Herpesvirus Infection Augments the Response to a Subsequent Fibrotic Challenge in the Lung Via the Recruitment of Fibrocytes and the Induction of Pro-fibrotic Factors

by

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To my family and Alberto

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LIST OF ABBREVIATIONS

γHV-68	murine gammaherpesvirus-68
AEC	alveolar epithelial cell
AM	alveolar macrophage
BAL	bronchoalveolar lavage
bFGF	basic fibroblast growth factor
BM	bone marrow
cAMP	cyclic adenosine monophophate
CCL	C-C motif ligand
CCR	C-C motif receptor
CD	cluster of differentiation
COPD	chronic obstructive pulmonary disease
COX	cyclooxygenase
cPLA	cytosolic phospholipase A
CXCL	C-X-C motif ligand
d.p.i.	days post-infection
EBV	Epstein-Barr virus
EIA	enzyme immunoassay
ELISA	Enzyme-Linked ImmunoSorbent Assay
EP	E-prostanoid

FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FLAP	5-lipoxygenase activating protein
GFP	green fluorescent protein
HCMV	human cytomegalovirus
HHV	human herpesvirus
i.p.	intraperitoneal
i.t.	intratracheal
i.v.	intravenous
IFN	interferon
IFNγR-/-	interferon-gamma receptor knock-out
Ig	immunoglobulin
IL	interleukin
ILD	interstitial lung disease
DPLD	diffuse parenchymal lung disease
IIP	idiopathic interstitial pneumonia
HRCT	high-resolution computer tomograph
IPF	idiopathic pulmonary fibrosis
LMP	latent membrane protein
LO	lypoxygenase
M-CSF	macrophage-colony stimulating factor
MIP	macrophage inflammatory protein
MOI	multiplicity of infection

MR	marker rescue
MRP ATP	multidrug resistance protein adenosine triphosphate
nd	not detectable
ns	not significant
PAI	plasminogen activator inhibitor
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PFU	plaque forming unit
PG	prostaglandin
PMN	polymorphonuclear leukocyte
PPAR	peroxisome proliferator-activated receptor
RT-PCR	reverse transcriptase-polymerase chain reaction
SCID	severe combined immunodeficiency
SDF	stromal cell-derived factor
SFM	serum-free media
SMA	smooth muscle actin
TGF	transforming growth factor
Th1	T helper 1
Th2	T helper 2
TNF	tumor necrosis factor
UIP	usual interstitial pneumonia
WT	wild-type

ABSTRACT

No effective treatment currently exists for pulmonary fibrosis. The cause of pulmonary fibrosis is unknown, and little is known about its pathobiology. While pulmonary fibrosis is likely multifactorial, evidence is accumulating to implicate gammaherpesviruses as cofactors in the pathogenesis of pulmonary fibrosis. We developed a murine model to test the hypothesis that latent gammaherpesvirus infection can augment the response to a subsequent pulmonary fibrotic challenge. Mice were infected intranasally with murine gammaherpesvirus (γHV-68) prior to an intratracheal fibrotic stimulus with either fluorescein isothiocyanate (FITC) or bleomycin. γHV-68 was latent in the lung by 14 days after infection. γHV-68 infection 14-70 days prior to the fibrotic stimulus augmented fibrosis measured 21 days later. Furthermore, latent γHV-68 infection induced fibrosis in response to a sub-threshold fibrotic challenge. Although FITC challenge initiated low-level lytic gene transcription by γHV-68, reactivation from latency was not necessary for viral-induced augmentation of fibrosis. Mechanisms that potentially contribute to latent γHV-68-induced augmentation of fibrosis were explored. Inflammation, CCL2, CCL12, transforming growth factor (TGF)-β1, and fibroctye number were increased in the lungs during γHV-68 latency. Inflammation, fibrocyte number, and TGF-β1 levels were further increased in the lungs of latently-infected mice that were administered FITC compared to mock-infected mice that received FITC. We found that γHV-68 infection can alter the phenotype of alveolar

epithelial cells (AECs) to promote a pro-fibrotic environment. Specifically, AECs isolated from latently-infected mice produced more CCL2, CCL12, TGF-β1 and cysteinyl leukotrienes (cysLTs) than AECs from uninfected mice. While the actions of CCL2 and CCL12 to recruit fibrocytes and the actions of TGF-β1 to promote fibrocyte differentiation were known, the effects of cysLTs on fibrocytes were unknown. Our studies demonstrate that cysLTs are critical mediators of fibrosis with both autocrine and paracrine effects on fibrocyte proliferation via cysLT1 receptors. In sum, gammaherpesvirus infection augmented subsequent fibrosis in mice via the induction of pro-fibrotic factors and the recruitment of fibrocytes. Our data complement existing human and animal literature supporting a role for gammaherpesviruses as cofactors in the pathogenesis of pulmonary fibrosis and provide new mechanistic insight into the disease pathogenesis.

CHAPTER ONE:

INTRODUCTION

Idiopathic Pulmonary Fibrosis: Definition and Clinical Problem

Idiopathic pulmonary fibrosis (IPF) is progressive and relentless lung scarring of unknown etiology recognized as the most lethal interstitial lung disease (ILD). Precise molecular mechanisms underlying the development of IPF and leading to the irreversible destruction of the lung are still unclear. As a result, there is currently no effective treatment for IPF.

In 2000, IPF was distinguished as a distinct clinical disorder by the American Thoracic Society and defined as "a specific form of chronic fibrosing interstitial pneumonia limited to the lung and associated with the histologic appearance of usual interstitial pneumonia (UIP) on surgical lung biopsy" [1]. IPF is classified as an ILD or, more accurately, as a diffuse parenchymal lung disease (DPLD) because IPF affects not only the interstitium, but also the airspaces, peripheral airways, and vessels along with their respective epithelial and endothelial linings [2, 3]. Among diffuse lung diseases, IPF belongs to a subgroup comprised of seven idiopathic interstitial pneumonias (IIPs) that each have unclear etiologies but are differentiated by specific clinical features and pathological patterns [4]. IPF is the most common type of IIP, and it is distinguished by its unique pattern of clinical, radiological, and pathological aspects of disease

manifestation and behavior [5]. Patients with the histologic appearance of UIP (normal lung alternating with patches of dense fibrosis in the form of collagen sheets) on a surgical lung biopsy along with compatible clinical and radiographic features are diagnosed with "definite" IPF [1, 2, 6]. Several high-resolution computed tomographic (HRCT) findings have been described that correlate with the histopathologic pattern of UIP [7-10]. A diagnosis of "probable" IPF is assigned if several clinical and radiologic criteria are met in the absence of a surgical lung biopsy [1, 6].

IPF is a chronic disease that occurs in the absence of known provocation. During the disease course, there is progressive scarring within the lungs, and patients experience progressively impaired pulmonary function [1, 2]. Individual clinical courses can be highly variable, and the natural history of the disease can take at least 2 forms. Patients can experience a slowly progressive disease, characterized by steady worsening of symptoms, lung function, and gas exchange [11, 12] or they can experience an acute respiratory deterioration termed an acute exacerbation noted by rapid worsening of symptoms over a short time frame (usually less than 1 month) [13]. Acute exacerbations can have extremely high mortality rates. Both the development of IPF and the onset of acute exacerbations are idiopathic but may involve occult infection, toxic exposures, genetics, aspiration, disordered coagulation and complications of comorbidities [13]. Some patients remain stable for extended periods, but long-term survival with IPF is not expected [14]. Using the consensus definition of IPF, the median survival time is between 2 and 5 years from the time of diagnosis [15-17].

The incidence of IPF is rare, occurring in approximately 10.7 per 100,000 men and 7.4 per 100,000 women. The prevalence is estimated at 20.2 men per 100,000 and

13.2 women per 100,000 [18, 19]. About 5 million people worldwide have the disease, and the incidence of IPF increases with age [2]. Two-thirds of all cases arise in patients over 60 years of age, and IPF occurs infrequently in those younger than 40. It rarely affects children [1]. Familial IPF accounts for 0.5 to 2% of all cases of IPF while most patients present with sporadic cases of the disease [2].

In the absence of effective therapy, the American Thoracic Society/European Respiratory Society consensus document suggests initial treatment with prednisone and azathioprine or cyclophosphamide [1]. More recently, studies have suggested there are possible therapeutic effects from non-immunosuppressive agents such as *N*-acetylcysteine and anticoagulation in IPF patients [6, 20, 21]. In addition, several new pharmacologic agents (e.g. interferon-gamma, pirfenidone, etanercept, imatinib, and bosentan) have been investigated in clinical trials [22, 23].

IPF Pathobiology

Accumulating evidence from clinical studies of human patients as well as laboratory research using animal models has allowed some understanding about how IPF starts and progresses. Fibrotic lung disease likely results from a dysregulated healing response to injurious events within the lung. Although the precise temporal sequence of events and mechanisms of disease are not understood, several common pathobiologic characteristics are recognized. These include damage and loss of type 1 alveolar epithelial cells followed by hyperplastic expansion of type 2 alveolar epithelial cells [24]; variable chronic inflammatory cell infiltration [25]; a predominant type 2 cytokine profile [26]; induction of pro-inflammatory cytokines, such as interleukin (IL)-8 and tumor

necrosis factor-alpha (TNF-α) [27, 28]; induction of fibroblast growth factors, such as basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF)[29]; induction of differentiation molecules such as transforming growth factor-beta 1 (TGF-β1) [26, 30]; an altered fibroblast phenotype characterized by exuberant proliferation and the transition to alpha-smooth muscle actin (α-SMA)-positive myofibroblasts [31]; excessive deposition of extracellular matrix proteins [32]; derangements in eicosanoid synthesis, including increased leukotriene synthesis and diminished prostaglandin production [33, 34]; diminished activation of plasminogen and altered coagulation cascades [35, 36], and recruitment of bone-marrow derived fibrocytes [37-39].

Alveolar epithelial cells (AECs)

AEC injury is a universal feature of the pathobiology of fibrotic lung disease [40-42]. Type 1 and type 2 alveolar cells constitute the epithelial component of the alveoli. Type 1 AECs cover more than 90% of the alveolar surface area of the peripheral lung where they interface with pulmonary capillaries to provide a surface of minimal thickness for gas exchange [40]. Type 2 cells are multifunctional cells that serve as progenitors for type 1 alveolar cells, and are found in the corners of alveoli. In the normal human lung, there are numerous contacts between AECs and the mesenchymal-derived fibroblasts lying below them that contribute to epithelial and mesenchymal cell homeostasis [43].

During a normal wound repair response after type 1 AECs are lost, type 2 AECs proliferate and differentiate into type 1 cells to cover the denuded basement membrane and replace the damaged type 1 AECs [44]. Meanwhile, fibroblastic cells are highly activated to serve multiple functions that are regulated by injured and regenerating AECs

and inflammatory cells [44]. Through the production of a number of profibrotic and anti-fibrotic factors capable of inducing fibroblast migration, proliferation, and activation, AECs are crucial to regulation of fibroblast-derived clot formation and extracellular matrix formation, as well as contraction of the wound by myofibroblasts. These factors include PDGF, TGF-β1, TNF-α, endothelin-1, connective tissue growth factor, and osteopontin [40]. After tissue remodeling is complete, AEC signaling primarily through prostaglandin E2 (PGE_2) suppresses fibroblast migration, proliferation, and activation, as well as collagen synthesis [45-47]. Remaining type 2 AECs and fibroblasts apoptose.

In IPF lungs, there are remarkable disturbances in physiological AEC phenotype and behavior. Alveolar reepithelialization is disordered in such a way that type 2 AECs proliferate hyperplastically, type 2 AEC activation leads to the migration, proliferation, and activation of mesenchymal cells that produce abnormal amounts of extracellular matrix, and normal apoptosis is deficient [48]. Epithelial-mesenchymal transition has been proposed as one mechanism for the loss of epithelial-mesenchymal homeostasis [49]. Abnormal levels of the fibroproliferative regulatory factors produced by AECs (i.e. PDGF, TGF-β1, TNF-α, endothelin-1, connective tissue growth factor, and osteopontin among others) all play a role in the development of pulmonary fibrosis [50, 51]. Recent data also suggest that expression of cyclooxygenase enzymes, COX-1 and COX-2, responsible for PGE_2 production is reduced in the bronchiolar epithelium of IPF lungs [52]. Reduction of AEC PGE_2 production as well as a reduction of AEC-mediated fibroblast production of PGE_2 could be additional reasons why injured AECs seem to be unable to stop fibrosis once it becomes aberrant [46].

Fibroblasts

Fibroblasts are the mesenchymal cells primarily responsible for maintaining extracellular matrix in human body tissues. They are also primarily responsible for synthesizing excessive amounts of extracellular matrix proteins such as collagen 1, collagen 3, and fibronectin deposited during IPF. An overabundance of these $CD45^-$ collagen 1^+ cells is a hallmark of fibrotic lung disease. Fibroblasts may accumulate in the fibrotic lung from several sources. Resident lung fibroblasts may expand, AECs may undergo EMT, or circulating bone-marrow-derived fibrocytes may enter the lung and differentiate into fibroblasts. Cell-cell and cell-matrix interactions, as well as a multitude of stimulatory, inhibitory, and differentiation factors, including TGF-β1 and IL-13, can affect the ability of fibroblasts to proliferate and produce matrix proteins [44]. TGF-β1 and IL-13 can exert proliferative and/or differentiative effects [53-58]. TGF-β1 induces expression of α-SMA, acting as a differentiation factor for fibroblast to myofibroblast transformation. Myofibroblasts are instrumental for lung contraction and alveolar collapse; they also produce enhanced amounts extracellular matrix [32, 59, 60]. Collections of myofibroblasts are found in the hallmark lesions of UIP, the fibroblastic foci. Fibroblastic foci are typically found at sites of AEC injury where there is basal lamina destruction and epithelial sloughing and repair. The number of fibroblastic foci is inversely correlated with disease survival and response to therapy [61, 62]. In contrast, fibroblast proliferation and extracellular matrix deposition are inhibited by IL-10 [63], interferon-gamma (IFN-γ) [64, 65], and PGE_2 [66-69]. PGE_2 can inhibit the differentiation of fibroblasts to myofibroblasts induced by TGF-β1, but fibrotic disease can reduce the ability of lung fibroblasts to produce autocrine PGE_2 and may reduce the

ability of fibroblasts to respond to PGE_2 via loss of E-prostanoid (EP) 2 receptors [70, 71].

Fibrocytes

Recent clinical and experimental evidence suggests that mesenchymal cells which circulate within the bloodstream contribute to fibrotic disease. The majority of collagen-producing cells within the fibrotic lesions of a mouse model of bleomycin-induced pulmonary fibrosis that had received bone marrow (BM) transplants from green fluorescent protein (GFP)-expressing donors were GFP-expressing BM-derived cells [72]. These BM-derived cells are likely circulating mesenchymal cell precursors termed fibrocytes [37]. Fibrocytes, originally cultured from leukocyte fractions of human blood, are adherent cells which express both mesenchymal and leukocyte markers [37]. Fibrocytes express leukocyte-associated antigens including common leukocyte antigen CD45, pan-myeloid antigen CD13, hematopoietic stem cell antigen CD34, and class II major histocompatability complex antigens [73, 74]. They proliferate *in vitro* and synthesize the fibroblast products collagen 1, collagen 3, and fibronectin. Fibrocytes migrate to sites of injury in a diverse group of lung diseases [38, 39, 75]. They are recruited to bleomycin- [39] or fluorescein isothiocyanate (FITC)-treated [38] lungs in mouse models of pulmonary fibrosis, and the number of fibrocytes recruited to the lung correlates with fibrotic disease severity [38, 39]. Fibrocytes comprise about 0.5% of peripheral blood leukocytes in normal subjects [73], and that level is increased in patients with IPF [76, 77]. Consistent with a pathogenic role for these cells, adoptive transfer of fibrocytes worsens fibrotic outcomes [78]. The recruitment of fibrocytes to the lung

precedes the development of rampant fibrosis [38, 39]. They rapidly enter sites of tissue injury, synthesize extracellular matrix, and express fibrogenic cytokines such as IL-1β, TNF-α, chemokine (C-C motif) ligand (CCL) 2, CCL12, chemokine (C-X-C motif) ligand (CXCL) 1, PDGF-α, TGF-β1 and (macrophage-colony stimulating factor) M-CSF [37]. Fibrocytes can express α-SMA and can contract collagen gels [79]. Fibrocytes have been shown to be recruited to the lungs by CXCR4, CCR7 and CCR2-mediated signals [38, 39, 80]. Thus, fibrocytes are important effector and regulatory cells in the host response to infection/injury.

Inflammatory cells

Unknown factors causing insult(s) to the lung activate an inflammatory cascade that includes the recruitment of inflammatory cells preceded and followed by the release of a myriad of factors that could impact the quality of injury and the healing response to the injury. Chronic inflammation can lead to an imbalance in the production of chemokines, cytokines and growth factors, and can disrupt cellular recruitment. The identity of the injurious agent affects the resulting immunological response and the proportions of inflammatory cells involved, but it is likely that macrophages and neutrophils play significant modulatory roles in the fibrogenic process at various stages of the disease. Alveolar macrophages (AMs) both promote and limit pulmonary fibrosis [81, 82]. AMs can promote fibrosis by secreting TNF-α, TGF-β1, or IL-13 [83-85], and they are potent producers of proinflammatory chemokines CCL2 and CCL12 [80, 86]. In a mouse model of pulmonary fibrotic disease, AMs have been shown to be "alternatively activated" by Th2 cytokines and chronically recruited to areas of epithelial hyperplasia

and fibrosis [87]. These "alternatively activated" AMs express arginase. Arginase metabolism of L-arginine to L-ornithine, L-proline, and polyamine promotes fibroblast proliferation, collagen production, and ultimately, fibrosis. In contrast, during a normal healing response, AMs can limit fibrosis by releasing PGE_2 or IFN-γ [64, 65, 88]. Although neutrophil numbers in the lower respiratory tract of IPF patients are not elevated, neutrophils in IPF lungs appear to have a more activated phenotype [89-91]. Studies suggest these IPF neutrophils release higher concentrations of oxidants, and thus cause more epithelial cell injury [89].

