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Viral infection and aging as cofactors for the development of pulmonary fibrosis

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Abstract

Idiopathic pulmonary fibrosis (IPF) is a disease of unknown origin and progression that primarily affects older adults. Accumulating clinical and experimental evidence suggests that viral infections may play a role, either as agents that predispose the lung to fibrosis or exacerbate existing fibrosis. In particular, herpesviruses have been linked with IPF. This article summarizes the evidence for and against viral cofactors in IPF pathogenesis. In addition, we review mechanistic studies in animal models that highlight the fibrotic potential of viral infection, and explore the different mechanisms that might be responsible. We also review early evidence to suggest that the aged lung may be particularly susceptible to viral-induced fibrosis and make recommendations for future research directions.

Keywords

collagen; Epstein-Barr virus; gammaherpesvirus; lung; murine gammaherpesvirus-68; senescence

Idiopathic pulmonary fibrosis: clinical presentation & potential causes

Idiopathic pulmonary fibrosis (IPF) and its histologic presentation, usual interstitial pneumonia (UIP), is a chronic parenchymal disorder of the lungs that is characterized by progressive loss of pulmonary function, probably due to fibroblast hyperplasia and excess collagen deposition destroying normal lung tissue. It is thought to occur after some inciting event causes injury to the alveolar surface and leads to dysregulated repair. This dysfunctional repair is characterized by loss of type 1 alveolar cells and expansion of type 2 cells [1], induction of proinflammatory cytokines with a Th2 predominance [2], the synthesis of profibrotic factors such as TGF-β1 [2,3], alterations in eicosanoid regulation skewing the balance towards leukotriene synthesis and away from prostaglandin synthesis [4,5], decreased plasminogen activation [6], the recruitment of bone marrow-derived fibrocytes [7–9], the occurrence of epithelial–mesenchymal transition [10–12], fibroblast proliferation, the transformation of these fibroblasts to α-smooth muscle actin producing

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myofibroblasts [13], and finally, deposition of abundant extracellular matrix. Unfortunately, IPF continues to carry a high morbidity and mortality, with median survival times of 3 years from the time of diagnosis. This is largely due to a lack of effective therapies to halt disease progression.

Particular challenges arise when studying IPF owing to the various disease phenotypes exhibited by patients. While the majority of patients present after their fifth decade, they can present in three different ways: chronically with stability over many years; with a steady progression of symptoms and worsening of lung function; or with a rapid decline in respiratory status over a few weeks with extremely high mortality rates termed an acute exacerbation [14,15]. Unfortunately, we have yet to determine what initiates the fibrotic process, why incidence of fibrosis increases with age [16], or why acute exacerbations occur. Several associations have been found between pulmonary fibrosis and inhaled toxins, chronic aspiration, certain drugs and genetic mutations in telomerase or surfactant protein C (SPC) [17–22]; however, these risk factors do not apply to most patients.

An intriguing possibility is the hypothesis that occult viral infections act as cofactors in the pathogenesis of pulmonary fibrosis [23]. Most viruses, such as Epstein–Barr virus (EBV), which have been identified as potential culprits are fairly ubiquitous and infect the majority of the population at some point in their lives [24]. Gammaherpesviruses like EBV can alternate between an aggressively replicating lytic phase and a latent phase existing in the epithelial cells of the lung, as well as in B cells and macrophages, and perhaps providing a chronic low level of lung inflammation [24–26]. It is plausible that this chronic inflammation in a host with a genetic susceptibility to dysfunctional repair disrupts the normal healing response, leading to progressive fibrosis. It is also possible that these agents either make the host more susceptible to further injury caused by a separate acute trigger, or something in the host environment, such as stress, coinfections or immunodeficiency, leads to reactivation of the virus to its more aggressive lytic phase, causing an acute deterioration.

This article will summarize the emerging evidence from clinical literature for and against the notion that viruses may contribute to some forms of IPF. In addition, data from animal models that support this theory will be explored. Finally, it will also explore the potential differences that exist in aged and young individuals, which may offer insights into why fibrosis is predominantly a disease of the aged.

Clinical evidence linking viral infections with IPF

Hepatitis C virus

Several studies have suggested a link between infection with the small, enveloped, positive-sense RNA virus, hepatitis C virus (HCV) [27], and IPF. In the first study, 28.8% of Japanese IPF patients had serum antibodies reactive against HCV, whereas the control patient group showed a much lower prevalence (3.7%) [28]. A subsequent Italian study demonstrated that 13% of IPF patients were seropositive for HCV [29], whereas the seropositivity in a control group of over 4600 blood donors was very low (0.3%). However, this group went on to show that HCV seroconversion was more prevalent in patients with lung disease in general. A control group of 130 patients with noninterstitial lung disease showed a 6.1% prevalence of HCV antibodies, suggesting that there was no statistical difference between the lung disease group and the IPF group [29]. More recently, a retrospective analysis demonstrated that of 6150 HCV-positive patients, 15 eventually developed IPF over the mean observational time frame of 8 years [30]. The cumulative incidence of IPF in the HCV-positive patients went up with age. The incidence was 0.3% after 10 years of infection and increased to 0.9% after 20 years of infection. Their study determined that age, liver cirrhosis and smoking all increased the incidence of IPF in HCV-

positive patients. By contrast, none of the 2050 patients surveyed with hepatitis B virus infection developed IPF. While HCV is not generally believed to replicate in the lung, at least one study has documented HCV RNA in the pulmonary parenchyma [31]. Furthermore, Idilman *et al.* have reported on increases in neutrophil counts in the lung lavage of patients with HCV [32]. Thus, it is possible that HCV infection may result in changes to the inflammatory cell composition within the lung alveoli, and that this may enhance the development of fibrosis within the lung. Taken together, it is interesting to speculate that the inflammatory changes which allow HCV to induce a fibrotic disease in the liver (cirrhosis) over time might also contribute to a fibrotic pathology in the lung. However, it is also possible that these studies reflect regional differences in HCV infection rates (with Japanese patients being particularly prone to HCV) [29], because a British study failed to find an association between IPF and HCV [33].

