta\)4\(^+\) populations in gated CD8\(^+\) T cells ± SD (n=3-4 mice per group) at 30 dpi.
Figure E2 Fibrocytes in young and old MHV68 infected mice.

Percentage of fibrocytes was evaluated by FACS in lung cell suspensions from young and aging MHV68 infected mice at 15 dpi. Results are shown as the mean percentage of CD45⁺ CXCR4⁺ Collagen I⁺ cells (n=3).
Figure E3. Dual immunofluorescent staining in lung sections of aging mice infected at 250 dpi for Pro-surfactant-C (green) and the markers of ER stress BiP (red) and XBP1 (red). Yellow cells in the merge column indicate type II cells supporting ER stress. Nuclei were visualized by DAPI staining (blue).
Figure E4 Memory Phenotype T cells in MHV68 infected mice.

Flow cytometry analyses of CD8 T cells in spleens of young and old mice uninfected or infected with MHV68 at 30dpi. Representative dot blots are shown in gated CD8⁺ cells. (A) Percentage of memory CD8⁺ T Cells of the effector (CD44^{high} CD62L^{low}) and central (CD44^{high} CD62L^{high}) phenotype were higher in naïve and virus infected old mice, respectively. (B) Similarly, percentage of activated memory (CD44^{hi} CD69⁺) CD8 T cells was higher in naïve and virus infected old mice.