TGF-β1

TGF-β1 is the most potent profibrotic molecule described to date [92]. TGF-β1 is the most powerful known promoter of extracellular matrix secretion; it causes differentiation of fibrocytes to fibroblasts, and it is the most potent promoter of fibroblast to myofibroblast differentiation [26, 58]. TGF-β1 induction of differentiation to myofibroblasts requires Smad signaling and is dependent on cell adhesion and integrin signaling through focal adhesion kinase [93]. Evidence even suggests that TGF-β1 inhibits epithelial cell proliferation and causes human alveolar epithelial to mesenchymal transition [94]. Active TGF-β1 is difficult to assay in human tissues, but immunohistochemical study has shown that TGF-β1 production is increased in the AECs and macrophages of IPF patients compared to controls [95]. In a mouse model of bleomycin-induced pulmonary fibrosis, microarray analysis of whole lung mRNA demonstrates upregulation of TGF-β1-responsive genes [96]. Overexpression of active

TGF-β1 in the lung leads to a vigorous fibrotic response, and inhibition of TGF-β1 abrogates bleomycin-induced fibrosis [97].

TGF-β1 is synthesized and secreted as an inactive precursor that requires activation for receptor binding and subsequent stimulus of signal transduction pathways. Activation is achieved when mature TGF-β1 is released from a latency-associated peptide [98, 99]. Cleavage can occur via proteases such as plasmin or thrombin, or it can occur through interactions with proteins like avβ6, an integrin highly-expressed in epithelia of poorly-healing wounds [100].

TNF-α

TNF-α is a pro-inflammatory cytokine up-regulated in a variety of different human inflammatory and fibrotic pulmonary pathologies [101, 102]. Its role in fibrosis is controversial, but *in vivo* studies indicate that TNF-α is predominantly a pro-fibrotic effector [102]. TNF-α is known to be important for the recruitment and activation of inflammatory cells by regulating adhesion molecule expression and altering the endothelium. The degree to which inflammation is necessary for human fibrotic disease remains unclear, but a strong inflammatory response is critical to development of subsequent fibrotic disease in most animal models of fibrosis [102]. Induction of TNF-α expression causes fibrosis in rat lungs, neutralization of TNF-α diminishes bleomycin-induced murine fibrosis [101, 103], and over-expression of TNF-α correlates with worse fibrosis in FITC-induced murine fibrosis [104].

CCL2 and CCL12

CCL2 (monocyte chemoattractant protein-1) and CCL12 (monocyte chemoattractant protein-5) are potent chemotactic ligands for chemokine (C-C motif) receptor 2 (CCR2)-mediated recruitment of inflammatory cells and fibrocytes during pulmonary fibrosis [38, 105]. CCL2 expression is increased in the lungs of patients with IPF, and CCL12, a murine homologue of human CCL2, is the CCR2 ligand most responsible for fibrocyte recruitment and fibroproliferation in mice [80, 106]. CCL2 and CCL12 are crucial to the development of pulmonary fibrosis in mice; mice deficient in CCR2 are protected from pulmonary fibrosis [38, 104].

Eicosanoids: leukotrienes and prostaglandins

The actions of leukotrienes (LTs) promote [33, 107, 108], while the actions of prostaglandins (PGs) (specifically PGE_2 and prostacyclin) inhibit [66-68, 109-118], the major pathobiologic features of pulmonary fibrosis including fibroblast chemotaxis, fibroblast proliferation and collagen synthesis.

Derangements of eicosanoid synthesis are present in fibrotic diseases in humans and animal models of pulmonary fibrosis. IPF patients exhibit underproduction of PGE_2 [34] and overproduction of LTs [33]. Levels of both classes of LTs-cysLTs (LTC_4, LTD_4 and LTE_4) and LTB_4 are elevated. AMs are a primary source of LT synthesis and contribute to increased production of LTs in IPF lung homogenates [33]. Animal models of fibrotic lung disease are also characterized by increased LT production following lung injury. Mice genetically deficient in LT production (5-lipoxygenase (5-LO)-/- mice) are protected from bleomycin-induced pulmonary fibrosis [119].

Conversely, reduced PGE_2 levels have been reported in bronchoalveolar lavage (BAL) fluid and AM conditioned media from patients with IPF [120, 121]. Fibroblasts from IPF patients are unable to upregulate the COX-2 enzyme responsible for PG synthesis, and are deficient in PGE_2 production [34, 122, 123]. Fibroblasts isolated from rat lungs following bleomycin-induced pulmonary fibrosis also have reduced PGE_2 synthesis [124]. Pharmacologic (administration of indomethacin) [125] or genetic (gene-deletion of COX-2) [126] reduction in PGE_2 synthesis in the lung augments bleomycin-induced fibrosis in mice.

Eicosanoid synthesis and receptors

All cells are capable of arachidonic acid release and its metabolism to bioactive eicosanoids [127]. Free arachidonic acid is liberated from membrane phospholipids via cytosolic phospholipase A_2 ($cPLA_2$). Once liberated, free arachidonic acid can be metabolized via either of two major pathways [128]. The 5-LO pathway gives rise to LTs primarily in leukocytes [129]. In contrast, the cyclooxygenase (COX) pathway yields prostanoid products, including PGs. This pathway is active in bone marrow (BM)-derived cells as well as structural cells [128, 130, 131]. The initial step in the PG pathway involves the conversion of arachidonic acid to PGH_2 via either the COX-1 or COX-2 enzymes [131-133]. LT generation from arachidonic acid involves 5-LO and the 5-LO activating protein (FLAP). Specific enzymes serve to convert LTA_4 into bioactive cysLTs and LTB_4 [127]. Similarly, specific synthases convert PGH_2 into its bioactive prostanoid products [127].

The actions of PGs and LTs are mediated via their interactions with cell surface

seven transmembrane-spanning receptors that couple to G proteins and subsequent intracellular signaling pathways [134, 135]. For cysLTs, two cognate receptors are known as cysLT1 and cysLT2 [135]. The PGs each have unique receptors as well. There are 4 PGE_2 receptors designated EP1-4 [134]. The functions of the EP receptors are dictated by the intracellular signaling machinery coupled to each receptor. EP2 and EP4 have been identified as the EP receptors which mediate inhibitory actions of PGE_2 on fibroblasts via their ability to increase cyclic adenosine monophosphate (cAMP) [136-139]. EP2 and EP4 have been shown to be down-modulated on fibroblasts during fibrotic responses in mice [139]. Similarly, fibroblasts from IPF lungs have also been shown to have defects in EP2 expression and/or cAMP production [69, 71].

The apparent multifactorial etiology of IPF

Despite growing information about pathobiological elements and ongoing research driven by the need for therapy, the initiating or injurious agents of IPF remain unknown, and it is not understood why the fibrosis is dysregulated and progressive [140]. A wide range of potentially injurious factors have been implicated in the initiation of IPF. In both experimental and occupational settings, exposure to dusts, fibers, and fumes have long been recognized as causing fibrotic lung diseases, but factors such as diagnostic difficulty, the infrequent occurrence of IPF, and variation in susceptibility to exposures have limited the recognition of an association between exposures and IPF [141, 142]. Case-control studies have yielded more information about potential risk-factors. A disproportionate number of patients with IPF have smoked heavily [143, 144]. Population-based studies have linked IPF to older men who work in dust-exposed

occupations, and to the manufacturing industry [18, 145]. Lung mineralogic analysis of IPF patients has revealed excess silica and metals [146, 147]. These studies are among many that lack ideal controls, but they all support the hypothesis that IPF is a heterogenous disorder that is caused by a variety of insults and depends on individual susceptibility. In that vein, genetic variations regulating the processes of epithelial particle uptake, epithelial cell injury, inflammation, and fibrosis are likely crucial to IPF pathogenesis [148]. Abnormalities such as mutations in telomerase, surfactant protein C, or tissue remodeling-related pathways have been associated with IPF, but no single fibrosis susceptibility gene has been identified [142, 148-152].

Viruses as co-factors in the development of IPF

An emerging hypothesis is that occult infections may play a pathogenic role as co-factors for the development of IPF or acute exacerbations. It is possible that the chronic presence of an inflammatory agent like a virus in a genetically susceptible host disrupts the normal healing response thus making the lung highly susceptible to a separate injurious trigger. Viruses are intriguing candidates for a role in IPF because of their ubiquitous prevalence in humans and because of the nature of their lifecycle. Some viruses exist as an antigenic stimulant in the epithelial cells of the lung in an actively replicating and potentially injurious lytic phase while other viruses persist in a latent phase for an entire lifetime. It is interesting to note that some viruses, such as Epstein-Barr virus (EBV), which has been linked to IPF, are viruses that are known to infect the majority of the population at some point in life [153]. This raises the interesting question of why some people may develop IPF in response to this infection while others may not.

Clearly, there is no easy answer, but it is likely that differential host responses to the virus may alter the pathogenesis. For instance, latent EBV infection is most often found in B cells [153]; however, in patients with IPF, EBV can be found in lung tissue, including epithelial cells [154, 155]. Alternatively, stress, drug exposures or immunodeficiency may be responsible for viral reactivation in some patients, but not others. Studies of viral respiratory tract infection in patients with IPF suggest an increased prevalence of past infection [5, 156]. Furthermore, there is some suggestion that IPF patients have been infected with specific subtypes of virus particularly associated with the induction of somatic mutation [156]. Human hepatitis C, adenovirus, and cytomegalovirus infections have also been found in association with IPF.

Hepatitis C

Two studies have suggested a link between infection with the hepatitis C virus (HCV) and IPF. Hepatitis C virus is a small, enveloped, positive-sense single-stranded RNA virus in the Flavivirus family [157]. Replication within hepatocytes causes a form of hepatitis, but the virus may be able to enter other cell types [158]. Ueda *et al*. were able to show a significant difference between the percentage of Japanese IPF patients who had serum antibodies to HCV (28.8%) and the corresponding percentage of control subjects (3.66%) [159]. A subsequent Italian study confirmed that 13% of patients with IPF were seropositive for HCV [160]. In a control group of 4614 blood donors, the prevalence of HCV antibodies was lower (0.3%). However, in a control group of 130 patients with non-interstitial lung disease, HCV antibody prevalence was 6.1%. While this was significantly higher than the blood donor group, the incidence of HCV in

patients with non-interstitial lung disease and IPF were not statistically different. It should also be noted that a British study failed to find an association between IPF and HCV [161]. One possible explanation for these findings is that there may be geographical differences in the prevalence of HCV infection, as the infection is more commonly seen in Japan and Mediterranean countries than it is in northern Europe [160]. Given that HCV is not known to replicate in the lung, it is not clear whether these associations suggest that HCV is pathogenic in IPF, or rather if they indicate that IPF patients develop HCV cross-reactive antibodies.

Adenovirus

Human adenoviruses have been suggested as etiological co-factors in the progression of interstitial lung disease [162-164]. Adenoviruses are medium-sized, non-enveloped, icosahedral, double-stranded DNA viruses. They are relatively resistant to chemical and physical agents, and as a result, they can remain infectious outside of the body for extended periods of time. Adenovirus infections typically cause respiratory symptoms and can be shed for long periods of time post-infection [162]. Kuwano *et al*. examined 19 patients with IPF, 10 patients with interstitial pneumonia associated with collagen vascular disease, and 20 patients with sarcoidosis using nested polymerase chain reaction (PCR) and *in situ* hybridization for the adenovirus gene product, E1A [163]. E1A DNA was present in 3 out of 19 (16%) cases of IPF, in 5 of 10 (50%) cases of interstitial pneumonia associated with collagen vascular disease, and in 2 of 20 (10%) cases of sarcoidosis [163]. While these data are not suggestive of a correlation between adenovirus infection and pulmonary fibrosis, Kuwano *et al*. found that the incidence of

E1A DNA was considerably higher in patients who had been treated with corticosteroids (67%) compared to those patients left untreated (10%). This finding raises the interesting possibility that corticosteroids, a common therapy for IPF, may make patients more susceptible to adenovirus infection or reactivation. However, studies investigating the titre of anti-adenoviral immunoglobulin (Ig) G in IPF patients have failed to demonstrate an elevation above normal [165]. Despite this, it is important to note that E1A has been shown to upregulate production of TGF-β1 and to induce lung epithelial cells to express mesenchymal markers [166]. This mechanism has been implicated in the architectural remodeling that occurs in chronic obstructive pulmonary disease (COPD). It is possible that a similar mechanism could contribute to extracellular matrix deposition and remodeling in those IPF patients who may also have adenovirus infections.

It is also conceivable that adenovirus infections could serve as exacerbating agents for patients with established lung fibrosis, although it is difficult to determine the frequency of this happening from published clinical literature. Furthermore, recent studies using an animal model of FITC-induced fibrosis were unable to demonstrate significant exacerbation of FITC-fibrosis within the first 7 days post-mouse adenoviral infection [167]. One note of caution in the interpretation of these experiments, however, is that human and mouse adenoviruses do show different tropisms, with human adenoviruses being predominantly respiratory pathogens.

Human cytomegalovirus (HCMV)

HCMV, a betaherpesvirus, is a widespread opportunistic pathogen that persists in healthy individuals [168]. HCMV infects the respiratory tract, and it has been

evaluated with regards to IPF. Dworniczak *et al*. studied 16 patients, newly diagnosed with IPF and never treated, compared to 16 adult healthy volunteers [169]. HCMV DNA copy number in BAL cells, blood leukocytes, and serum was calculated by real-time PCR, and the prevalence of the HCMV DNA positive subjects in the patient group (75%) did not differ significantly from the prevalence of positive subjects in the control group (69%). IPF patients did show significantly higher DNA copy numbers in their blood compared to controls, however [169]. Also, the viral copy number in the BAL cells of both IPF patients and healthy volunteers was elevated relative to respective viral copy numbers in blood leukocytes suggesting an important role for the lungs (perhaps as a viral reservoir) in the pathobiology of HCMV. Consistent with this idea, a subsequent study by Tang *et al*. did note higher levels of HCMV DNA in IPF lung tissue compared to control samples [170]. Similarly, in a study by Yonemaru *et al*., HCMV IgG and complement fixation titers were found to be elevated in the serum of patients with IPF when compared to several other disease-specific controls [165]. In a retrospective study of lung transplant recipients, 102 patients were screened by urine test for evidence of HCMV infection on the day of transplant. Only 5 patients were found to be HMCV+ prior to transplant, and all 5 of the patients were IPF patients [171]. Despite testing positive, none of these 5 patients exhibited symptoms of HCMV disease suggesting that viral infections in this population can be occult. The increased incidence of HCMV infection prior to transplant correlated with an increased risk of HCMV infection post-transplant as well. In sum, there is evidence that suggests an association between the incidence of HCMV infection and the incidence of IPF, but a mechanism by which HCMV may affect fibrosis remains to be elucidated.

Epstein-Barr virus (EBV)

The virus that has been associated most strongly with IPF is EBV. EBV is a gammaherpesvirus that is present in all populations, infecting more than 95% of humans within the first decades of life [172]. An association between EBV infection and IPF was first established when elevated levels of IgA and IgG against EBV antigens were measured in a serological study of 13 patients with IPF [173]. In contrast, 12 patients with interstitial lung disease of known cause had normal EBV serological profiles. This finding led to further research on EBV in the context of IPF. An immunohistochemical study indicated that EBV replicates within epithelial cells of the lower respiratory tract in IPF patients [174]. Consequently, Stewart *et al.* sought to confirm the presence of EBV DNA in the lung tissue of IPF patients using PCR. They found that EBV was present in the lungs of patients with IPF at a significantly higher percentage (48%) than in the lungs of control subjects (14%) [175].

A couple of studies have associated the presence of active and latent EBV markers with IPF. Kelly *et al*. investigated the occurrence of productive EBV replication by analyzing for the presence of a EBV gene rearrangement termed WZhet [176]. 61% of EBV DNA-positive lung tissue biopsies from IPF patients were positive for WZhet. Buffy coat analysis for WZhet was positive in 16 of 27 IPF patients compared to none of 32 lung transplant recipients and 1 of 24 normal blood donors. Tsukamoto *et al.* then determined that the presence of EBV latent membrane protein 1 (LMP1) is linked with more rapid disease progression. From a group of 29 patients, they found that patients positive for LMP1 died more quickly than patients who tested negative for EBV [177].

It should be noted that not all studies have found an association between EBV and

IPF. In 1997, Wangoo *et al*. published findings contrary to previous reports when they did not detect any EBV DNA in the lungs of IPF patients [178]. Also, in 2005, an Italian study by Zamo *et al*. failed to find evidence of either EBV or human herpesvirus (HHV)-8 DNA in their tissue banks of IPF samples [179]. Whether these discrepancies reflect geographical distribution, technical sensitivities, or disease heterogeneity is still unclear.

Although EBV had been detected with more frequency in the lungs of IPF patients than in the lungs of control patients in most previous studies, many members of each IPF cohort analyzed did not test positive for EBV infection at all. Tang *et al.* went on to test the hypothesis that at least one herpesvirus could be detected in the lungs of all IPF patients. They identified one or more of four herpesviruses—EBV, HCMV, HHV-7, and HHV-8—in 32 of 33 patients with IPF and in 9 of 25 controls [170]. They found two or more herpesviruses in 19 of 33 IPF patients and in 2 of 25 controls. These data strongly support the notion that at least one herpesviral infection accompanies the development of IPF.

Tang *et al.* draw other conclusions from their study that suggested susceptibility to viral infection and IPF depends on a genetic or acquired predisposition. Co-infection occurred more frequently in patients with the sporadic form of IPF compared to those with the familial form. Familial IPF is characterized by the incidence of IPF in two or more members of an immediate family [180]. This led the authors to suggest that a patient with familial IPF may require less viral influence to trigger a progressive fibrotic response than a patient with sporadic IPF. In addition, Tang *et al*. note that the increased frequency of HHV-8 in the lungs of the IPF cohort is particularly interesting. In the United States, HHV-8 infection is predominantly found in patients with human

immunodeficiency virus (HIV) infection and Kaposi's sarcoma [181], and all of the subjects tested negative for HIV in this study.