TT virus

TT virus (TTV) is a nonenveloped, single-stranded, 3.8-kb circular DNA virus [34,35]. Infection of humans is common with TTV, and many people believe the infection to be harmless [36]. However, there is intriguing evidence to suggest that the presence of TTV infection in patients with IPF may worsen their disease course. To determine the prevalence of TTV in IPF, the sera of 33 patients with IPF were tested for the presence of TTV DNA and viral DNA was detected in 12 out of 33 (36.4%) of IPF patients [37]. More importantly, the survival rate was lower for IPF patients with TTV than it was for the cohort of IPF patients without TTV at both 3 and 4 years postdiagnosis [37]. Whether or not TTV increases the incidence of acute exacerbations and/or modifies fibrotic pathogenesis requires further study in large cohorts of patients.

Adenovirus

Adenoviruses are medium-sized, nonenveloped, icosahedral, dsDNA viruses that are relatively stable and can remain infectious outside of the body for extended periods of time [38]. These viruses are known primarily for causing respiratory infections in humans and the viral gene product, E1A, has been shown to upregulate production of the profibrotic mediator, TGF-β, and to induce lung epithelial cells to express mesenchymal markers [39]. As such, adenoviruses have been studied for an association with IPF, but with no positive correlations. The adenovirus E1A gene product was identified in three out of 19 (16%) cases of IPF, in five out of ten (50%) cases of interstitial pneumonia associated with collagen vascular disease, and in two out of 20 (10%) cases of sarcoidosis [40]. Furthermore, the titer of antiadenoviral IgG in IPF patients did not show any elevations over that seen in control patients [41]. In addition, animal models that have explored the potential for adenovirus infections to exacerbate established pulmonary fibrosis were not able to demonstrate significant exacerbation within the first 7 days post-mouse adenoviral infection [42]. One note of caution in interpreting the animal data, however, is that the tropisms of human and murine adenoviruses are quite different, with human adenoviruses being more predominant respiratory pathogens than the mouse viruses.

Human cytomegalovirus

A member of the herpesvirus family, human cytomegalovirus (HCMV) is a widespread pathogen that causes a subclinical or asymptomatic infection in most individuals early in life [43]. Although it can cause severe clinical disease in immunocompromised patients, it usually persists in a latent state in monocyte precursors and tissue stromal cells of healthy hosts. Though often latent, it has been implicated in a number of chronic inflammatory disorders, vascular diseases and malignancies [44]. In regards to pulmonary fibrosis, Magro *et al.* found evidence of HCMV in the sera of nine out of 19 patients with IPF (nine others had evidence of parvovirus infection) and *in situ* hybridization revealed evidence of HCMV

RNA in pulmonary cells without the cytopathological changes of active HCMV infection [45]. This supports the theory that HCMV establishes latency in the lungs of IPF patients. Dworniczak *et al.* studied the bronchoalveolar lavage (BAL) fluid and blood of 16 patients with newly diagnosed IPF and 16 healthy volunteers [46]. Using real-time PCR, there was no difference in the prevalence of HCMV DNA-positive patients in the IPF group (75%) and the control group (69%). However, there was a significantly higher number of viral DNA copies in the blood of the IPF group. In addition, both patient populations had significantly higher HCMV DNA copies in the BAL fluid compared with their blood, further supporting the idea that the lungs are a significant reservoir for viral latency. Yonemaru *et al.* also found higher serum levels of HCMV IgG and complement fixation titers in 43 IPF patients compared with 35 healthy controls, 22 patients with sarcoidosis and 17 patients with chronic obstructive pulmonary disease [41]. Finally, Tang *et al.* found the presence of HCMV DNA in IPF lung tissue more often than in control subjects; however, in 33 patients with IPF, only 21% had evidence of HCMV [47]. Thus, while there is some evidence suggesting a link between HCMV infection and IPF, proof of direct association is largely lacking.

Epstein–Barr virus

Possibly the strongest association between a virus and pulmonary fibrosis exists with EBV, a gammaherpesvirus that is known to infect more than 95% of humans within their first decade of life [24]. The connection was first discovered in 1984, when a serological study of 13 patients with IPF found elevated levels of EBV-specific IgA and IgG antibodies [48]. This was in contrast to 12 patients with fibrosis of known cause with normal EBV serologies. Further studies have found evidence of EBV viral capsid-specific IgA in the BAL fluid of 60% of IPF patients versus 22% of controls and EBV DNA was present in the lung tissue of 48% of IPF patients compared with 14% of controls [49,50]. In addition, EBV, a virus known to establish latency in B cells and the spleen, was found to actively replicate within alveolar type 2 epithelial cells of the lower respiratory tract in IPF patients [25]. Kelly *et al.* noted that 11 out of 18 (61%) EBV-positive DNA lung tissue biopsy specimens from IPF patients showed evidence of a rearranged form of EBV, known as WZhet, indicative of productive replication [51]. In another study, when the alveolar epithelial cells of patients with IPF were positive for EBV latent membrane protein-1, there was a much poorer prognosis [52]. Malizia *et al.* offered insights into a potential mechanism by providing evidence that infection of type 2 human alveolar epithelial cells with EBV *in vitro* led to increased expression of both active and total TGF-β, a key profibrotic mediator [53].

Tang *et al.* hypothesized that presence of any human herpesvirus in the lung, not just EBV, might show a strong association with IPF. When evaluating four human herpesviruses (HHVs), HCMV, EBV, HHV-7 and HHV-8, they found evidence of at least one herpesvirus in 32 of 33 patients with IPF compared to nine of 25 controls and evidence of at least two herpesviruses in 19 of 33 IPF patients and only two of 25 control patients [47]. While these data are strongly suggestive of a role for herpesviruses as a cofactor in the development of IPF, they also indicate that there must be some genetic or acquired predisposition in certain patients leading to the development of IPF. This was supported by the fact that familial IPF patients, patients with IPF in another immediate family member, were less likely to have evidence of coinfection with two or more herpesviruses than sporadic IPF patients [47]. The authors speculated that this was because patients with familial IPF were already predisposed to IPF and needed less of a viral stimulus to trigger a fibrotic response.

Lawson *et al.* suggested that this predisposition in familial IPF patients may be secondary to abnormal processing of mutated proteins, such as SPC, leading to endoplasmic reticulum (ER) stress, activation of the unfolded protein response (UPR) and apoptosis of alveolar

epithelial cells [54]. After demonstrating increased markers of ER stress and apoptosis in cultured cells expressing a known mutated SPC (L188Q), they evaluated lung biopsies from 23 IPF patients, 13 with familial forms of the disease and three of which had the SPC mutation (L188Q). Using immunostaining, they found elevated markers of ER stress and the UPR in alveolar epithelial cells lining areas of fibrosis in all patients including those without the known SPC mutation. They also found evidence of three herpesvirus antigens (EBV, CMV or HHV-8) in alveolar epithelial cells of fibrotic regions in the same distribution seen for the ER stress markers in 15 out of 23 patients evaluated versus none of ten controls. It is important to note that the viral antigens detected were associated with chronic herpesvirus infection. Performing dual fluorescence microscopy studies on the samples that were herpesvirus positive, they also found that UPR markers and herpesvirus proteins localized to the same cell population. The authors suggested that since herpesvirus had been shown to induce ER stress *in vitro* [55], that ER stress and UPR activation was a common final pathway for both abnormal protein processing and chronic herpesvirus infections to induce apoptosis in alveolar epithelial cells. This is interesting in light of previous studies implicating alveolar epithelial-cell apoptosis in the pathogenesis of lung fibrosis (reviewed in [56]).

Although the aforementioned studies largely suggest a relationship between EBV and IPF, it is difficult to explain why many of the IPF patients in these studies did not have evidence of EBV infection in the lung. In addition, there have been two studies to date which have failed to find evidence of EBV DNA in the lungs of IPF patients [57,58]. Whether these discrepancies are secondary to technical differences, variation in patient population or a heterogeneous disease process is difficult to say. In addition, it should be noted that gene-expression profiling of patients with IPF experiencing acute exacerbations did not find evidence of infectious gene signatures (such as type 1 interferon production) [59], which could be taken as another indicator that acute viral infections are not prominent during IPF disease exacerbation. Further skepticism about the role that viral infections may play in IPF comes from the observation that many of the clinical features of IPF (e.g., lower lobe predominance and peripheral involvement) do not fit the spatial lytic replication patterns of many viruses, although the spatial and temporal heterogeneity of latent viral persistence in less well understood. An unanswered question is whether or not an occult infection (perhaps very low level acute or latent infection that escapes detection) could alter the lung environment in such a way as to promote fibrogenesis.

Unfortunately, appropriate human studies have several limitations:

- All of the identified studies were retrospective analyses;
- Identifying test subjects at risk for pulmonary fibrosis for prospective studies is problematic as the natural history of pulmonary fibrosis is still poorly defined;
- We do not yet know what virus to look for and we may not have the sensitivity to detect virus in at risk patients prior to the development of fibrosis;
- It is not clear that viral replication contributes to disease development;
- If replication is required, effective antiviral therapies are not available for many potential viruses associated with IPF.

These issues have led many investigators to explore the use of animal models to fill in the mechanistic gaps left by human studies and to experimentally demonstrate the profibrotic effects of viruses on the lung.

Animal models exploring gammaherpesvirus-induced lung fibrosis

Naturally occurring cases of gammaherpesvirus-induced lung fibrosis

Pulmonary fibrosis is not a human-specific disease. Cases of pulmonary fibrosis arising naturally in both horses and cats and even a single donkey have been reported [60–64]. Feline pulmonary fibrosis has histopathological features consistent with human IPF, including interstitial fibrosis with myofibroblast/fibroblast foci, honeycombing with alveolar epithelial metaplasia and minimal interstitial inflammation [62]. No studies have been performed to evaluate for the presence of virus in these cases, although a herpesvirus has been detected in a case report of feline interstitial pneumonia [65]. Equine multinodular pulmonary fibrosis is a progressive disease associated with significant interstitial fibrosis but with preservation of alveolar architecture. Unlike the feline studies, virology studies were performed on 24 horses with fibrosis and 23 controls. Using genus-specific PCR, evidence of equine herpesvirus-5 was found in all 24 horses with fibrosis compared with zero controls [63]. These naturally occurring cases strongly suggest that herpesviruses are causally associated with lung fibrosis in other species.