Rationale for mouse models of fibrosis

Collectively, the analyses of IPF lung tissue above create a rationale to study the association between viral infections and the occurrence of IPF, but do not provide evidence for a causal relationship between viruses and IPF. Demonstrating causation in humans requires detection of a virus in the lungs prior to clinical manifestations of IPF (which is clinically implausible) or evidence that an anti-viral therapy confers anti-fibrotic effects. The latter has been attempted with some success in a limited number of case studies, but no large trials have been conducted to date [170, 182]. While at least 4 different viruses have been correlated with IPF, the most striking observations have linked EBV infection of lung tissue with the presence of IPF. Again, it is important to note that the pathogenesis of IPF is complex and multifactorial. In reality, IPF is likely a spectrum of diseases that result from a variety of genetic abnormalities and/or environmental factors. As mentioned above, the data regarding the association of viruses, even EBV, with IPF is controversial. What is particularly intriguing, however, is that these clinical observations are somewhat strengthened by emerging evidence in animal models which demonstrate that gammaherpesvirus infections can be linked to the development of, or the exacerbation of, experimentally-induced fibrosis. In addition, murine models of pulmonary fibrosis have enabled identification of pathogenic cells and mediators that are believed to be important in human fibrotic disease, and they have facilitated further exploration of a pathogenic role for viruses in humans [183].

Murine models of fibrosis

A variety of murine models have been developed to study the pathobiology of fibrosis, and they have recently been reviewed [184].

Bleomycin

The bleomycin model of pulmonary fibrosis is the best-characterized murine model in use today. The drug was originally isolated from *Streptomyces verticillatus* [185]. This antibiotic was subsequently found to be effective against squamous cell carcinomas and skin tumors [186]; however, its usefulness as an anti-neoplastic agent was limited by dose-dependent pulmonary toxicity resulting in fibrosis [187]. Bleomycin has been shown to induce lung injury and fibrosis in a wide variety of experimental animals including mice, rats, hamsters, rabbits, guinea pigs, dogs, and primates over a range of doses induced via intraperitoneal (i.p), intravenous (i.v.), subcutaneous, or intratracheal (i.t.) delivery [187]. The delivery of bleomycin via the i.t. route (generally 1.25–4 U/kg, depending on the source) has the advantage that a single injection of the drug produces lung injury and resultant fibrosis in rodents [188-190]. I.t. delivery of the drug to rodents results in direct damage initially to AECs. This event is followed by the development of neutrophilic and lymphocytic pan-alveolitis within the first week [191]. Subsequently, alveolar inflammatory cells are cleared, fibroblast proliferation is noted, and extracellular matrix is synthesized [192]. The development of fibrosis in this model can be seen biochemically and histologically by 14 days after bleomycin administration with maximal responses generally noted around 21-28 days after bleomycin administration

[192-195]. The accumulation of collagen in the lung is measured both by histological and biochemical techniques, most notably via accumulation of hydroxyproline, which is almost totally derived from collagen in the lung and thus serves as a surrogate for whole lung collagen content [196]. Beyond 28 days, however, the response to bleomycin is more variable. Original reports suggest that bleomycin delivered intratracheally may induce fibrosis that progresses or persists for 60–90 days [190, 197, 198]; however, other reports demonstrate a self-limiting response that begins to resolve after this period [195, 199, 200].

The fibrotic response to bleomycin in mice is strain-dependent. C57Bl/6 mice are more susceptible to bleomycin-induced fibrosis than are BALB/c mice [192, 201]. This likely reflects strain-dependent differences in the expression of the inactivating enzyme, bleomycin hydrolase [202]. The advantages of the bleomycin model are that it is well characterized, that it has clinical relevance, and the fact that multiple delivery routes are possible for the induction of fibrosis. The disadvantage is that the disease may be self-limiting in mice.

FITC

The FITC-induced model for pulmonary fibrosis was originally described in 1995 by Roberts *et al*. [203] and involved the i.t. administration of FITC (0.007 mg per g body weight dissolved in phosphate-buffered saline (PBS)) delivered to BALB/c mice. Instillation of FITC resulted in a marked infiltration of mononuclear cells and neutrophils within the lung interstitium centered primarily around respiratory bronchioles with focal evidence of edema and AEC hyperplasia. Protein leak was also noted in the BAL fluid

within the first week indicating acute lung injury. Several months post-FITC, patchy, focal destruction of the normal lung architecture with interstitial fibrosis was noted. Anti-FITC-specific antibodies were detected in treated mice by 7 days post-FITC and persisted for at least 6 weeks, suggesting that the immune response to this hapten may be crucial to the development of the disease [203].

The model was further characterized by Christensen *et al*. who demonstrated that both C57Bl/6 and BALB/c mice were susceptible to FITC-induced fibrosis, with BALB/c mice showing a greater degree of fibrosis to a given dose of FITC [204]. In addition, the protocol was modified slightly. 14 mg of FITC was dissolved in 10 ml of PBS and the solution was vortexed and sonicated for 30 seconds at 50% power prior to the instillation of 50μl per mouse. More extensive sonication resulting in a finer particulate being dispersed to the lung results in significantly more acute lung injury and increased mortality [204]. However, our laboratory has noted that the effective dose of FITC can vary up to 3-fold depending on the lot (Moore laboratory, unpublished observations). Similar to findings in the bleomycin model, FITC was demonstrated to induce fibrosis by 21 days in CD4-depleted mice, severe combined immunodeficient (SCID) mice, and recombinase activating gene knockout mice despite the abolition of the anti-FITC Ig response [204] demonstrating that specific T cell immunity is not required for the development of FITC-induced fibrosis. More recent investigations utilizing the FITC model of fibrosis have demonstrated that like bleomycin, the fibrotic response to FITC is dependent on CCR2 signaling [104]. Release of CCL12, and to a lesser extent CCL2 [205], in the injured lung results in the recruitment of CCR2-expressing circulating

fibrocytes [206] to the lung and the augmentation of fibrosis. FITC also induces the production of IL-13 in the lung, which is essential for the fibrotic response as well [53].

One distinct advantage of the FITC model is the ability to visualize the areas of the lung where deposition occurs via immunofluorescence imaging for the characteristic green color of the FITC. Fibrotic changes have only been noted in areas of FITC deposition [203, 204] suggesting that focal injury to the alveolar epithelial cells, and perhaps persistent conjugation of the FITC to stable proteins such as elastin, may perpetuate the remodeling response [203]. A second advantage to the FITC model is that the response is persistent (for at least 6 months) and does not appear to be self-limiting in the way that bleomycin has been reported to be [207]. This persistence makes the FITC model ideal for long-term studies such as viral-induced augmentation or exacerbation of fibrosis.

Other mouse models of pulmonary fibrosis

There are other well-characterized mouse models of pulmonary fibrosis induced by irradiation and silica, but the development of fibrosis in those models takes place over a longer time frame than in the bleomycin- and FITC- induced fibrosis models [184]. Due to those time restrictions, we chose not to use the irradiation and silica models for our studies.

Murine gammaherpesvirus-68 (γHV-68)

The human herpesviruses identified to be prevalent in IPF lung tissue have limited infection capability in mice. Thus, investigators have utilized a natural murine pathogen

called murine gammaherpesvirus-68 (γHV-68), that has been characterized as genetically and biologically closely related to human gammaherpesviruses. γHV-68 is a large, double-stranded DNA virus, and its genome is largely colinear with EBV and HHV-8 [208]. There is evidence that these viruses each infect the respiratory tract and can persist in B cells as well as lung epithelial cells [208-212]. γHV-68, EBV, and HHV-8 are all members of the *Gammaherpesvirinae* subfamily because of their ability to establish life-long latency in a host [213]. Infection of mice by the i.n. route results in acute viral replication in AECs [214]. The lytic infection is controlled within 10-12 days, although low-level infectious virus persistence in the lung can be demonstrated by polymerase chain reaction (PCR) or *in situ* hybridization [214]. Macrophages also harbor latent γHV-68 [215], and fibroblasts are permissive for γHV-68 replication *in vitro* [216] γHV-68 encodes several gene products that have cellular homologs, including homologs of cyclin D, Bcl-2, and the IL-8 receptor [208].

γHV-68 infection is a cofactor for bleomycin-induced murine fibrosis

In 2002, Lok *et al.* used γHV-68 to demonstrate that gammaherpesviruses could serve as cofactors in the development of pulmonary fibrosis [217]. BALB/c mice infected intranasally with γHV-68 one week prior to intratracheal administration with the fibrotic stimulant bleomycin later developed pulmonary fibrosis even though BALB/c mice are normally resistant to bleomycin. Mice infected with γHV-68 but not challenged with bleomycin did not develop fibrosis. The gammaherpesvirus infection alone was not sufficient to cause fibrosis, but Lok *et al.* proposed that a viral infection made the previously protected lungs susceptible to fibrotic disease upon the event of an exogenous

injury. These results are enticing, but it should be noted that the bleomycin was delivered during the peak of lytic viral infection. It is difficult to infer from these studies whether chronic latent infection with γHV-68 might also predispose the lung to subsequent fibrotic responses. Also, the mechanism(s) for how γHV-68 infection augmented the subsequent fibrotic response to bleomycin were not defined. These are questions that are addressed in my studies.

γHV-68 infection in Th2-biased mice causes multi-organ fibrosis

Ebrahimi *et al*. showed that γHV-68 causes fibrosis in IFNγ receptor (R)-/- mice [218]. IFN-γ is a T helper type 1 (Th1) cytokine with anti-viral and anti-fibrogenic properties. It down-regulates the expression of both type 1 and type 3 collagens and fibronectin [219-222]. The cytokine profile of IFNγR-/- mice is Th2-biased resulting in a cytokine imbalance similar to that observed in the lungs of IPF patients [223]. This study suggests that a herpesvirus infection delivered to lungs skewed towards a profibrotic cytokine environment is sufficient for fibrogenesis. In this case, the fibrogenesis was not limited to the lung only. The mice developed multi-organ fibrosis (liver, lung, spleen and lymph nodes). The development of multi-organ fibrosis correlated with an overproduction of pro-fibrotic mediators such as TNF-α, TNF-β, IL-1β, TGF-β1, lymphotactin, and macrophage inflammatory protein (MIP)-1β. These mediators were elevated on day 14 after infection whereas the anti-fibrotic chemokines IP-10 (CXCL10) and MIG (CXCL9) were significantly reduced. The authors noted that γHV-68 gene expression may have influenced the cytokine imbalance. These results are fascinating in light of the fact that γHV-68 infection in wild-type mice shows no signs of causing multi-

organ fibrosis and suggest that the outcome of viral infection may be critically dependent on the cytokine milieu at the time of infection.

Mora *et al*. extended studies in the IFNγR-/- model of herpesvirus-induced fibrosis by characterizing the disease seen in the lungs and examining possible pathogenic effects of the virus that contribute to fibrosis. They showed that γHV-68 induces epithelial damage and inflammatory responses leading to alveolar remodeling and ultimately to unresolving progressive interstitial fibrosis resembling human IPF [182]. More recently, Mora *et al*. published data implicating AMs as integral profibrotic effectors in IFNγR-/- mice [87]. These studies suggested that AMs were chronically recruited to areas of epithelial hyperplasia and fibrosis and that these AMs displayed signs of alternative activation, a process known to be driven by Th2 cytokines. In addition, there is evidence that microvascular injury has a role in the pathogenesis of IPF [224], and Mora *et al*. detected viral-induced vasculitis accompanied by red blood cell extravasation compatible with hemorrhage. With regards to viral-induced vasculitis, it is interesting that Magro *et al*., who proposed microvascular injury as a pathogenic mechanism for IPF, also found evidence of CMV and parvovirus B19 infection in IPF patients. In fact, Magro speculated that endotheliotropic viral infections, such as CMV and B19 may be precursors for the microvascular injury which is noted in IPF [224]. Similar mechanisms may be responsible for the γHV-68-induced damage noted in the studies by Mora *et al.* γHV-68 has previously been reported to cause vascular damage [225]. Finally, Mora notes enhanced expression of TGF-β1 in the γHV-68-infected IFNγR-/- mice, and it is likely that this pro-fibrotic cytokine may make a significant contribution to the fibrosis in this model [182]. In wild-type mice, however, IFN-γ

signaling would be expected to inhibit transcription of the TGF-β gene [226].

The authors attribute some of the TGF-β1 dysregulation to epithelial cell damage. Interestingly, type 2 alveolar cells are a target of γHV-68, and Mora *et al*. speculate that epithelial cell infection and injury may be triggering surfactant abnormalities as well as dysregulated epithelial cell repair [182]. An association between the frequency of polymorphic variants of surfactants and IPF has been documented previously [227]. Furthermore, another series of studies on IPF has found some evidence that a link between EBV and p53 expression leads to modifications in epithelial cell repair and apoptosis [228, 229]. Flano *et al*. have reported chronic low level reactivation of γHV-68 in the lung [230]. It is possible that reactivation is a repetitive trigger in the setting of the IFNγR-/- mice that contributes to the progressive disruption of lung epithelial cells and unresolving fibrosis. In support of this, prevention of chronic reactivation of γHV-68 to the lytic phase through the use of anti-viral drugs reduces fibrosis in IFN-γR-/- mice [230, 231].

γHV-68 as an exacerbating agent for established fibrosis

While most IPF patients have a slow, progressive disease, some patients have an acute deterioration in function which carries a poor prognosis. In the placebo arm of a study of 32 patients who died from IPF-related causes, Martinez *et al*. reported that 47% suffered an acute deterioration, and 27% of the acute deteriorations were associated with infection [232]. McMillan *et al*. from our laboratory were recently able to model acute exacerbations of fibrosis in a murine model of FITC-induced pulmonary fibrosis. Wild-type mice infected with γHV-68 after the establishment of fibrosis (day 14) developed a

significantly worse fibrotic response than fibrotic mice that were mock-infected with saline [167]. The recruitment of fibrocytes to the lung and increased CCR2 ligand production were associated with viral exacerbation of FITC-induced fibrosis [167].

This finding is especially interesting in light of the work done in Th2-biased mice discussed above because γHV-68 exacerbation of fibrosis in wild-type mice occurred despite a strong Th1-biased anti-viral immune response. In fact, γHV-68 was able to exacerbate FITC-induced fibrosis even in Th2-deficient (IL-4 and IL-13-/-) mice [167]. In these experiments looking at exacerbation of established fibrosis, γHV-68 infection was lytic. The pro-fibrotic actions of γHV-68 may vary greatly depending on whether the infection precedes or follows the fibrotic stimulus, and the contribution of Th2 cytokines to the pathogenesis may vary as well depending on the timing of infection. I was pleased to participate in these studies which examined the ability of γHV-68 to exacerbate established fibrosis.

Concluding remarks

While the studies that have been done with γHV-68 can only suggest that similar human viruses found in the lungs of IPF patients have a pathogenic role in fibrosis, they currently offer our best approach to investigate what role viruses play in IPF. The work of Lok, Ebrahimi, and Mora provide clues about the role of lytic γHV-68 infection in subsequent fibrosis. These results are enticing, but they leave many questions to be addressed about γHV-68 infection that precedes or follows fibrosis. First, it is difficult to infer from these studies whether chronic latent infection with γHV-68 might also predispose the lung to subsequent fibrotic responses or even whether latent γHV-68

infection can be established in the lungs. Since most humans harbor latent herpesviruses for their lifetime, and IPF occurs at advanced age, it will be important to determine what effect, if any, long-term latent γHV-68 has on fibrosis in naïve mice. A latent infection does not appear to create a Th2 bias equal to that of IFN-γR-/- mice, but any bias may predispose a latently-infected individual to a fibrotic trigger from an unrelated factor.

The effect of latent γHV-68 infection on the recruitment of cells and the production of profibrotic factors should be investigated in naïve mice and in EBV-infected human lungs. If γHV-68 causes the recruitment of fibrocytes as suggested by McMillan *et al*., then this mechanism may be able to explain the enhancement of fibrosis noted by Lok *et al*. when the infection preceded the administration of bleomycin by 7 days [217]. In fact, if γHV-68 infection in the lung is associated with prolonged recruitment of fibrocytes even after the virus has established latency, this could explain why herpesviral infections may predispose persons to an enhanced fibrotic response upon a second challenge.

It will be helpful to more carefully study the cell types that harbor long-standing viral infection because the prolonged injury caused to such a cell type is likely responsible for increasing susceptibility to fibrosis. Flano *et al*. found that long-term latency in the lung is maintained primarily in B cells, but they also showed that γHV-68 maintains persistent replication in the lung at least 3 months after infection suggesting that cell types besides B cells may be reinfected [230]. In fact, Stewart *et al*. have suggested that lung epithelial cells may be long-term reservoirs of persistent viral infection [211]. Given that there is evidence that γHV-68 can reactivate, it seems important to better understand the chronic reactivation that can occur during herpesviral

infections. It is possible that repeated activation may lead to repeated rounds of epithelial cell damage, cytokine release and fibrocyte recruitment that creates progressive pathology. Additionally, epigenetic changes caused by infection should also be explored. Genetic deficiencies in LT production and expression of the CCR2 receptor have been shown to be protective against fibrosis in animal models independent of viral infection [104, 119]. As viral infections are known to induce the production of both LTs and chemokines, it may be important to understand how significant such mediators are to a virus-induced predisposition to fibrosis. It is also possible that the additional inflammatory cells recruited by the lytic or latent virus may alter the fibrotic milieu.

In summary, evidence from both clinical studies and animal models suggest that viruses, especially gammaherpesviruses, may be co-factors for the development or exacerbation of lung fibrosis. Based on the information reviewed above, the goal of my studies was to determine whether a preceding viral infection could augment the development of pulmonary fibrosis. Specifically, we wanted to model a preceding latent lung infection with a murine gammaherpesvirus to determine how such an infection could alter the development of lung fibrosis. Our results suggest a pathogenic role for the recruitment of fibrocytes and elucidate the roles that CCL2, CCL12, cys LTs and TGF-β1 may play in the exaggerated fibrotic responses.

CHAPTER TWO:

MATERIALS AND METHODS

Mice

C57Bl/6 and 5-LO-/- mice backcrossed to a C57Bl/6 background were purchased from The Jackson Laboratories, Bar Harbor, ME. Due to limited availability of 5-LO-/- mice backcrossed to a C57Bl/6 background, however, some experiments were performed with 5-LO-/- (129-Alox5$^{tm1Fun/J}$) [233] and strain-matched (129SvEv) wild-type (WT) mice bred in the University of Michigan Unit for Laboratory Animal Medicine from breeders originally obtained from Jackson Laboratories. Both female and male mice were used, and they were studied between 2-5 months of age. Mice were housed under specific pathogen-free conditions and were monitored daily by veterinary staff. Mice were euthanized by CO_2 asphyxiation.