MHV-68 infection augments fibrotic responses in mouse models of pulmonary fibrosis

Although no natural murine models of pulmonary fibrosis exist, both bleomycin and fluorescein isothiocyanate (FITC) have been well described in the literature to cause pulmonary fibrosis in mice [66]. These experimental models have been essential in determining the key pathologic features of pulmonary fibrosis, including the cells and mediators involved. Given their success, investigators have turned to murine modeling to identify the potential role of viruses in the pathogenesis of pulmonary fibrosis. Murine gammaherpesvirus (MHV)-68 is a member of the gammaherpesvirus family that is closely related to HHVs and has a genome similar to HHV-8 and EBV [67]. It infects the respiratory tract with an initial lytic phase before setting up latency in both B cells and lung epithelial cells by day 14 postinfection [42,67–72]. With its similarity to EBV, MHV-68 has been used in several murine lung fibrosis studies. Collectively, these animal models have provided the strongest evidence to date of the profibrotic potential of gammaherpesvirus infections in the lung.

Initially, Lok *et al.* gave Balb/c mice, known to be resistant to bleomycin-induced fibrosis, intranasal MHV-68 8 days prior to intraperitoneal bleomycin to determine if viral infection could increase the fibrotic response of these mice to bleomycin [73]. There were four groups of mice: those given virus and bleomycin; virus alone; bleomycin alone; and a control group. Histologically, the group given both virus and bleomycin had higher fibrosis and inflammation scores than the other groups with thickening of the alveolar wall and complete loss of lung architecture. In addition, the collagen content as measured by hydroxyproline was significantly higher in the bleomycin/virus group when compared with bleomycin alone and trended higher compared with virus alone [73]. The authors suggested that while this gammaherpesvirus alone did not cause fibrosis in young mice, actively replicating MHV-68 served as a cofactor, augmenting the fibrotic response to another insult. However, the mechanisms by which this might occur were not yet known.

More recently, our laboratory found that latent virus could augment the fibrotic response to a subsequent insult [72]. As stated previously, MHV-68 exists in a latent state in lung epithelial cells by day 14 postinfection [71,72]. Intranasal MHV-68 was administered and allowed to reach latency in the lungs before challenging the mice with an additional fibrotic stimulus, either bleomycin or FITC. Our results demonstrated an increase in collagen in the infected mice compared with controls, whether the fibrotic challenge occurred at 14, 21, 35, 42 or 70 days postinfection [72]. Of particular interest was the observation that the presence

of latent MHV-68 infection could augment a subsequent fibrotic challenge to a dose of FITC that was not fibrogenic when administered alone. Although low level reactivation of MHV-68 to lytic replication occurred after FITC administration, subsequent studies with an *ORF72* mutant virus suggested that reactivation of MHV-68 was not necessary to augment fibrosis in these young mice [72]. These studies are consistent with the 'two-hit hypothesis' of fibrogenesis, which suggests that two insults, if encountered individually may not be sufficient to induce fibrosis. Yet, previous infection with a gammaherpesvirus (hit 1) may predispose the lung to develop fibrosis upon exposure to the FITC or bleomycin stimulus (hit 2). Understanding how hit 1 can alter the lung environment to favor fibrosis is the goal of the mechanistic studies discussed below.

Mechanisms by which latent MHV-68 infection may augment fibrosis

Our studies revealed that latent MHV-68 infection increased fibrocyte accumulation in the lung and also augmented the inflammatory response to FITC [72]. Fibrocytes are bone marrow-derived cells that share characteristics common to both leukocytes and mesenchymal cells [7]. They are recruited to the lung by chemokines CCL2, CXCL12 and CCL12 [8,9,74], and contribute to the fibrotic process by differentiating into myofibroblasts and secreting profibrotic factors [9,75]. We and others have determined that latently infected alveolar epithelial cells expressed higher levels of CCL2 and CCL12, providing a mechanism for increased recruitment of fibrocytes to the lungs [72,76]. Furthermore, latent infection with MHV-68 increased epithelial cell production of TGF-β, a potent fibrotic mediator and cysteinyl leukotrienes, well-known profibrotic lipids [72,76]. The presence of excess cysteinyl leukotrienes in the lung may increase mesenchymal cell proliferation [77,78], and the presence of TGF-β, would be expected to activate fibroblast to myofibroblast transformation [3]. Taken together, these results provide a potential mechanism by which a latent herpesvirus infection can alter the lung environment and thereby act as a cofactor in fibrosis caused by a subsequent epithelial-cell injury.

Gammaherpesvirus-induced exacerbation of existing fibrosis

Herpesvirus may also be a key element in the etiology of acute exacerbations of pulmonary fibrosis. Associated with extremely high mortality rates, these rapid deteriorations in pulmonary function often have no clear etiology and histology shows evidence of diffuse alveolar damage or organizing pneumonia plus usual interstitial pneumonitis [14]. When examining the placebo arm of a clinical trial, Martinez *et al.* reported that 15 out of 32 patients who died of their fibrosis, did so abruptly, and five of those 15 patients were found to have an infectious source of their decompensation [15]. These data strongly support the theory that infections can exacerbate fibrosis and raise the possibility that at least some IPF exacerbations may be secondary to occult viral infections, which are not readily identifiable.

We were successful in modeling a rapid exacerbation of pulmonary fibrosis using MHV-68 infection in mice with already established pulmonary fibrosis. Mice with established FITC-induced fibrotic responses (14 days) were infected with MHV-68 and lungs were harvested on day 21 (7 days after infection and during the peak of lytic viral replication) [42]. Wild-type mice infected with MHV-68 had significantly more collagen deposition than those mock-infected with saline [42]. In addition, histology in the virus-infected mice showed evidence of interstitial edema, intra-alveolar hemorrhage and alveolar epithelial denudation all consistent with findings of diffuse alveolar damage seen with acute exacerbations in humans with IPF [14]. When compared with mice given virus alone, mice given virus after fibrosis had been established had an increased viral titer that was associated with increased expression of mRNAs encoding lytic viral genes. This showed that FITC-injured lungs were more susceptible to increased viral replication suggesting possible deficiencies in host defense. Finally, similar to our previous latent infection model, our acute viral exacerbation

model had evidence of upregulation of CCL2 and CCL12 production and fibrocyte recruitment, corroborating this mechanism for increased fibrosis in the lung in the setting of lytic as well as latent infection [42].

MHV-68 infection alone causes fibrosis in IFN-γ receptor-deficient mice

Although the aforementioned work demonstrates the ability of the virus to act as a cofactor in the fibrotic process, other studies have shown viral infection alone to be a sufficient trigger to cause fibrosis in a predisposed host. It is known that some patients with pulmonary fibrosis have evidence of a cytokine imbalance skewed towards a type 2 immune response (Th2) [79]. IFN-γ is a Th1 cytokine with antiviral properties and it is known to decrease collagen and fibronectin expression [80]. IFN-γ receptor-deficient mice produce a Th2-biased cytokine profile and it has previously been shown that IL-4 (a Th2 cytokine) promotes, while IFN-γ suppresses, fibrotic responses [81]. Ebrahimi *et al.* were the first to demonstrate that MHV-68 can independently cause fibrosis in IFN-γ receptor-deficient mice (IFN-γR$^{-/-}$) [82]. The resultant disease process was not just limited to the lungs but caused multiorgan fibrosis in the liver, lung, spleen and lymph nodes. It was associated with both an increase in production of profibrotic mediators (TNF-α, TGF-β, IL-1β, lymphotactin and CCL4) and a decrease in production of antifibrotic chemokines (CXCL10 and CXCL9) [82].

Further characterizing the pulmonary fibrosis caused by MHV-68 in IFN-γR$^{-/-}$ mice, Mora *et al.* found evidence of severe inflammation initially around blood vessels followed by chronic lymphocytic infiltrates, which continued on day 180 postinfection [83]. Like human IPF, this inflammation was associated with increased collagen deposition, areas of severe fibrosis located in the peripheral regions of the lungs, increased TGF-β production and the presence of myofibroblasts in fibroblastic foci. Furthermore, viral antigen persisted in type 2 epithelial cells and led to continued hyperplasia and hypertrophy of these cells, a marker of repair and remodeling after epithelial cell damage. It also led to surfactant abnormalities, primarily upregulation of surfactant protein-D expression [83]. The authors suggested that infection of type 2 epithelial cells led to dysfunction and apoptosis of these cells, leading to alterations in surfactant production causing further epithelial-cell injury and fibrosis.

Mora *et al.* also found evidence of macrophage alterations in the lungs of MHV-68-infected IFN-γR$^{-/-}$ mice [84]. They showed these macrophages were recruited to sites of extensive fibrosis and displayed alternative activation profiles consistent with type 2 macrophages [84]. Unlike type 1 macrophages, these type 2 macrophages expressed mediators such as found in inflammatory zone 1, the lectin, Ym1/2 and IGF-1, all actively involved in cell repair, healing and proliferation. They also secreted fibronectin, a component of the extracellular matrix. Finally, the type 2 macrophages in these MHV-68-infected mice displayed an increased production of arginase 1, which increases production of polyamines and L-proline promoting cell proliferation and collagen production. Similar findings have been found in human IPF lungs and are felt to also contribute to the fibrotic pathology [85]. Taken together, these data suggest that MHV-68 infection of Th2-biased mice promoted fibrosis through recruitment and alternative activation of macrophages [84].

While the aforementioned studies suggest that a Th2-biased lung environment can predispose to MHV-68-induced fibrosis, it does not follow that a Th2 environment is required for fibrosis. Studies using the viral exacerbation model from our laboratory demonstrated augmentation of fibrosis during acute infection despite a strong Th1 cytokine profile [42]. In fact, in our model, MHV-68 infection exacerbated FITC-induced fibrosis even in Th2-deficient, IL-4/IL-13$^{-/-}$ mice [42]. It is likely, however, that the mechanisms by which herpesviruses may augment fibrosis differ in Th1 versus Th2 environments. Mora *et al.* found that by giving an antiviral drug and preventing MHV-68 reactivation in latently infected IFN-γR$^{-/-}$ mice, they could prevent severe fibrosis [86]. Severe fibrosis was also