Viral infection

Mice were anesthetized with ketamine and xylazine. 5 x 10^4 plaque forming units (PFU) of γHV-68 clone WUMS (American Type Culture Collection, Manassas, VA), 5 x 10^4 PFU ΔORF72 (a v-cyclin mutant virus described previously [234]), or 5 x 10^4 PFU of v-cyclin marker rescue virus ("MR") were suspended in 20 µl saline and delivered i.n. to each mouse. The "MR" virus was generated by transfection of v-cyclin viral DNA with a

LacZ reporter, thus reconstituting an essentially "wild-type" virus for use as a control with ΔORF72. Other mice were mock-infected with 20 μl of saline.

In experiments where epithelial cells were infected *in vitro*, cells were infected with γHV-68 at a multiplicity of infection (MOI) of 0.005 or 0.05 for 3d or 7d before supernatants were harvested for a luciferase assay used to measure active TGF-β1.

In experiments where fibrocytes or fibroblasts were infected *in vitro*, 4×10^5 fibrocytes or fibroblasts per well were infected with γHV-68 at a MOI of 0.01 or 0.1 for 48h. RNA was isolated from some wells to analyze viral gene expression by real-time reverse transcriptase (RT)-PCR, and cell lysates were made from others wells. 10 μg of protein from each lysate was run and analyzed by Western blotting.

Virus plaque assay

3T12 cells (American Type Culture Collection) were cultured in DMEM with 4% fetal calf serum (FCS), harvested using trypsin digestion, and added to 12-well plates at 8.33×10^4 cells per well. The cells were incubated overnight to confirm <70% confluence. Two lung lobes of infected mice were homogenized in 2 ml DMEM with Complete® protease inhibitor cocktail (1 tablet/7 ml media; Roche, Mannheim, Germany) and Triton X-100 (7μl/7ml media; Sigma, St. Louis, MO) and centrifuged at 1500 rpm for 5 minutes at 4°C. Dilutions were made in DMEM from the supernatant, and the pellet was discarded. 250 μl of each dilution was placed on the 3T12s; the plates were rocked 10 times and then incubated at 37°C and rocked every 15 min for one hour. The inoculum was not removed, and an overlay of 0.75% carboxymethylcellulose and 2x MEM with 10% FCS was placed on cell monolayers. The plates were incubated at 37°C.

On day 6, the overlay was removed, and the cells were fixed and stained with 70% methanol and 0.35% methylene blue for plaque enumeration.

Semiquantitative real-time RT-PCR

Semiquantitative real-time RT-PCR was performed on an ABI Prism 7000 thermocycler (Applied Biosystems, Foster City, CA). Gene-specific primers and probes were designed using Primer Express software (PerkinElmer/PE Applied Biosystems). The sequences for all primers and probes used can be found in Table 1. The reaction mixture contained 300 ng of RNA, 12.5 µl of TaqMan 2x Universal PCR Master Mix (Applied Biosystem; Roche), 0.625 µl of 40x MultiScribe and RNase Inhibitor Mix (Applied Biosystem; Roche), 250 nM FAM probe, and forward and reverse primers at 300 nM in a final volume of 25 µl. For each experiment, samples from mice were run in duplicate or triplicate. The average cycle threshold (C_T) was determined for each mouse from a given experiment. Relative gene expression (using the formula $2^{-\Delta\Delta CT}$) was calculated using the comparative C_T method [235], which assesses the difference in gene expression between the gene of interest and an internal standard gene (β-actin) for each sample to generate the C_T. In some experiments, the average of the control sample was set to 1, and the relative gene expression for each experimental sample was compared with that.

Table 1. Primers and probes for semiquantitative real-time RT-PCR

Gene	Oligo	Sequences
M3	F. primer	AGTGGGCTCACGCTGTACTTGT
	R. primer	TGTCTCTGCTCACTCCATTTGG
	Probe	CATGGGCAAGTGTTCATCTTAGCC
DNApol (ORF9)	F. primer	ACAGCAGCTGGCCATAAAGG
	R. primer	TCCTGCCCTGGAAAGTGATG
	Probe	CCTCTGGAATGTTGCCTTGCCTCCA
gB (ORF8)	F. primer	CGCTCATTACGGCCCAAA
	R. primer	ACCACGCCCTGGACAACTC
	Probe	TTGCCTATGACAAGCTGACCACCA
CCL2	F. primer	GGCTCAGCCAGATGCAGTTAAC
	R. primer	CCTACTCATTGGGATCATCTTGCT
	Probe	CCCCACTCACCTGCTGCTACTCATTCAC
CCL12	F. primer	TGGCTGGACCAGATGCG
	R. primer	GACGTGAATCTTCTGCTTAACAACA
	Probe	TGAGCACCCCAGTCACGTGCTGTTA
cysLT1	F. primer	CTGAGGTACCAGATAGAGGCTGATC
	R. primer	CTTGGTGCCTTGGAGGTACATT
	probe	TTCCTGCTTTGGCTTCTCAAGGGCTG
cysLT2	F. primer	TGCTGAGTGTGGTGCGTTTC
	R. primer	CCAGGCACTCCTGACACTGGTG
	probe	ACAGTCCACCCCTTCCGGATGTTCC
β-actin	F. primer	CCGTGAAAAGATGACCCAGATC
	R. primer	CACAGCCTGGATGGCTACGT
	Probe	TTTGAGACCTTCAACACCCCCAGCCA

FITC and bleomycin injections

FITC inoculation was performed i.t. as described previously [104]. Briefly, mice were anesthetized with ketamine and xylazine. The trachea was exposed and entered with a needle under direct visualization. FITC (28 mg, Sigma) was dissolved in 10 ml of sterile PBS, vortexed extensively, and sonicated for 30 seconds. This slurry was

transferred to multiuse vials and vortexed extensively before each 50 µl aliquot was removed for i.t. injection using a 23-gauge needle. For bleomycin experiments, a single 50-µl injection containing 0.025 U of bleomycin (Sigma) diluted in normal saline was injected i.t. as above.

Hydroxyproline assay

Total collagen measurements were made via hydroxyproline assay [104]. Hydroxyproline is a useful surrogate for collagen content because elastin is the only mammalian protein besides collagen that contains hydroxyproline. Every third amino acid in collagen is hydroxyproline, and low levels of hydroxyproline are found in elastin. Mice were euthanized and perfused via the heart with saline. Individual lung lobes were removed, taking care to avoid the large conducting airways. The isolated lobes were homogenized in 1 ml of PBS, and hydrolyzed by the addition of 1 ml of 12 N hydrochloric acid (HCl). Samples were then baked at 110° C overnight. Aliquots (5 µl) were then assayed by adding chloramine T solution for 20 min followed by development with Erlich's reagent at 65°C for 15 min. Absorbance was measured at 550 nm, and the amount of hydroxyproline was determined against a standard curve generated using known concentrations of hydroxyproline standard (Sigma).

Collagenase digests of whole lung

Lungs were excised, minced, and enzymatically digested for 30 min using 15 ml/lung of digestion buffer (complete media; 1 mg/ml collagenase, Roche; and 30 µg/ml DNase, Sigma). The cell suspension and undigested fragments were further dispersed by

repeated passage through the bore of a 10 ml syringe without a needle. The total cell suspension was pelleted and any contaminating erythrocytes were eliminated by lysis in ice-cold NH_4Cl buffer (0.829% NH_4Cl, 0.1% $KHCO_3$, and 0.0372% Na_2 EDTA, pH 7.4). The pellet was resuspended in 5 ml of SFM and dispersed by 20 passages through a 10 ml syringe. The dispersed cells were filtered through a Nytex filter (Sefar, Depew, NY) to remove clumps. The total volume was brought up to 10 ml with complete media. An equal volume of 40% Percoll (Sigma) in complete media was added, and the cells were centrifuged at 3000 rpm for 20 min with no brake. The cell pellets were resuspended in complete media and leukocytes were counted on a hemocytometer in the presence of trypan blue. Cells were >90% viable by trypan blue exclusion.

Flow cytometry

Murine leukocytes recovered from collagenase digests or BAL were incubated for 15 min on ice with Fc block (1:100 dilution, clone 24G2; BD PharMingen, San Diego, CA) before surface staining with CD45-PerCPCy5.5 (1:500 dilution, BD PharMingen) followed by fixation/permeabilization using the BD PharMingen Cytofix/cytoperm kit according to manufacturer's instructions. Cells were then blocked with goat IgG [(1:2000 dilution) Jackson ImmunoResearch, West Grove, PA] before staining with rabbit anti-mouse collagen 1 (1:400 dilution, Rockland Immunochemicals, Gilbertsville, PA) or rabbit IgG (1:4500 dilution, Jackson ImmunoResearch) that served as an isotype control. Experiments done to assess fibrocyte recruitment to FITC were conducted by staining with a different collagen 1 antibody (rabbit anti-mouse; Accurate Chemical & Scientific, Westbury, NY) using secondary Ab [donkey anti-rabbit PE-secondary (1:200 dilution,

Jackson ImmunoResearch)] alone as a control. Cells were analyzed on the flow cytometer (FACScan; BD Biosciences, Mountain View, CA).

Human fibrocytes were stained with anti-human CD45 FITC (BD Biosciences) and following a blocking step, collagen was stained using anti-human pro-collagen I antibody from Santa Cruz Biotechnology (Santa Cruz, CA) followed by a donkey anti-goat PE secondary antibody from Jackson ImmunoResearch.

Histology

Lungs from mice were inflated with 10% neutral buffered formalin, fixed overnight and dehydrated in 70% ethanol prior to paraffin embedding. Thin sections of lung were then cut and stained with hematoxylin and eosin.

ELISA

CCL2, CCL12, TNF-α, and TGF-β1 were measured in lung homogenates or alveolar cell supernatants by specific ELISA using DuoSet® ELISA Development System kits from R&D Systems (Minneapolis, MN). The measurements for cysLTs and LTB_4 were performed on lung homogenates and alveolar cell and fibrocyte supernatants according to manufacturer's instructions using EIA kits obtained from Cayman Chemical (Ann Arbor, MI). The lower limit of detection was 10 pg/ml. Lipids were first extracted from lung homogenates using C_{18} Sep Pak cartridges according to our previously published protocol [119]. In some fibrocyte, fibroblast, AM, or AEC cultures, 5 μM Ca^{2+} ionophore (A23187) was added for 1h in (serum-free media) SFM as a maximal stimulus for LT synthesis.

AEC purification

Type II AECs were isolated using dispase and DNase digestion of lower lungs as previously described [236, 237]. Bone marrow-derived cells were removed via anti-CD32 and anti-CD45 magnetic depletion. Mesenchymal cells were removed by overnight adherence in a petri dish. After this initial plating, AECs were isolated from the remainder of the non-adherent cells by putting the non-adherent cells on fibronectin-coated tissue culture plates. Purity was >94% as determined by staining with cytokeratin and vimentin.

Fibroblast and fibrocyte isolation

Murine lungs were perfused with 5 ml of normal saline and removed using aseptic conditions. Lungs were minced with scissors in complete media. Lungs from a single animal were placed in 15 ml of media in tissue culture flasks. Mesenchymal cells were allowed to grow out of the minced tissue, and when cells reached 70% confluence they were passaged using trypsin digestion. Mesenchymal cells were grown for 14 days before being harvested by trypsin digestion. Cells were stained with anti-CD45 antibodies coupled to magnetic beads (Miltenyi Biotec; Bergisch Gladbach, Germany). Labeled cells were then sorted by binding the cell population to LS-positive selection columns using a SuperMACS apparatus (Miltenyi Biotec) according to manufacturer's instructions. Cells were then washed extensively. Fibrocytes ($CD45^+$ cells) were retained on the column and were removed by flushing the column with buffer once it is removed from the magnetic field. Fibroblasts ($CD45^-$ cells) were isolated by collecting the orginal flow-through after magnetic separation.

For isolation of human fibrocytes, 20 ml of peripheral blood was collected in heparinized vacutainers from consenting normal volunteers. Whole blood was diluted 1:1 with 0.9% normal saline. 30 ml of the diluted whole blood was layered onto 15 ml of Ficoll-Hypaque in a 50 ml conical tube and centrifuged at 1200 rpm for 45 minutes. The buffy coat layer was removed, washed 3 times in SFM and the cell pellet was then cultured in complete media containing 20% FCS for 14d. At this time point, adherent cells were >95% fibrocytes as determined by flow cytometry for CD45 and pro-collagen 1 expression. Fibrocytes were trypsinized and replated in SFM for proliferation assays or Western blot analysis. These experiments were approved by the University of Michigan Institutional Review Board.

BAL

Alveolar cells were obtained via *ex vivo* lung lavage using a previously described protocol [238]. Briefly, these cells were collected in lavage fluid consisting of complete medium and 5 mM EDTA. The cells were enumerated by counting on a hemocytometer before use. In some experiments, alveolar cells were enriched by a 1h adherence step in SFM before being cultured for 24h in complete media. The adherent fraction consisted largely of AMs in untreated mice and of AMs, fibrocytes and neutrophils post-FITC. In other experiments, BAL cell pellets were cultured for 14d *ex vivo*.

TGF-β1 bioassay

In some experiments, mink lung epithelial cells stably transfected with a plasminogen activator inhibitor (PAI)-1 promoter-driven luciferase gene were left

untreated or infected with γHV-68 at a MOI of 0.005 or 0.05. Following 48-72 h of infection, cell lysates were prepared and luciferase activity was measured in a luminometer using the Promega luciferase assay kit (Madison, WI) according to manufacturer's instructions. This assay measured active TGF-β1.

Proliferation Assays

In some experiments, murine fibrocytes (2 $x10^5$ per ml) were plated in 96-well flat-bottomed tissue culture dishes and were cultured for 8-32 h in SFM before the addition of 10 μCi of [^{3}H]-thymidine for an additional 16 hours. For experiments with γHV-68, fibrocytes or fibroblasts were purified from lung mince cultures from WT C57Bl/6 mice on day 14. 5 x 10^3 cells/well were left untreated or infected *in vitro* at a MOI of 0.1 or 1 in complete media for 24-72h, and proliferation was measured via ^{3}H-thymidine incorporation. Following the labeling period, cells were harvested onto glass fiber filters using an automated cell harvester and filters were counted using a β-scintillation counter. In some experiments, fibrocytes from WT or 5-LO-/- mice were cultured in the presence or absence of LTD_4 or LTC_4 at indicated doses. Some experiments also included the cysLT1 antagonists, Ly171883 (1μM) or MK571 (10 nM). Human fibrocytes were cultured at 2 $x10^5$ cells/ml in SFM or SFM containing 10 nM LTD_4.

Western blots

5 x 10^5 primary culture fibrocytes or fibroblasts per well were plated in 6-well tissue culture plates overnight. The cells were washed with cold PBS, and 125-200 μl of

cold lysis buffer [1% w/w NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M NaH_2PO_4, 0.02 M EDTA, 0.05 M NaF, 0.002 M NA_3VO_4, 1:100 dilution of Calbiochem Protease Cocktail Set III (Calbiochem; La Jolla, CA)] was added to each well. Lysates were assayed for total protein concentration using the D_C Protein Assay (Bio-Rad; Hercules, CA). 4 or 10 μg of protein from each lysate were run on a 4-20% Tris-Gly gel (Invitrogen; Carlsbad, CA). For some experiments, blots were probed with rabbit anti-mouse collagen 1 antibody (Cedar Lane; Hornby, Ontario), monoclonal α-SMA (Sigma), and monoclonal β-actin (Sigma) as a loading control. Blots were stripped for 1h at room temperature in Restore™ Western Blot Stripping Buffer (Thermo Scientific; Rockford, IL) between β-actin and α-SMA immunoblotting. In other experiments, lysates were analyzed for expression of cysLT1, cysLT2 or β-actin using methods that have previously been described [239]. Antibodies against human cysLT1 (rabbit polyclonal, sc-25448) and murine cysLT2 (goat polyclonal, sc-27097) were purchased from Santa Cruz Biotechnology. Appropriate secondary antibodies conjugated to peroxidase were purchased from Pierce Biotechnology (Rockford, IL).

Reagents used

Complete media is DMEM (Lonza; Walkersville, MD) with 10% FCS, 1% penicillin-streptomycin, 1% L-glutamine, and 0.1% Amphotericin B (Lonza). SFM is DMEM with 10% bovine serum albumin (Sigma), 1% penicillin-streptomycin, 1% L-glutamine, and 0.1% Amphotericin B (Lonza). CysLT1 antagonists MK571 and Ly171883 were purchased from BIOMOL (Plymouth Meeting, PA). LTD_4 was purchased from Cayman Chemical.

Statistical analyses

When analyzing three or more groups, statistical significance was measured by ANOVA. For comparison between data from two groups, data were analyzed by Student's *t* test. A $p < 0.05$ was considered significant.

CHAPTER THREE:

RESULTS

A role for latent virus in the development of fibrosis

γHV-68 infection is latent in the lung by 14 days post-infection (d.p.i.)

To test the effect of latent gammaherpesvirus infection on subsequent pulmonary fibrosis, we first confirmed when lytic virus was cleared from the lungs of normal mice infected with γHV-68. After i.n. inoculation with 5×10^4 PFU, we were not able to detect productive γHV-68 infection in the whole lung by a standard plaque assay by 14 d.p.i (Fig. 1A). Using real-time RT-PCR, we next measured lytic and latent viral gene expression in the whole lung over the first three weeks post-infection. We found that expression of a lytic viral gene, DNApol, declined substantially to nearly undetectable levels by 14 d.p.i (Fig. 1B). By 30 d.p.i., DNApol transcript was undetectable in the lungs of all mice tested (not shown). Conversely, M3, a viral gene expressed during both lytic and latent infection was readily detectable for at least 30 d.p.i. (Fig. 1C and data not shown). Detection of M3 in the absence of DNApol is indicative of latent infection by 14 d.p.i. in the lung.

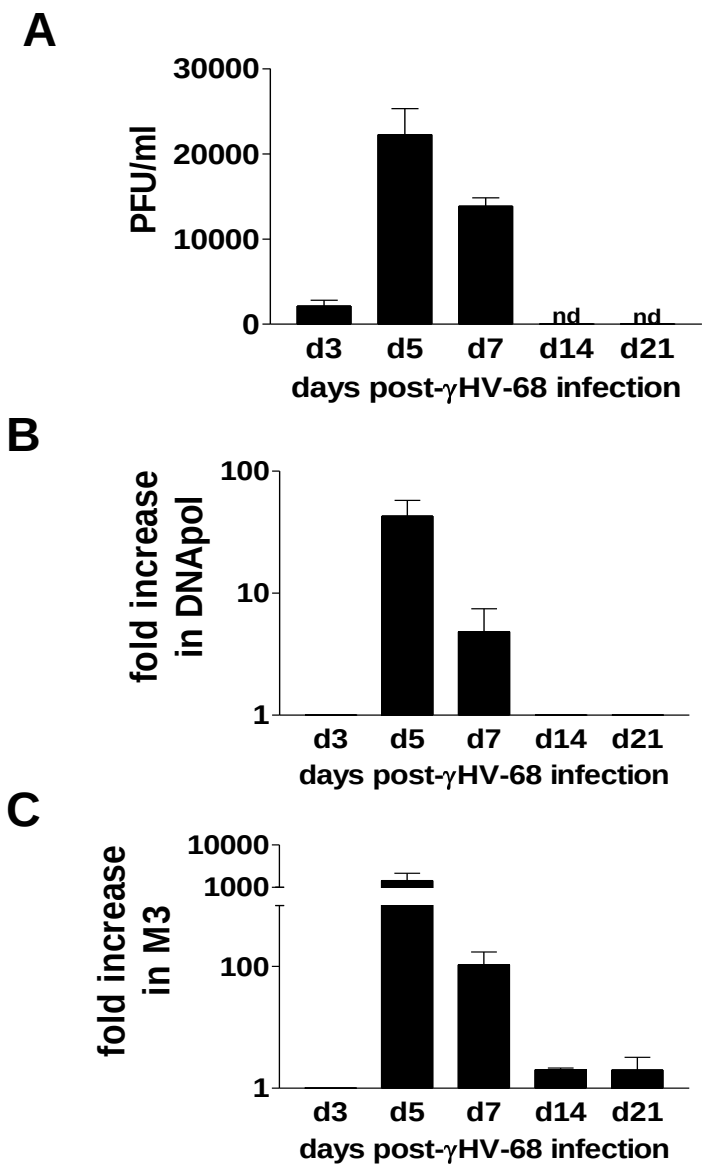


Figure 1: γHV-68 infection is latent in the lung by 14 d.p.i.
WT C57Bl/6 mice were given an i.n. infection with 5 x 10^4 PFU γHV-68. Subsequently, lytic and latent γHV-68 infection was measured in the lungs. Error bars represent standard error of the mean between individual mice. A. Viral plaque assay demonstrates that there is no active viral replication by 14 d.p.i (nd=not detectable, n=3). B. Real-time RT-PCR demonstrates that gene expression of the lytic viral gene DNApol decreases to nearly undetectable levels by 14 d.p.i (n=4). Viral gene expression 3 d.p.i. was set at 1, and viral gene expression on subsequent days is expressed in comparison for (B) and (C). C. Real-time RT-PCR demonstrates that gene expression of the latent viral gene M3 is detectable beyond 14 d.p.i (n=4). It should be noted that M3 is also expressed during lytic infection and remains present during latent infection.

Latent γHV-68 augments FITC- or bleomycin-induced pulmonary fibrosis

Mice were inoculated i.n. with 5 x 10^4 PFU γHV-68 or were mock-infected with saline on day 0. 14, 21, 30, 45, and 70 d.p.i., γHV-68-infected mice and mock-infected mice were administered i.t. FITC or saline. Lungs were harvested 21 days after the i.t. injection, and lung hydroxyproline content was assayed to measure collagen content. Established latent γHV-68 infection at all time-points resulted in significantly higher hydroxyproline levels in FITC-treated mice than preceding mock infection in FITC-treated mice (Fig. 2B-F). γHV-68 infection alone did not increase lung hydroxyproline content above the level of saline-treated mice (Fig. 2A). As expected, latent γHV-68 in the lung does not cause pulmonary fibrosis without a separate fibrotic insult. In an attempt to confirm our findings, we tested whether latent γHV-68 had the same effect on a murine model of pulmonary fibrosis induced by bleomycin. We observed that i.n. infection with γHV-68 70d prior to bleomycin-induced pulmonary fibrosis also significantly augments hydroxyproline levels in the lung (Fig. 3). These results demonstrate for the first time that either short-term or long-term γHV-68 latency in the lung significantly augments a subsequent fibrotic stimulus.

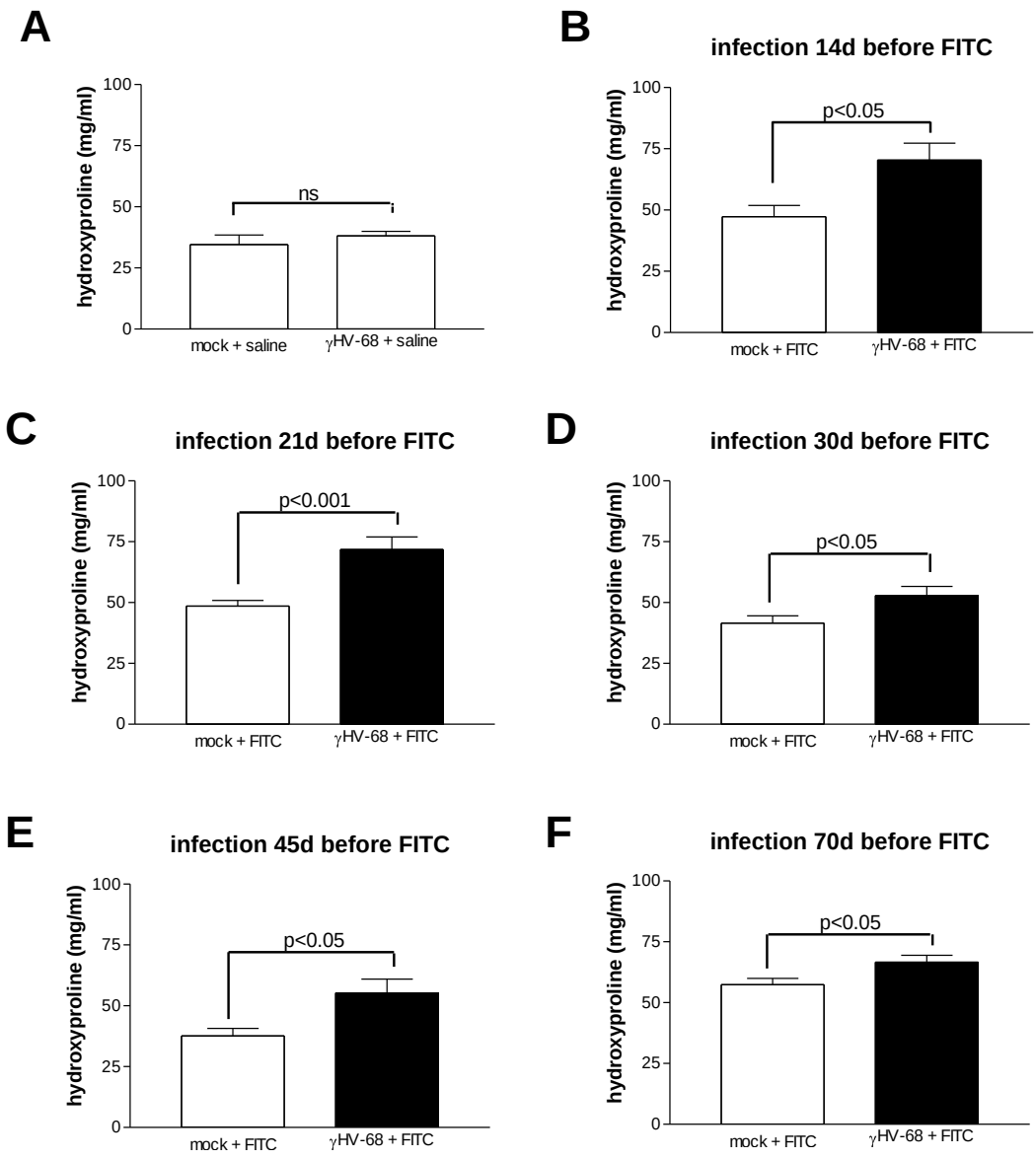


Figure 2: Latent γHV-68 augments FITC-induced pulmonary fibrosis
WT C57Bl/6 mice were inoculated i.n. with 5 x 10^4 PFU of γHV-68 or were mock-infected with saline on day 0. A. 14 d.p.i., mice of both groups were administered i.t. saline. Lungs were harvested 21d after saline i.t. administration. A hydroxyproline assay of the lungs demonstrates that γHV-68 infection alone does not augment collagen levels in the lung compared to lungs of mock-infected mice. 14 (B), 21 (C), 30 (D), 45 (E), and 70 (E) d.p.i., γHV-68-infected mice and mock-infected mice were administered i.t. FITC. In each case, lungs were harvested 21d after FITC administration. Hydroxyproline assays of the harvested lungs demonstrate that γHV-68 infection at each time-point prior to FITC causes a significant augmentation in collagen levels in fibrotic lungs. Data represent n=4-18 mice per group collected from multiple experiments.

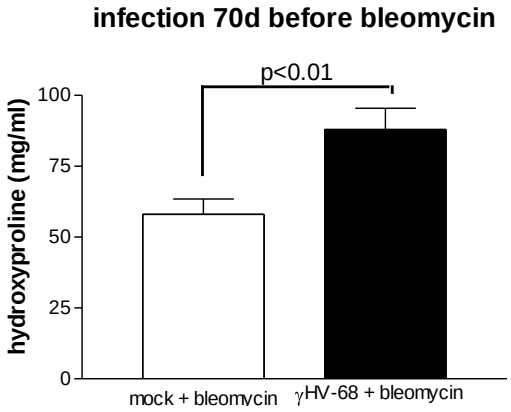


Figure 3: Latent γHV-68 augments bleomycin-induced pulmonary fibrosis
WT C57Bl/6 mice were inoculated i.n. with 5 x 10^4 PFU γHV-68 or mock-infected with saline on day 0. 70 d.p.i., γHV-68-infected mice and mock-infected mice were administered i.t. bleomycin. Lungs were harvested 21d after bleomycin administration. Hydroxyproline assays of the harvested lungs demonstrate that γHV-68 infection prior to bleomycin-induced fibrosis causes a significant augmentation in collagen levels in fibrotic lungs (n=6 mice per group).

Latent γHV-68 infection can augment a sub-threshold fibrotic stimulus

It is noteworthy that in a couple of experiments we performed with latent γHV-68-induced augmentation of pulmonary fibrosis, the usual dose of FITC did not cause a statistically significant increase in hydroxyproline levels compared to control mock-infected mice given saline i.t. Despite the ineffectiveness of FITC in these cases, the presence of latent γHV-68 at the time of the FITC challenge resulted in significantly higher hydroxyproline levels after the administration of FITC (Fig. 4). This result is particularly intriguing because it demonstrates in our model that latent γHV-68 can cause pulmonary fibrosis even in a scenario where a subsequent insult is not sufficient to cause measureable levels of fibrosis on its own.

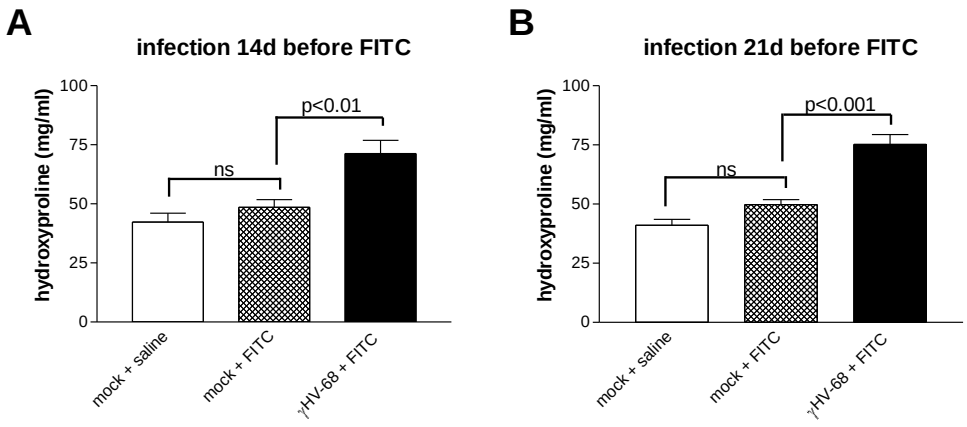


Figure 4: γHV-68 latency induces fibrosis even with sub-threshold FITC stimulus
Like the experiments described for Figs. 2 and 3, WT C57Bl/6 mice were inoculated i.n. with 5 x 10^4 PFU γHV-68 or mock-infected with saline on day 0. 14 d.p.i. (A) and 21 d.p.i. (B), mice were administered i.t. saline or i.t. FITC at the usual dose. Lungs were harvested 21d after i.t. injections. A hydroxyproline assay of the lungs demonstrates that FITC alone (checkered bars) does not augment collagen levels in the lung compared to mice given saline i.t (white bars) in either of these experiments. The lungs of FITC-treated mice infected 14d or 21d previously with γHV-68 (black bars) displayed a significant augmentation in collagen levels compared to mock-infected mice treated with FITC (n=3-11).

FITC induces a low-level reactivation of γHV-68

To understand why the virus augments fibrosis, we first wanted to determine if the fibrotic stimulus was reactivating latent γHV-68 to undergo lytic replication. Thus, we infected mice i.n. with γHV-68 on day 0, and then mice were given FITC 30 d.p.i. We harvested lungs immediately prior to FITC inoculation or 7d after FITC to measure the expression of the lytic viral genes, DNApol and gB. By comparing the lungs harvested at the two time-points, we observed a small increase in the expression of both the lytic genes 7d after FITC was administered (Fig 5). FITC induced reactivation of lytic viral RNA transcription to levels two orders of magnitude below the levels of lytic

viral RNA transcription at the peak of viral gene expression 3-5 days after the primary infection (Fig. 5 and data not shown). While these data support the possibility that FITC induces γHV-68 reactivation, the induction of lytic gene expression by FITC is modest.

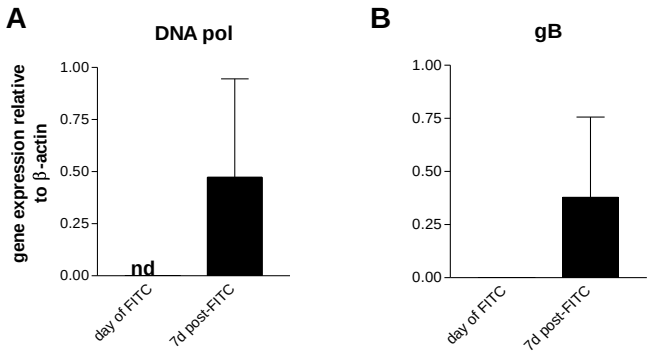


Figure 5: FITC induces a low-level reactivation of γHV-68
WT C57Bl/6 mice were given an i.n. injection with 5 x 10^4 PFU γHV-68 on day 0. On day 30, mice were administered i.t. FITC. The lungs of some mice were harvested on day 30 prior to FITC administration, and the expression of γHV-68 genes was measured. The lungs of the remainder of the mice were harvested on day 37 for analysis of viral gene expression. Real-time RT-PCR demonstrates that levels of gene expression of DNApol (A), and gB (B) are increased 7d after FITC administration compared to mice that were not administered FITC. Viral gene expression is presented relative to the expression level of a house-keeping gene, β-actin, which was set at 1 for each mouse (n=4 mice per group, nd=not detectable).

Reactivation of γHV-68 is not necessary to augment fibrosis

To determine whether modest reactivation of lytic viral gene expression by FITC-treatment was responsible for the augmentation of fibrosis, we next tested whether a mutant γHV-68 virus without a v-cyclin gene contained in open reading frame 72 (ΔORF72) could augment fibrosis. As a result of the mutation, ΔORF72 has a greatly diminished ability to reactivate from latency (approximately 1000-fold reduced ability to reactivate) [240]. Mice were inoculated i.n. with 5 x 10^4 PFU of ΔORF72, 5 x 10^4 PFU

of a v-cyclin marker-rescue virus ("MR", essentially a WT control), or were mock-infected with saline on day 0. 14 or 21 d.p.i., the mice were administered i.t. FITC. Lungs were harvested 21 days after the i.t. injection and lung hydroxyproline content was assayed. There was no significant difference between the augmentation of fibrosis in mice infected with either the ΔORF72 or the "MR" virus 21 or 14 days before the administration of FITC (Fig. 6). Thus, we conclude that lytic reactivation is not necessary for latent γHV-68 to augment FITC-induced pulmonary fibrosis.