avoided if IFN-γR$^{-/-}$ mice were infected with a strain of MHV-68 defective in a reactivation gene [86]. More recently, this same group has shown that if you block nuclear factor-κB signaling in the infected host cells using a virus that expresses a mutant dominant inhibitor called IκBαM, you can disrupt the ability of the virus to establish latency (and thus persistence) in the IFN-γR$^{-/-}$ mice [76]. In this circumstance, the reduced viral persistence at later time points correlated with reduced fibrosis, reduced chemokine secretion, reduced alternative activation of macrophages and diminished expression of the αvβ6 integrin necessary to activate TGF-β [76]. What was interesting was that this IκBαM virus showed no defect in early lytic replication or inflammation; however, this virus was not able to establish latent or persistent infection, and thus, was not able to maintain the environment necessary to promote fibrosis [76]. Ongoing viral replication/reactivation may be necessary to maintain viral persistence in these Th2-biased mice [86]. This is in contrast to our data showing that viral reactivation was not necessary to augment fibrotic responses to FITC in wild-type mice [72]. Rather, we hypothesize that viral-induced changes in profibrotic mediator secretion may account for the fibrotic phenotype in this situation. Perhaps viral reactivation and chronic replication or cellular injury are necessary for MHV-68 to cause fibrosis independent of an additional fibrotic stimuli. This hypothesis is supported by a recent study indicating a candidate gene for fibrosis susceptibility in familial IPF, *ELMOD2*. This gene has been found at lower levels in the lungs of IPF patients and is responsible for antiviral responses [87,88]. Potentially, these patients are more susceptible to viral insults, and as a result, may accumulate an increased risk for pulmonary fibrosis.

The aging lung is prone to fibrosis

Idiopathic pulmonary fibrosis is a disease of the elderly. Incidence, prevalence and mortality of the disease increase with age with the highest prevalence in patients over the age of 75 years [16]. In a recent retrospective study looking at a database of 135 patients with surgical lung biopsy-proven UIP versus other interstitial pneumonias, age was found to be a significant independent predictor of UIP [89]. Possibly, differences in the environment of the aging lung are responsible for elderly patients' susceptibility to pulmonary fibrosis.

These differences were investigated by looking at bleomycin-induced fibrosis in a senescence-accelerated mouse model commonly used for studying physiological and pathological responses during aging. Senescent-accelerated mice have a tendency to age more rapidly than wild-type mice and display features associated with the aging process such as memory deficits, cataracts and osteoporosis [90]. After treatment with intratracheal bleomycin, mice from a senescence prone strain-8 had increased fibrosis with increased TGF-β production and increased serum fibrocytes at day 7 compared with mice from a senescence-resistant strain [91]. The authors concluded that the increased propensity for fibrosis in the senescence-prone mice was due to increased fibrocyte recruitment to sites of lung injury. They also noted that reparative mesenchymal stem cells from senescence-prone mice migrate less efficiently to their chemotactic ligand, CXCL12 [91]. As CXCL12 is also a ligand for fibrocytes [9], it is interesting to speculate that aged mice may have an imbalance in the mobilization of protective (mesenchymal stem cell) and pathologic (fibrocytes) cell types in response to inflammatory stimuli.

Also implicated in fibrosis, the ER of all cells, including alveolar epithelial cells, is altered in the aged environment. There is an increase in oxidative stress and misfolding of proteins and a decrease in the number of protein folding chaperones, leading to abnormal protein accumulation, In addition, there is a decline in the ability of the ER to rid itself of these abnormally folded proteins through autophagy or lysosomal degradation. This can lead to increases in ER stress, which then activates the UPR. Many studies have shown that low-to-moderate levels of ER stress, which develop in response to a variety of insults, including

viruses [55], can increase stress resistance via an adaptive UPR that is actually protective of aging. However, with high, persistent levels of ER stress, the UPR generated induces apoptotic cell death [92]. Interestingly, alveolar epithelial cells of IPF patients have increased ER stress markers [54]. In addition, with aging, there is an upregulation of levels of C/EBP-homologous protein (CHOP), a major protein involved in the apoptotic pathway and downregulation in PKR-like ER-localized eIF2α kinase (PERK) and IRE1a, major proteins in the adaptive system skewing the UPR in the elderly towards apoptosis [92]. Thus, high ER stress and changes in the UPR lead to more programmed cell death in the alveolar epithelial cells of the elderly and may, therefore, predispose them to pulmonary fibrosis [92,93].

The aging lung is also susceptible to changes in the immune system known as immunosenescence. Innate immunity is relatively preserved over time with only mild decreases in the efficiency of neutrophils and natural killer cells and an increase in inflammatory cells and proinflammatory cytokines [94]. Within the adaptive immune system, B-cell differentiation, B-cell/T-cell interaction and B-cell antibody affinity are reduced. However, the most dramatic age-related changes occur in T cells, which are important for control of viral replication. There is involution of the thymus, resulting in decreased numbers of circulating naive T cells [94]. These naive T cells have shorter telomeres and decreased production of IL-2, which leads to a restricted repertoire and decreased proliferation. Also, aged mouse models have shown a shift from Th1 cytokine production to a predominantly Th2 phenotype with IL-4, IL-6 and IL-10 production [94]. However, it should also be noted that data in humans regarding Th2 cytokine skewing in the elderly are inconsistent [95]. In addition, in humans, there is an increased proportion of memory and effector T cells, including large clonal populations of end-stage CD8 cells that have lost CD28, a B-cell costimulatory molecule [96]. These populations of cells are largely directed against HCMV and EBV and are likely due to the increased viral reactivation and replication that occurs in the elderly [97]. They are felt to be responsible for poor responsiveness to vaccination in the elderly [98] and decreased immunity to other viral infections [99].