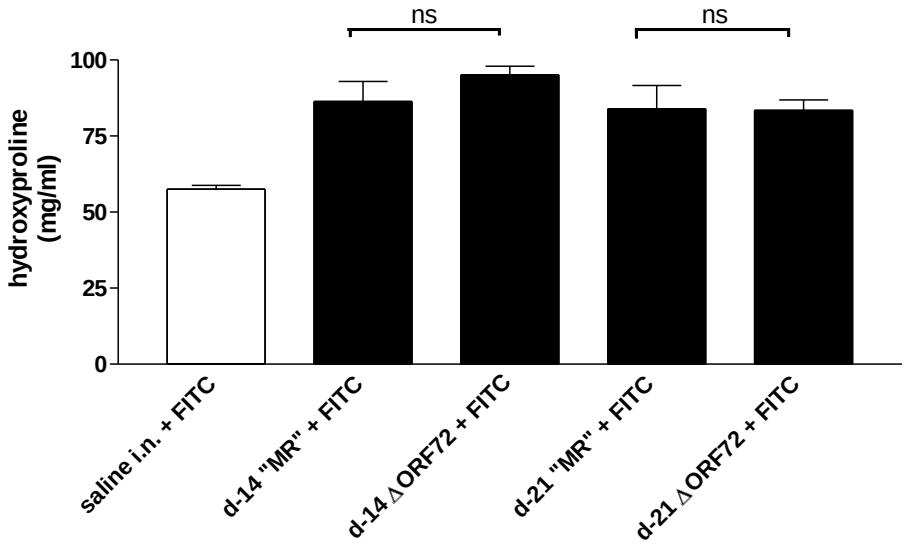
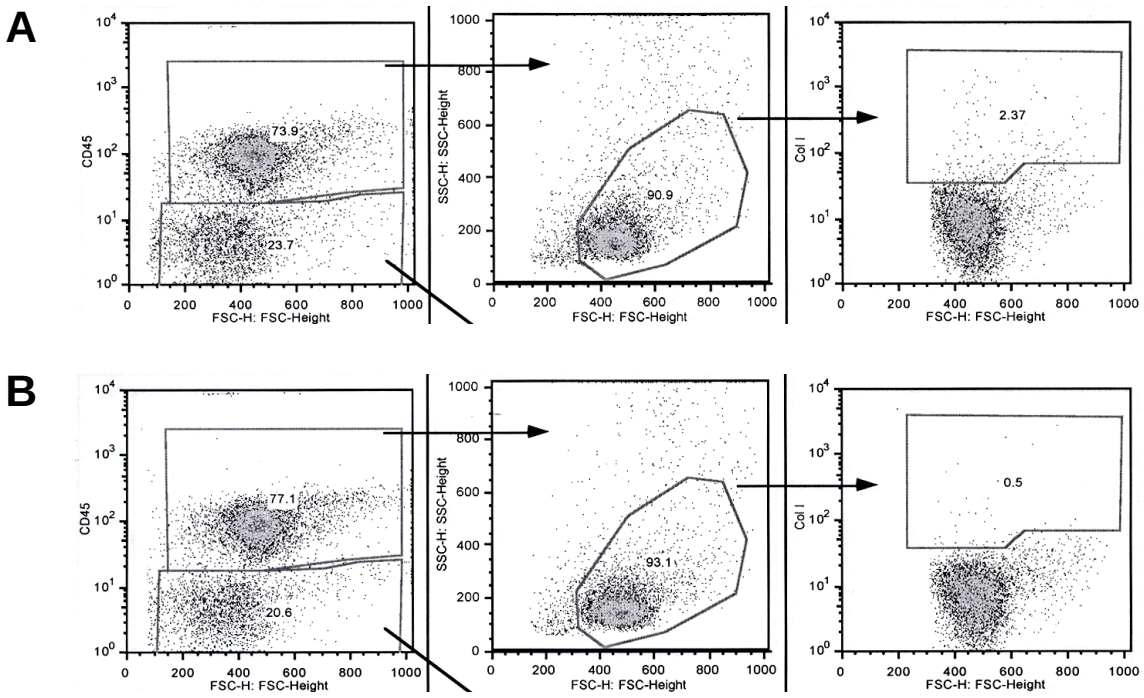


Figure 6: Reactivation of γHV-68 is not necessary to augment fibrosis
WT C57Bl/6 mice were inoculated i.n. with 5 x 10^4 PFU of ΔORF72, 5 x 10^4 PFU of a v-cyclin marker-rescue virus ("MR"), or were mock-infected with saline on day 0. 14 or 21 d.p.i., all of the mice were administered i.t. FITC. Lungs were harvested 21 days after the i.t. injection and lung hydroxyproline content was assayed. A hydroxyproline assay demonstrates that there was no significant difference between fibrosis in mice infected with either the ΔORF72 or the "MR" virus 21 or 14 days before the administration of FITC (n=5-6 mice per group).

γHV-68 infection induces fibrocyte accumulation in the lungs for at least 45 d.p.i.

To understand why latent virus increases fibrosis, we investigated how γHV-68 alters the lung environment prior to the fibrotic stimulus. We have already shown that i.n. γHV-68 infection results in an increase in fibrocyte numbers in the lung 5 d.p.i. at the height of the lytic infection [241]. Because fibrocytes are important mediators of fibrosis [38, 39], we sought to determine whether fibrocyte recruitment persists once γHV-68 establishes latency. Mice were infected i.n. with 5×10^4 PFU γHV-68, and lungs were harvested and digested with collagenase 7, 15, 30, 45, and 70 d.p.i. Lungs of uninfected mice were harvested and digested as controls. Subsequently, we stained the total cells from the digest for CD45 and collagen 1 and used flow cytometry to identify $CD45^+$ collagen 1^+ fibrocytes. To illustrate how we identified fibrocytes, examples of flow cytometry from these studies are shown in Figure 7A-B. We found that the absolute number of fibrocytes in the lung is significantly increased 15, 30 and 45 d.p.i (Fig. 7C). Taken together with our previous results, these findings suggest that latent viral infection is capable of recruiting fibrocytes to the lung, but the viral infection alone is insufficient to cause fibrosis.


A
B
CD45
FSC-H: FSC-Height
SSC-H: SSC-Height
Col I
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23.7
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2.37
77.1
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93.1
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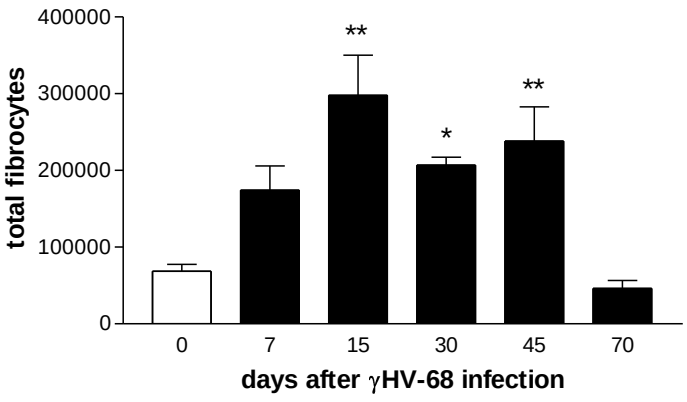

C
total fibrocytes
days after γHV-68 infection
400000
300000
200000
100000
0
0
7
15
30
45
70
**
*
**

Figure 7: Viral infection induces fibrocyte accumulation in the lungs for at least 45 d.p.i.

WT C57Bl/6 mice were infected i.n. with 5 x 10^4 PFU. The lungs of uninfected mice were harvested on day 0, and the lungs of virally-infected mice were harvested 7, 15, 30, 45, and 70 d.p.i. All lungs were digested with collagenase, and total cells from the digest were enumerated and stained for CD45 and collagen 1. Flow cytometry was used to determine the percentage of fibrocytes. A. Flow cytometric analysis of cells from the lung digest of one mouse 30 d.p.i (representative of analysis at each time-point post-infection). In the left panel, CD45+ cells were gated. In the middle panel, those CD45+ cells were gated to exclude dead cells. In the right panel, living CD45+ collagen 1+ were gated. B. An equal number of cells from each lung digest were stained with a rabbit isotype control antibody instead of the rabbit collagen 1 antibody. To account for non-specific binding, the percent of cells staining with the isotype control was set to ~0.5%. The shape of the gate was chosen to account for autofluoresence. The percentage of fibrocytes was calculated by subtracting the percentage of cells in the irrelevant control (B) from the specific collagen 1 staining percentage (A). C. The total number of fibrocytes in the lungs was then calculated by multiplying the percentage of fibrocytes by the total number of cells from a lung digest. Enumeration of fibrocytes demonstrates that fibrocytes accumulated to significantly higher levels in the lung 15, 30 and 45 d.p.i. compared to uninfected mice (n=4 mice per group; *=$p<0.05$, **=$p<0.01$ compared with uninfected).

γHV-68 infection does not change the number of fibroblasts in the lungs

After finding that latent γHV-68 infection had a significant effect on fibrocyte accumulation, we wondered if viral infection had an effect on the number of fibroblasts in the lung as well. Mice were infected i.n. with 5 x 10^4 PFU γHV-68, and lungs were harvested and digested with collagenase 7, 15, 30, 45, and 70 d.p.i. Lungs of uninfected mice were harvested and digested as controls. Subsequently, we stained the total cells from the digest and identified $CD45^-$ collagen 1^+ cells by flow cytometry to enumerate fibroblasts. Viral infection alone did not change the absolute number of fibroblasts significantly in the lung (Fig. 8). These results are consistent with our findings that viral infection alone does not induce fibrosis.

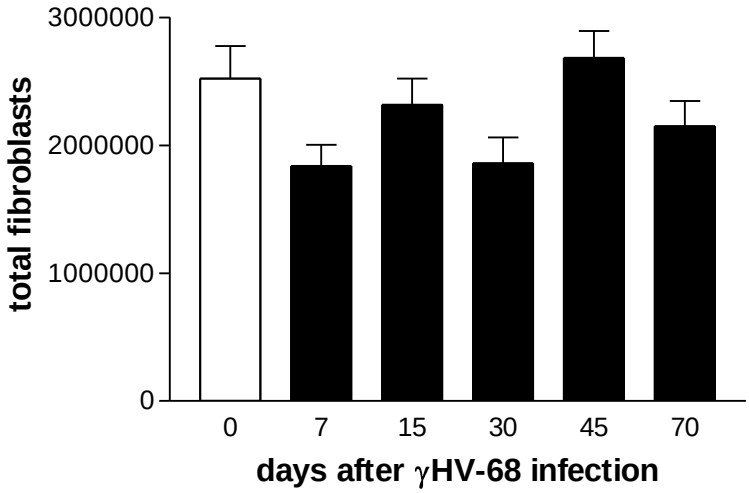


Figure 8: γHV-68 infection does not change the number of fibroblasts in the lungs
WT C57Bl/6 mice were infected i.n. with 5 x 10^4 PFU. The lungs of uninfected mice were harvested on day 0, and the lungs of virally-infected mice were harvested 7, 15, 30, 45, and 70 d.p.i. All lungs were digested with collagenase, and total cells from the digest were stained for CD45 and collagen 1. Flow cytometry was used to determine the percentage of fibroblasts. Enumeration of fibroblasts demonstrates that the number of fibroblasts is not significantly altered by latent γHV-68 infection (n=4 mice per group).

Cellular inflammation persists in the lungs of γHV-68-infected mice

Next, we counted the total number of lung leukocytes in collagenase-digested lungs prior to infection as well as 7, 14, 30, 45, and 70 d.p.i. Following infection, the total number of lung leukocytes was persistently higher than the total number of cells in the lungs of uninfected mice (Fig. 9). Even at 70 d.p.i., the total number of lung leukocytes remained nearly double the total number of lung leukocytes in the lungs of uninfected mice ($21.2 \times 10^6 \pm 1.32$ vs. $11.6 \times 10^6 \pm 0.27$, $p<0.01$). Thus, chronic inflammation persists during latent viral infection.

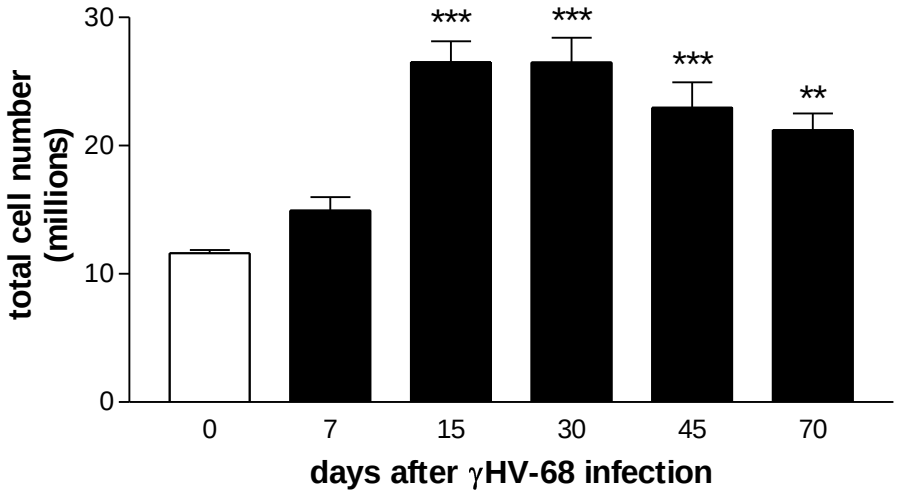


Figure 9: Cellular inflammation persists in the lungs of γHV-68-infected mice
WT C57Bl/6 mice were infected i.n. with 5×10^4 PFU. The lungs of uninfected mice were harvested on day 0, and the lungs of virally-infected mice were harvested 14, 21, 30, 45, and 70 d.p.i. All lungs were digested with collagenase, and total cells from the digest were counted demonstrating inflammatory cells accumulate at significantly higher levels in the lung 15, 30, 45, and 70 d.p.i. compared to uninfected mice (n=4 mice per group; **=$p<0.01$, ***=$p<0.001$ compared with uninfected).

CCL2 and CCL12 levels are increased in the lungs beyond the clearance of lytic γHV-68

CCL2 and CCL12 are potent chemotactic ligands for CCR2-mediated recruitment of inflammatory cells and fibrocytes during pulmonary fibrosis [38, 105]. Past studies by our group and others have suggested that γHV-68 induces CCL2 and CCL12 gene expression, and we wished to investigate whether latent infection caused a persistent increase in CCL2 and CCL12 protein production [241-243]. We infected mice i.n. with 5 x 10^4 PFU γHV-68 on day 0, and the lungs of the infected mice were harvested 5, 10, 15, 24, and 38 d.p.i. for the measurement of CCL2 and CCL12 in lung homogenates by ELISA. The lungs of uninfected mice were measured as controls. Figure 10 demonstrates that both CCL2 (A-C) and CCL12 (D-F) remain elevated during latent infection of the lung compared to uninfected controls.

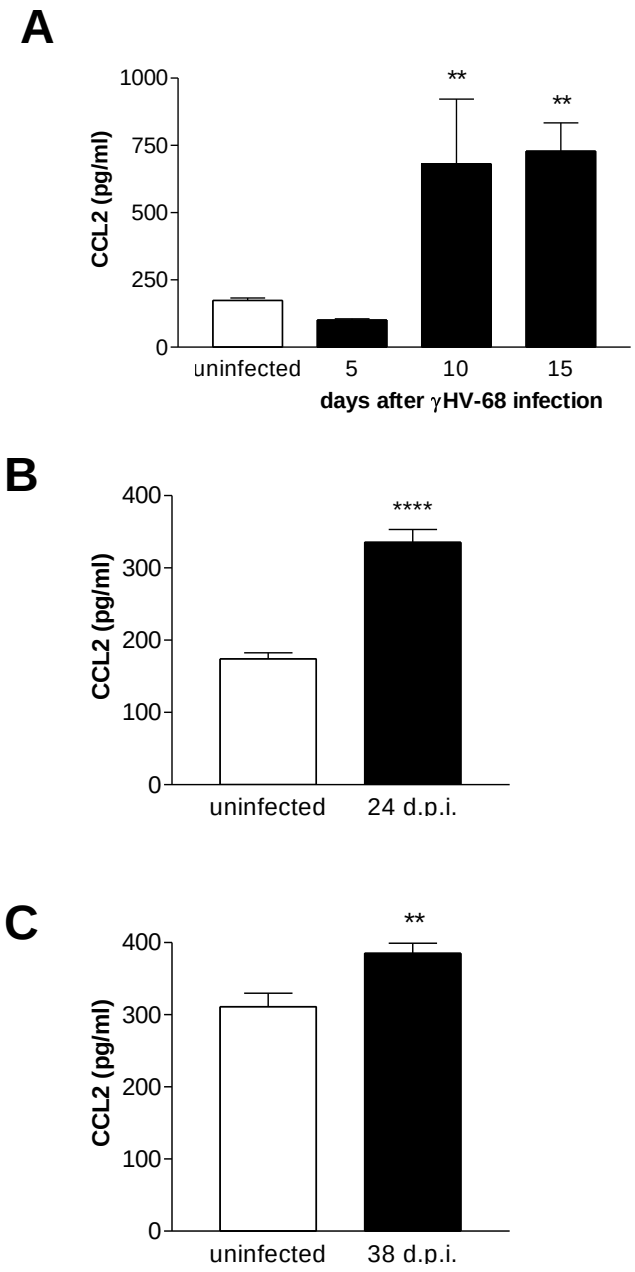


Figure 10 continued

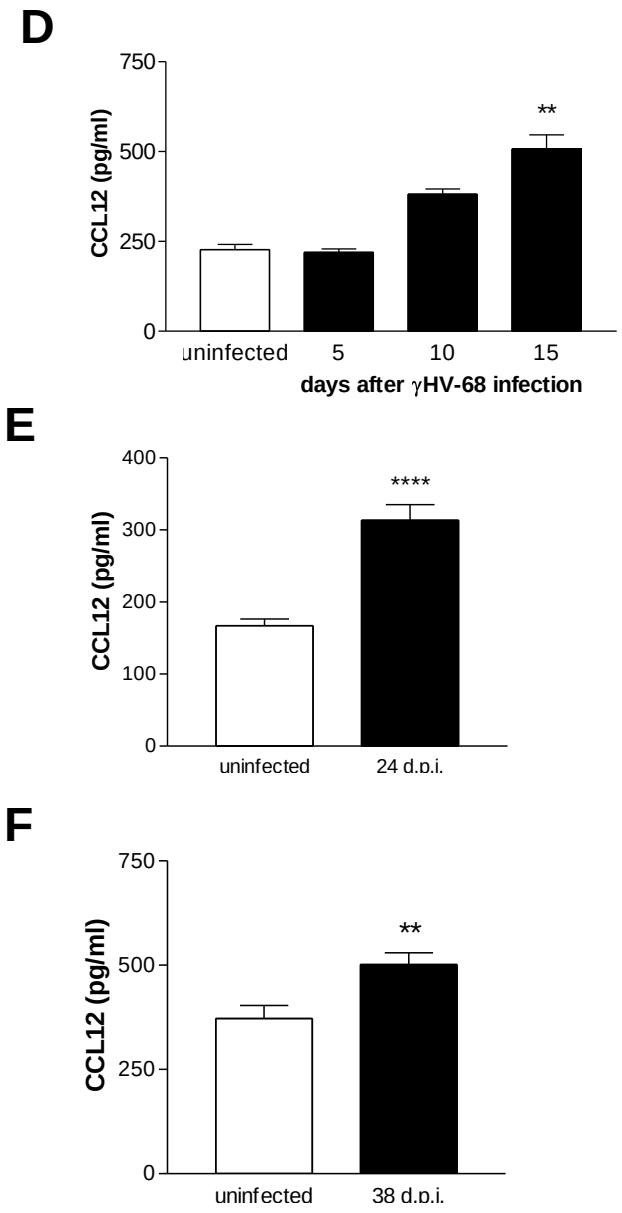


Figure 10: CCL2 and CCL12 levels are increased in the lungs beyond the clearance of lytic γHV-68

WT C57Bl/6 mice were infected i.n. with 5 x 10^4 PFU γHV-68 on day 0 or were left untreated. The lungs of γHV-68-infected mice were harvested 5, 10, and 15 d.p.i. along with lungs of untreated mice (white bar) (A and D). During other experiments, the lungs of γHV-68-infected mice were harvested 24 (B and E) or 38 (C and F) d.p.i. along with lungs of untreated mice (white bar). The amount of CCL2 (A-C) and CCL12 (D-F) in the lung homogenate was assayed by ELISA. The lungs of mice latently-infected with γHV-68 have significantly higher levels of CCL2 and CCL12 compared to the lungs of uninfected mice (n=3-10 mice per group; **=$p<0.01$, ****=$p<0.0001$ compared with uninfected).

TNF-α is not significantly increased in the lungs beyond the clearance of lytic γHV-68

We also hypothesized that the pro-inflammatory cytokine TNF-α is increased in the lung after γHV-68 infection in the context of the robust inflammation we observed. To test that hypothesis, we infected mice i.n. with 5 x 10^4 PFU γHV-68 on day 0, and the lungs of the infected mice were harvested 15, 21, 24, and 38 d.p.i. for the measurement of TNF-α in lung homogenates by ELISA. The lungs of uninfected mice were measured as controls. While there appears to be a trend towards an increase in TNF-α levels due to viral infection at the time-points we tested, the increase was not statistically significant in three of the four experiments we conducted. As a result, we conclude that TNF-α levels are not enhanced significantly during γHV-68 latency (data not shown).

TGF-β1 is increased in the lungs beyond the clearance of lytic γHV-68

We tested to see if there is more TGF-β1 in latently-infected lungs because TGF-β1 is the most potent pro-fibrotic factor identified to date. We infected some mice i.n. with 5 x 10^4 PFU γHV-68 on day 0, and some mice were left untreated. The lungs of the infected and uninfected mice were harvested 24 or 38 d.p.i. for the measurement of total TGF-β1 in lung homogenates by ELISA. The ELISAs demonstrated that the lungs of γHV-68-infected mice contained higher levels of total TGF-β1 than the lungs of control mice 24 and 38 d.p.i (Fig. 11). Interestingly, despite the induction of TGF-β1 expression during latent infection and the recruitment of fibrocytes, the viral infection alone is insufficient to initiate fibrosis.

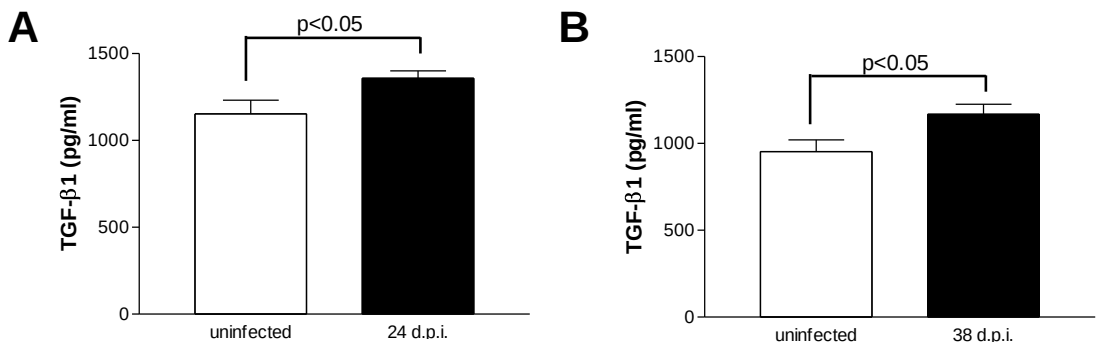


Figure 11: Total TGF-β1 levels are increased in the lungs beyond the clearance of lytic γHV-68

WT C57Bl/6 mice were infected i.n. with 5 x 10^4 PFU γHV-68 on day 0 or were left untreated. The lungs of γHV-68-infected mice were harvested 24 (A) or 38 (B) d.p.i. along with lungs of untreated mice. The amount of total TGF-β1 in lung homogenates was assayed by ELISA. The ELISAs demonstrate that the lungs of mice latently-infected with γHV-68 (black bars) have significantly higher levels of total TGF-β1 compared to the lungs of uninfected mice (white bars) 24 or 38 d.p.i (n=6-10 mice per group).

CysLT production is induced by lytic but not latent γHV-68

We also considered it important to test whether the cysLT production is elevated in the lung during viral infection since cysLT production is elevated in fibrotic lungs [33, 119]. We infected some mice i.n. with 5 x 10^4 PFU γHV-68 on day 0, and some mice were left untreated. The lungs of the infected and uninfected mice were harvested 4, 24 or 38 d.p.i. for the measurement of total cysLTs in lung homogenates by ELISA. There was no significant difference between the level of cysLTs in uninfected lungs and the level of cysLTs in the lungs harvested 24 or 38 d.p.i (Figs. 12B-C). There was a significant increase in the amount of cysLTs in infected lungs 4 d.p.i. compared to uninfected lungs, however (Fig. 12A). This suggests that lytic γHV-68 stimulates

production of cysLTs, but cysLT production does not persist at such levels during latency.

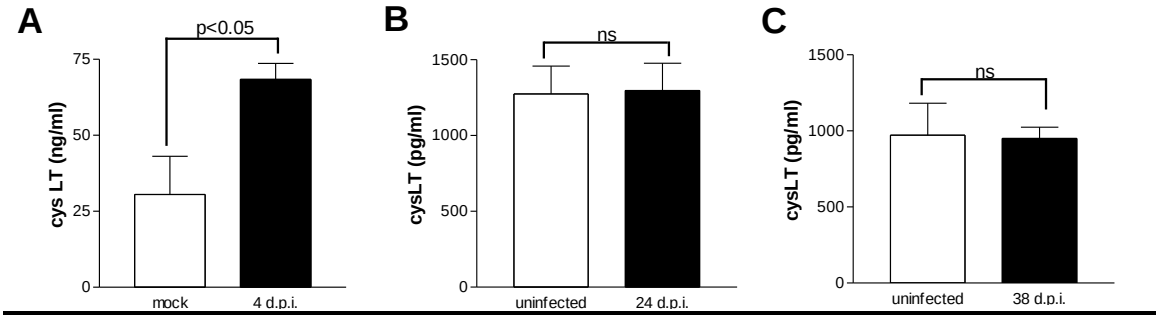


Figure 12: CysLT production is induced by lytic but not latent γHV-68
WT C57Bl/6 mice were infected i.n. with 5 x 10^4 PFU γHV-68 on day 0, were left untreated, or in one experiment, were mock-infected. The lungs of γHV-68-infected mice were harvested 4 (A), 24 (B), or 38 (C) d.p.i. along with lungs of mock-infected mice (A) or untreated mice (B and C). The amount of cysLTs in lung homogenates was assayed by ELISA. The ELISA represented by A demonstrates that the lungs of mice infected with lytic γHV-68 (black bar) contain significantly higher levels of cysLTs compared to the lungs of mock-infected mice (white bars) 4 d.p.i (n=3 mice per group). The ELISAs represented by B and C demonstrate that the lungs of mice infected with latent γHV-68 (black bars) do not contain significantly different levels of cysLTs compared to the lungs of uninfected mice (white bars) 24 or 38 d.p.i (n=5-9 mice per group).

Latent γHV-68 augments fibrocyte accumulation in the lung during FITC-induced pulmonary fibrosis

The data in figures 7, 9, and 10 all demonstrate that viral infection alone is able to induce the recruitment of inflammatory cells, including fibrocytes to the lung and that this is likely secondary to the induction of chemokines in response to viral infection. Viral infection alone is not fibrotic, however. Therefore we wished to determine whether the combined stimuli of latent viral infection plus a fibrotic insult would manifest in alterations in cellular recruitment, chemokine production, and pro-fibrotic factors. We

first investigated whether augmentation of fibrocyte accumulation is a mechanism of latent virus-induced augmentation of fibrosis. Mice were infected i.n. with 5×10^4 PFU γHV-68 or were mock-infected on day 0. 17 d.p.i., latently-infected mice and mock-infected mice were administered i.t. FITC. Lungs were harvested 7d or 21d after FITC administration. Subsequently, total pulmonary fibrocytes were enumerated as described previously. 7d after FITC i.t., latently-infected mice had approximately twice the number of fibrocytes in the lungs (344,900 ± 52,880) compared to mock-infected mice (170,400 ± 22,430) (Fig. 13A). 21d after FITC i.t., latently-infected mice had also had significantly more fibrocytes in the lungs (591,000 ± 98,880) compared to mock-infected mice (260,300 ± 55,650) (Fig. 13B). Thus, we conclude that the presence of latent γHV-68 augments fibrocyte accumulation in the lungs during FITC-induced pulmonary fibrosis to levels that are significantly higher than those achieved with the fibrotic stimulus alone.

We also examined the absolute numbers of fibroblasts present in these lungs. Surprisingly, we found no increase in fibroblast numbers when isolated by collagenase digest (data not shown), despite the fact that collagen content in the lung is increased at this time-point. These data likely reflect a technical inability to digest fibroblasts out of the fibrotic lung tissue at this time.

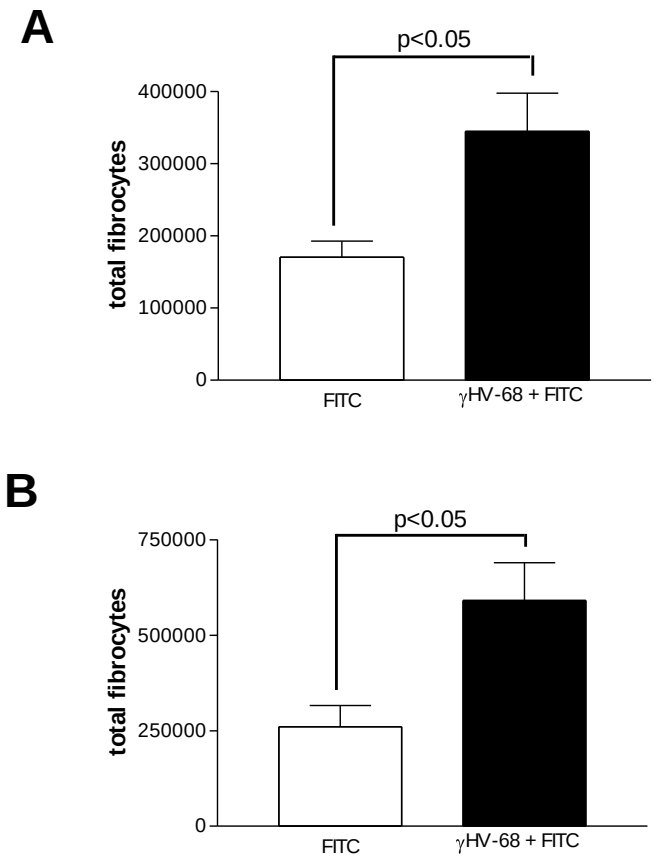


Figure 13: Latent γHV-68 augments fibrocyte accumulation in the lung during FITC-induced pulmonary fibrosis
WT C57Bl/6 mice were infected i.n. with 5 x 10^4 PFU γHV-68 or were mock-infected on day 0. 17 d.p.i., latently-infected mice and mock-infected mice were administered i.t. FITC. Lungs were harvested 7d (A) and 21d (B) after FITC administration, and total fibrocytes were enumerated as previously described. The lungs of mice previously infected with γHV-68 (black bar) have a significant increase in the accumulation of fibrocytes compared to the lungs of mock-infected mice (white bar) 7d (A) and 21d (B) after FITC administration (n=3-5).

Latent γHV-68 augments inflammation in the lung during FITC-induced pulmonary fibrosis

We next investigated whether augmentation of the number of inflammatory cells is a mechanism of latent virus-induced augmentation of fibrosis. Mice were infected i.n.

with 5 x 10^4 PFU or were mock-infected on day 0. 17 d.p.i., latently-infected mice and mock-infected mice were administered i.t. FITC. Lungs were harvested 7d or 21d after FITC administration and the total number of inflammatory cells were enumerated. 7d (Fig. 14A) and 21d (Fig. 14B) after FITC i.t., latently-infected mice had significantly more total cells in the lungs compared to mock-infected mice. We analyzed lung histology for evidence to support our inflammatory cell enumeration. For the histological studies, mice were infected or mock-infected 14d prior to FITC administration. 21d after FITC i.t., lungs were harvested and prepared for histology. Histological staining corroborates with our cell counts by displaying increased inflammation during the fibrotic response in the lungs of infected mice compared to mock-infected mice (Fig. 14C). It is particularly interesting that the virally-infected mice show focal clusters of mononuclear cells that are not seen in the mice challenged with FITC alone. Based on morphology, these cells look like leukocytes. These data provide evidence that the presence of latent γHV-68 augments and changes the nature of inflammation in the lungs during the development of FITC-induced pulmonary fibrosis.

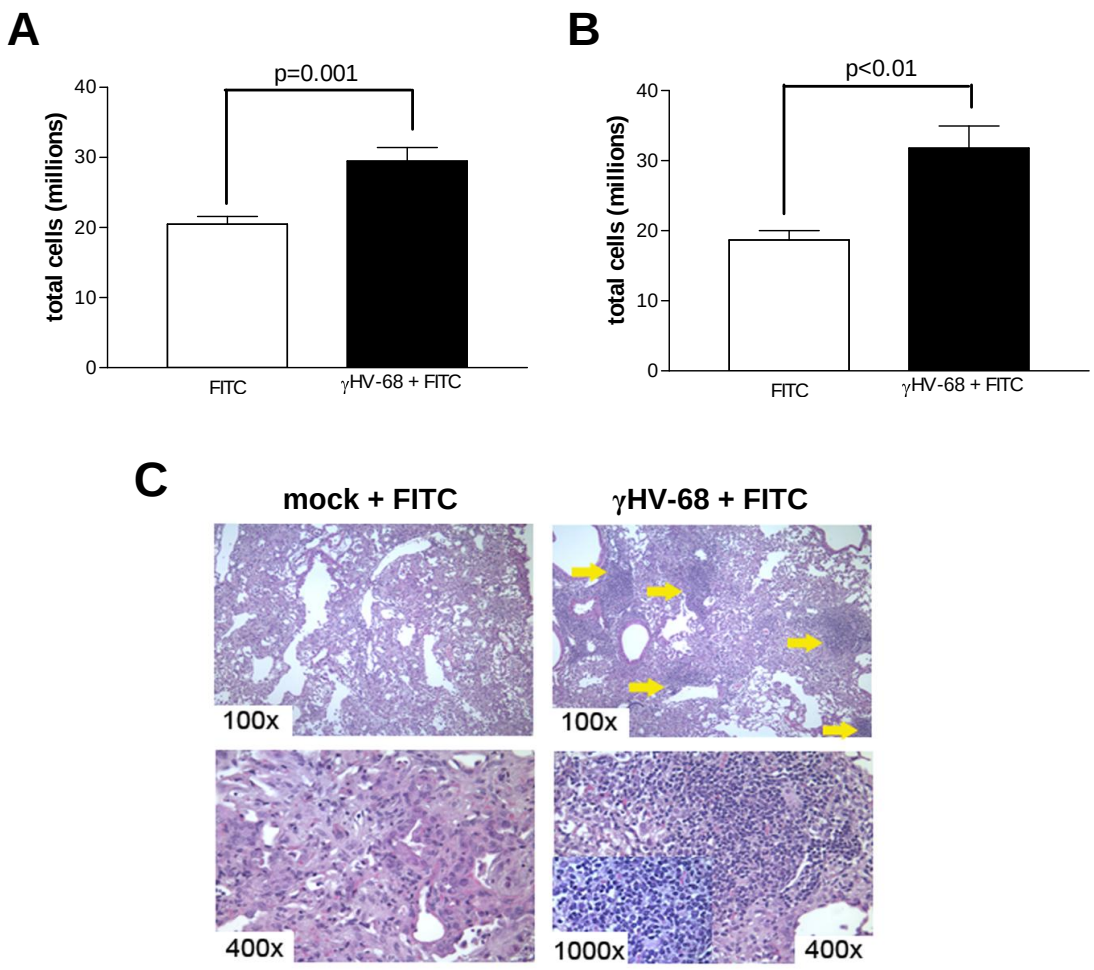


Figure 14: Latent γHV-68 augments inflammation in the lung during FITC-induced pulmonary fibrosis

A. and B. WT C57Bl/6 mice were infected i.n. with 5 x 10^4 PFU γHV-68 or were mock-infected on day 0. 17 d.p.i., latently-infected mice and mock-infected mice were administered i.t. FITC. Lungs were harvested and digested 7d (A) or 21d (B) after FITC administration, and total cells were enumerated. The lungs of mice previously infected with γHV-68 (black bar) display a significant increase in the accumulation of inflammatory cells compared to the lungs of mock-infected mice (white bar) 7d (A) or 21d (B) after FITC administration (n=3-5). C. Mice were given 5 x 10^4 PFU γHV-68 or saline 14 days prior to the FITC i.t. Lungs were prepared for histology 21 days post-FITC. Panels on the left side represent mice pre-treated with saline, and then challenged with FITC. Panels on the right side represent mice infected with γHV-68 prior to the FITC challenge. Staining with hematoxylin and eosin demonstrates that viral pre-infection causes increased numbers of inflammatory cells to enter the lung during the subsequent fibrotic response. It is particularly interesting that the virally-infected mice show focal clusters of mononuclear cells (yellow arrows) that are not seen in the mice challenged with FITC alone. The inset in the lower right panel is a 1000x magnification of one of these mononuclear foci.