Finally, genome scans of patients with familial pulmonary fibrosis show evidence of mutations in telomerase, a protective enzyme that synthesizes DNA to prevent telomere shortening [100]. Without telomerase, shortened telomeres initiate cell senescence, stop proliferation and lead to cell apoptosis to prevent against chromosomal instability. Mutations of telomerase reverse transcriptase (TERT), were first discovered in patients with dyskeratosis congenita, a congenital disorder sometimes associated with a form of pulmonary fibrosis. This led to further identification of a number of loss of function mutations in telomerase in approximately 15% of patients with familial pulmonary fibrosis. These mutations have also been found in sporadic cases of pulmonary fibrosis [100]. In addition, many patients with IPF without identified telomerase mutations have shorter telomeres than expected [101]. Whether this is secondary to unidentified genetic abnormalities or environmental factors is still unknown. Since aging is associated with telomere shortening and loss of telomerase function, it follows that predisposing genetic or environmental shortening of telomeres are exacerbated by aging and may trigger fibrosis [93,102]. Given the presence of increased circulating fibrocytes in response to fibrotic injury, the increased Th2 cytokine profile present, the dysfunction of telomerase, the increased ER stress causing apoptosis in airway epithelial cells and the presumed increase in susceptibility to viral infection and/or reactivation, it is possible that an aged lung provides the setting necessary for gammaherpesvirus alone to cause fibrosis. In fact, preliminary data presented by two groups (including our own) at the American Thoracic Society meetings have explored this question using MHV-68 in aged mice. After intranasal infection with MHV-68, mice aged 15–18 months, have increased lung fibrosis and produce more collagen

than mice 4 months infected with MHV-68 or saline controls [103,104]. Surprisingly, recent investigations by Yager *et al.* regarding MHV-68 immunity in aged mice showed no reactivation of MHV-68 or increase in latent viral load 1 year after infection, repudiating the theory that increased viral reactivation, and therefore increased chronic injury, in the aged mouse lung causes fibrosis [105]. In addition, these authors found clearance of *de novo* lytic infection with MHV-68 to be only slightly delayed in aged mice, suggesting that immunity to MHV-68 is not severely impaired with age. Thus, while it is still unclear by what mechanism aged mice are more susceptible to MHV-68-induced fibrosis, the answer does not appear to include viral reactivation or impaired lytic virus clearance.

These findings, along with our data regarding the ability of latent viral infection to augment fibrosis [72], suggest to us that the viral-mediated mechanism of fibrosis is likely to be the result of altered secretion of pro- and anti-fibrotic mediators, at least in wild-type mice. This is clearly an area that needs further investigation.

Expert commentary

Admittedly, there have been no studies that definitively prove causality between herpesvirus and pulmonary fibrosis and much of the clinical data regarding the association of viral infection and IPF has been difficult to interpret. This is largely because we still do not know what questions are the most important to ask. Does the virus need to be found in the lung? Can viral infections in other parts of the body still influence responses in the lung? Does the virus need to be replicating to cause disease? Does the virus need to be persistent? Are particular viruses able to infect particular cell types relevant to lung fibrosis? Must the virus be present at the time of fibrosis or could a preceding infection alter the lung environment? Do viral infections cause fibrosis, or are fibrotic lungs just more susceptible to infection? Do we have the technical ability to accurately detect appropriate viruses and, if so, what compartment should we sample? Clearly, we still do not have answers to all of these questions. However, significant progress has been made in recent years. On the whole, we believe a compelling set of clinical studies now suggest that herpesviruses in particular may be associated with IPF (Table 1). In addition, animal models have clearly demonstrated that MHV-68 can augment subsequent fibrotic responses in mice [72,73]. It is particularly interesting to us that the pre-existing viral infection can cause a fibrotic stimulus that on its own is particularly weak, to become more pronounced. Furthermore, we do not know exactly how long this viral-induced predisposition to fibrosis might last. The latest time point studied in the mice was infection on day 70 pre-FITC or prebleomycin [72]. If we can extrapolate these data to humans (and it should be stressed that this is speculative), it is possible that a viral infection acquired months to perhaps years prior to the inhalation of a toxic particle or gas may be the deciding factor in who develops fibrosis and who does not. It may also explain why the etiology of the disease has been so hard to discover. If hit 1 and hit 2 are temporally far apart, and neither insult on its own is pathogenic, it could explain why the disease is rarely caught early and why patients may not be able to piece together the natural history of infection and exposure leading to the development of their symptoms. The findings that MHV-68 can cause fibrosis on its own in Th2-biased mice also suggests that certain persons who have a tendency to develop Th2 responses in the lung may be more prone to viral-induced fibrosis [83]. This is intriguing given the finding that many IPF patients tend to be Th2 prone [79]. It is also interesting that aged mice are believed to be Th2 prone [94]. Thus, this could explain why aged mice develop fibrotic responses to the virus even in the absence of hit 2. It is also possible that in an aged or a Th2 environment, viral replication and persistence may be critical for fibrogenesis [76,86] whereas in young or Th1-biased mice, viral replication may be unnecessary for the augmentation or exacerbation of disease [72]. Such observations suggest that there may be multiple mechanisms whereby a virus can promote fibrogenesis. While these mechanisms are not fully known, animal

models have offered clues to potential mechanisms (Table 2). Given the possibility that aged or Th2 individuals may require viral replication, it will be important to develop and test antiviral drugs as potential therapies for IPF. Antiviral treatment in Th2-biased mice prone to gammaherpesvirus-induced fibrosis has already been shown to increase viral antigen clearance, decrease fibrosis and improve survival [83]. Furthermore, the studies in Th2-biased mice have also suggested that disruption of host factors necessary to establish viral latency and thus persistence may be a therapeutic option [76].

Five-year view

While it would certainly be premature to speculate that all cases of IPF have a viral origin, it would also be wrong to assume that none do. The animal models have clearly shown that gamma-herpesviruses can influence responses to other fibrotic stimuli [42,72,73], and in some cases cause fibrosis on their own [83,86]. Because gammaherpesviruses have infected the majority of the population, an important area for future investigation is to understand why some individuals are prone to fibrosis, while others are not. This is akin to trying to understand why EBV causes cancer or mononucleosis is some individuals, but not others [24]. Fibrosis may just be one more example in the spectrum of diseases that viruses like EBV can cause. One thing that seems clear from the clinical studies is that the most striking associations come when the virus is actually detected in the lung [47,51]. Generally, EBV is believed to be maintained as a latent infection in B cells. It is rarely detected in the lung tissue of control individuals, even though they may be seropositive [47,51]. Furthermore, analysis of rearranged forms of EBV indicating active replication in buffy coats from peripheral blood showed higher prevalence in IPF patients than controls, even though incidence of infection was not different between the groups [51]. We need to better understand what may lead to viral retention in the lungs, and perhaps other sites, in IPF patients. We also need to understand whether IPF patients are more susceptible to viral reactivation. Most likely, this will have to come from insights in the animal models. We speculate that studies of MHV-68 infection in numerous lung cell types will be fruitful areas of investigation. It will be important to determine whether latent, or even low-level lytic infection can influence cell proliferation, apoptosis or mediator secretion. Once a fingerprint for viral predisposition is obtained, translational studies can be planned for clinical specimens. Because at least some instances where MHV-68 causes fibrosis appear to require viral replication [51,86], an important goal for the next 5 years will be to test antiviral therapies in IPF patients. It is particularly interesting that at least one case study has reported that a 9-month course of an antiherpetic drug valacyclovir orally administered (2 g twice daily) stabilized the lung function of a patient with a history of familial IPF and evidence of EBV replication in the lung that was previously worsening [47]. We suggest that patients with evidence of EBV in the lung with IPF should be treated with antiherpetic agents and evaluated for disease progression. These types of trials may offer benefit to patients with forms of fibrosis that require viral replication. In addition, a goal for the next 5 years is to establish fibrotic mechanisms that may be important in the setting of latent (or nonreplicating) viral infection. Using viral mutants that cannot reactivate, antiviral drugs and UV-crosslinking of viral genomes prior to infection, we may be able to identify mediators or cellular changes that promote fibrosis in the absence of active replication. Those targets should then become the focus of new studies to test specific interventions aimed at blocking the viral-induced changes.

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Table 1

Viruses studied in association with idiopathic pulmonary fibrosis.

Virus	Studies showing an association with IPF disease development or progression	Studies that do not support a specific association with IPF disease
HCV	Increased serum antibodies against HCV in IPF [28] IPF incidence increases with time post-HCV infection [30]	HCV more prevalent in all cases of lung disease (including IPF) than controls [29] No association between IPF and HCV prevalence [33]
TTV	Survival rate for IPF patients with TTV is lower [37]	
Adenovirus		E1A gene product not detected at higher levels in IPF patients [40] No elevations in antiviral IgG in IPF [41]
HCMV	Increased HCMV in sera of IPF [45] Higher HCMV IgG in serum of IPF than controls [45] Increased HCMV serum IgG in IPF compared with controls [41] Increased HCMV DNA in lung tissue of IPF compared with controls [47]	No difference in prevalence of HCMV DNA in IPF and control patients [46]
EBV	Elevated titers of anti-IgG and -IgA in IPF compared with patients with fibrosis of known cause [48] Increased anti-IgA in BAL of IPF [49] Increased EBV DNA in IPF[47,49,50] lungs IPF patients with EBV LMP-1 in lung epithelial cells had worse prognosis [52] Increased presence or rearranged WZhet form of EBV in lung and buffy coat of IPF compared with controls [51]	No increased presence of EBV DNA in IPF patients compared with controls [57,58]

BAL: Bronchoalveolar lavage; EBV: Epstein–Barr virus; HCMV: Human cytomegalovirus; HCV: Hepatitis C virus; IPF: Idiopathic pulmonary fibrosis; membrane protein-1; TTV: TT virus.

Table 2

Potential mechanisms responsible for viral-enhanced fibrosis according to cell type.

Cell type	Profibrotic effects
Alveolar epithelial cells	Lytic destruction destroys lung architecture and allows fibroproliferation Increased apoptosis of infected epithelial cells Enhanced cysteinyl leukotriene production may increase fibrocyte proliferation Enhanced chemokine release may recruit fibrocytes and inflammatory Enhanced TGF-β production may increase myofibroblast differentiation and matrix production Surfactant abnormalities Epithelial cell hyperplasia and hypertrophy Increased expression of αvβ6 integrins to activateβTGF-
Macrophages	Alternative activation may increase arginase-1 l-proline and and promote fibroblast proliferation and collagen secretion Increased Th2 cytokine production may activate collagen production Increased chemokines may recruit more fibrocytes and inflammatory cells Increased TGF-β may promote myofibroblast differentiation and collagen production Decreased apoptosis may increase inflammatory mediator release
Mesenchymal cells	Increased fibrocyte recruitment, proliferation and collagen secretion Increased fibroblast migration, proliferation and extracellular matrix production Decreased apoptosis of fibrocytes and/or fibroblasts
B cells	Source of latent Epstein–Barr virus infection may continually reseed the lung Increased profibrotic mediator secretion
T cells	Altered Th1 vs Th2 cytokine skewing Aging may inhibit immunosurveillance and increase viral reactivation Increased profibrotic mediator secretion
Neutrophils	Increased recruitment of neutrophils or increased activation of these cells may lead to exuberant lung damage