Latent γHV-68 does not significantly augment CCL2 and CCL12 levels in the lung during FITC-induced pulmonary fibrosis

We hypothesized that augmentation of CCL2 and CCL12 contributed to latent virus-induced augmentation of the fibrocyte accumulation and inflammation during FITC-induced fibrosis. Mice were infected i.n. with 5×10^4 PFU γHV-68 or were mock-infected on day 0. 17 d.p.i., latently-infected mice and mock-infected mice were administered i.t. FITC. Lungs were harvested 7d or 21d after FITC administration, and CCL2 and CCL12 were assayed in the lung homogenate by ELISA. 7d and 21d after FITC i.t., neither CCL2 levels nor CCL12 levels were significantly different when comparing the lungs of virally-infected mice and mock-infected mice (Fig. 15). Thus, latent γHV-68 does not further increase FITC-induced CCL2 and CCL12 production in the lungs.

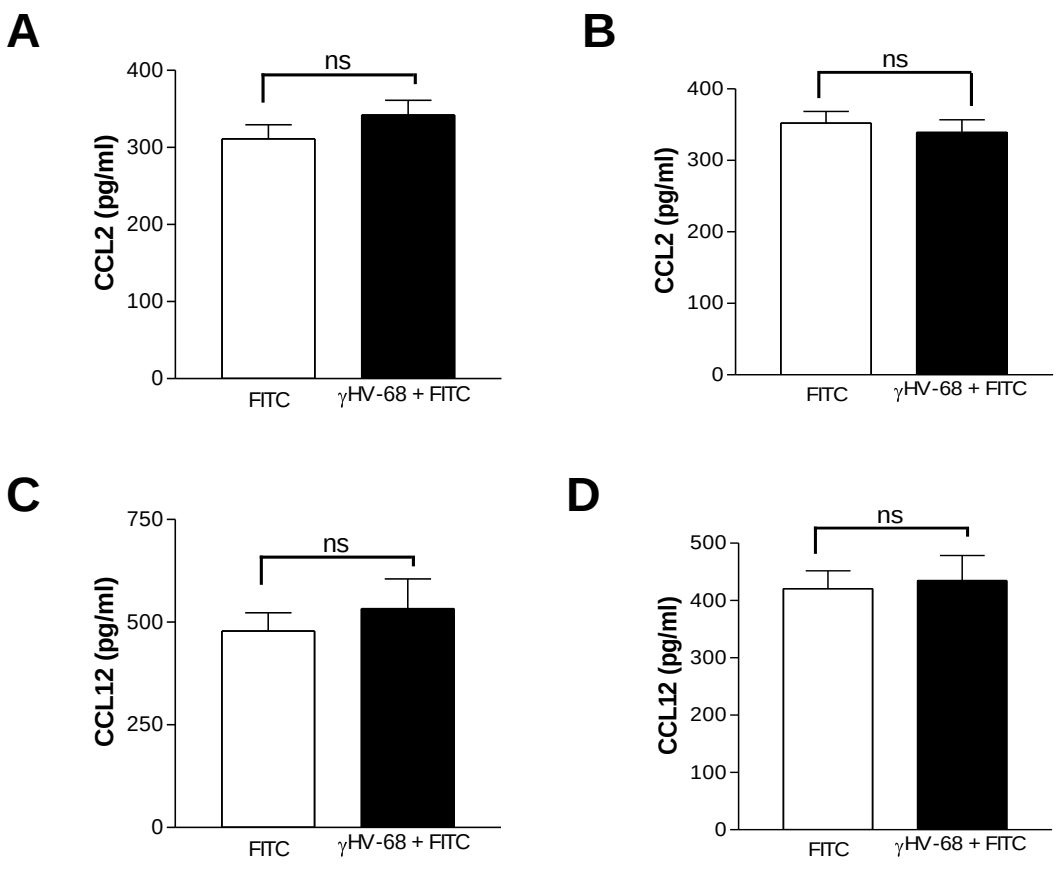


Figure 15: Latent γHV-68 does not significantly augment CCL2 and CCL12 levels in the lung during FITC-induced pulmonary fibrosis

WT C57Bl/6 mice were infected i.n. with 5 x 10^4 PFU γHV-68 or were mock-infected on day 0. 17 d.p.i., latently-infected mice and mock-infected mice were administered i.t. FITC. Lungs were harvested and digested 7d (A and C) and 21d (B and D) after FITC administration, and the amount of total CCL2 (A and B) or CCL12 (C and D) in the lung homogenate was assayed by ELISA. The amounts of CCL2 or CCL12 in the lungs of mice previously infected with γHV-68 (black bar) are statistically not different than the amounts of those chemokines in the lungs of mock-infected mice (white bar) 7d or 21d after FITC administration (n=5-10).

Latent γHV-68 augments TGF-β1 in the lung 7d but not 21d after the instillation of FITC

We next investigated whether augmentation of TGF-β1 is a mechanism of latent virus-induced augmentation of fibrosis. Mice were infected i.n. with 5×10^4 PFU γHV-68 or were mock-infected on day 0. 17 d.p.i., latently-infected mice and mock-infected mice were administered i.t. FITC. Lungs were harvested 7d or 21d after FITC administration, and the amount of total TGF-β1 (includes both latent and active forms) in the lung homogenate was assayed by ELISA. 7d after FITC i.t., the level of total TGF-β1 was significantly higher in lungs of latently-infected mice compared to lungs of mock-infected mice (Fig. 16A). 21d after FITC i.t., the level of total TGF-β1 was not statistically different in lungs of latently-infected mice compared to lungs of mock-infected mice (Fig. 16B). These findings suggest that the presence of latent γHV-68 augments levels of TGF-β1 in the lungs early in the development of FITC-induced pulmonary fibrosis, but latent γHV-68 does not further increase the level of total TGF-β1 at a time-point when fibrosis has been established.

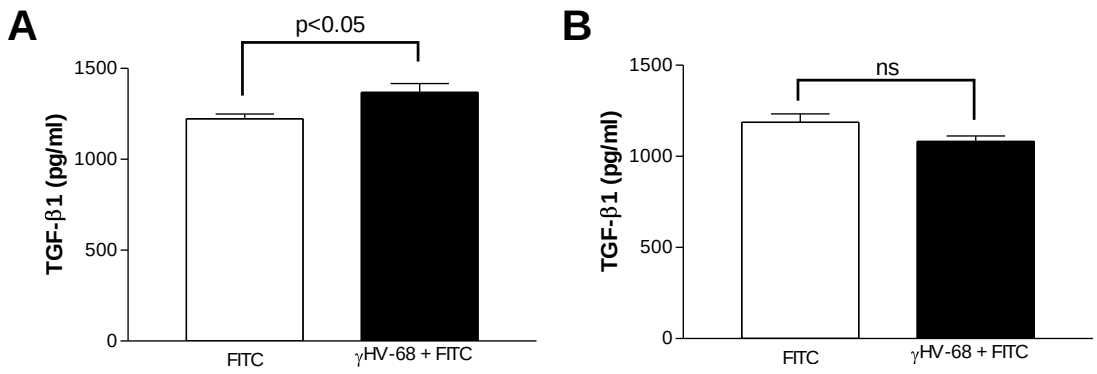


Figure 16: Latent γHV-68 augments TGF-β1 in the lung 7d but not 21d after the instillation of FITC

WT C57Bl/6 mice were infected i.n. with 5 x 10^4 PFU or were mock-infected on day 0. 17 d.p.i., latently-infected mice and mock-infected mice were administered i.t. FITC. Lungs were harvested 7d (A) or 21d (B) after FITC administration, and the amount of total TGF-β1 in the lung homogenate was assayed by ELISA. A. 7d after FITC administration, the lungs of mice previously infected with γHV-68 (black bar) contain significantly higher levels of TGF-β1 compared to the lungs of mock-infected mice (white bar)(n=7-8). B. 21d after FITC administration, the amount of TGF-β1 contained in the lungs of mice previously infected with γHV-68 (black bar) is not significantly different compared to the lungs of mock-infected mice (white bar)(n=5-8).

AECs are latently-infected at least 21d after i.n. injection with γHV-68

AECs have been shown to be a reservoir of latent γHV-68 for at least 54 d.p.i. *in vivo* [214], and we were interested in investigating whether latent γHV-68-induced alteration of AECs plays a role in the mechanisms of fibrotic augmentation we had observed. First, we tested whether murine AECs are infected by γHV-68 in our *in vivo* model. We infected mice i.n. with 5 x 10^4 PFU γHV-68 or mock-infected mice with saline on day 0, and AECs were purified from the lungs of the infected mice 14 and 21 d.p.i. Using real-time RT-PCR, we measured viral mRNA expression levels in the purified AECs. Viral gene expression was not detectable in the AECs purified from mock-infected mice, but the AECs purified from infected mice expressed the latent viral

gene M3 as well as lower levels of the lytic viral gene gB (Fig. 17). This pattern of viral gene expression is consistent with a latent infection of the lung at 14 and 21 d.p.i.

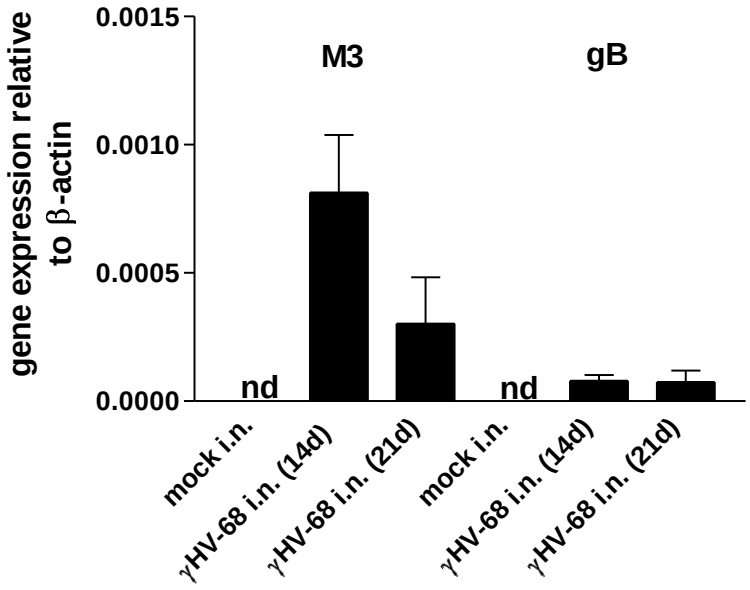


Figure 17: AECs are latently-infected at least 21d after i.n. injection with γHV-68
WT C57Bl/6 mice were infected i.n. with 5 x 10^4 PFU γHV-68 or were mock-infected on day 0. AECs were purified from the lungs of the infected mice 14 and 21 d.p.i. for analysis of viral gene expression. Real-time RT-PCR demonstrates that viral genes M3 and gB are expressed in the AECs of mice given γHV-68 i.n. Neither M3 nor gB gene expression was detectable in the AECs of mock-infected mice. Viral gene expression is presented relative to the expression level of a house-keeping gene, β-actin, which was set at 1 for each mouse (n=3-8 mice per group, nd=not detectable).

Latently-infected AECs express higher levels of CCL2 and CCL12

Next, we investigated if AECs contributed to excessive CCL2 and CCL12 generation that we observed during γHV-68 infection. We infected mice i.n. with 5 x 10^4 PFU γHV-68 or mock-infected mice with saline on day 0, and AECs were purified from the lungs of the infected mice 14 and 21 d.p.i. Using real-time RT-PCR, we measured chemokine mRNA expression levels in the purified AECs. CCL2 gene expression

increased markedly in latently infected AECs by 21 d.p.i (Fig. 18A). On the other hand, CCL12 mRNA levels increased significantly by 14 d.p.i (Fig. 18B). We conclude that AECs contribute to the increased levels of CCL2 and CCL12 in lungs of latently infected mice. Both chemokines are known to recruit CCR2-expressing fibrocytes.

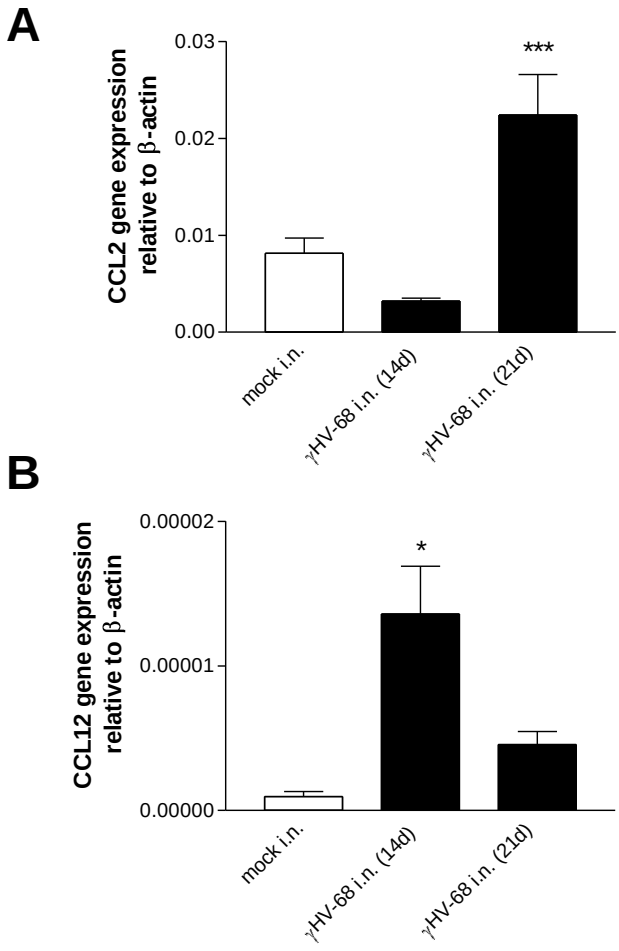


Figure 18: Latently-infected AECs express higher levels of CCL2 and CCL12
WT C57Bl/6 mice were infected i.n. with 5 x 10^4 PFU γHV-68 or were mock-infected on day 0. AECs were purified from the lungs of the infected mice 14 and 21 d.p.i. for analysis of chemokine gene expression. Real-time RT-PCR demonstrates that CCL2 gene expression is significantly increased in AECs harvested 21 d.p.i. (A) while CCL12 gene expression is significantly increased in AECs harvested 14 or 21 d.p.i (B). (n=3-8 mice per group; *=$p<0.05$, ***=$p<0.001$ compared with mock infected).

γHV-68 infection induces TGF-β1 production in AECs

We also investigated whether AECs contribute to virus-induced generation of TGF-β1. We infected mice i.n. with 5 x 10^4 PFU of γHV-68 or mock-infected mice with saline, and AECs were purified from the lungs of the γHV-68-infected and mock-infected mice 14 d.p.i. AECs were then cultured for 24h, and the culture supernatants were assayed for total TGF-β1 by ELISA. The level of total TGF-β1 was significantly higher in supernatants from AECs isolated from latently-infected mice compared to supernatants from AECs isolated from mock-infected mice (Fig. 19A). Our ELISA measures total TGF-β1 which would include both latent and active forms of this growth factor. While it is assumed that an increase in total TGF-β1 would be indicative of a rise in active TGF-β1, we used another method to investigate if γHV-68 infection augments active TGF-β1 production directly. *In vitro,* we infected mink lung epithelial cells stably transfected with a reporter construct encoding a luciferase gene with a PAI-1 promoter. Active TGF-β1 expression corresponds with the amount of luciferase expressed. Epithelial cells were infected at a MOI of 0.005 or 0.05. Using a luciferase assay, we found that mink lung epithelial cells infected for 3d and 7d *in vitro* produced significantly more active TGF-β1 than uninfected mink lung epithelial cells (Fig. 19B). Thus, we conclude that latent γHV-68 infection can augment TGF-β1 production in AECs, and also that γHV-68, at least in the lytic phase, augments the production of active TGF-β1. Furthermore, in conjuction with our previous results (Fig. 16), these data suggest that local concentrations of TGF-β1 may be elevated locally in the areas of latently-infected AECs at time-points when whole-lung levels of TGF-β1 are not noticeably increased.

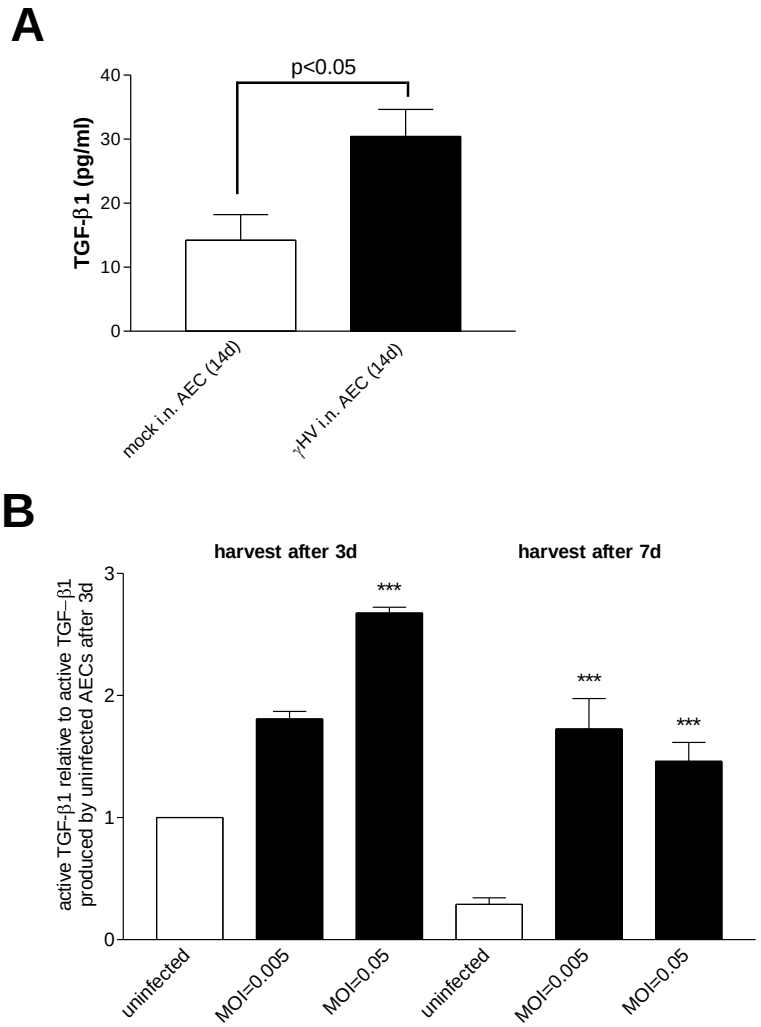


Figure 19: γHV-68 infection induces TGF-β1 production in AECs

A. AECs from latently infected mice produce more total TGF-β1 than AECs from mock-infected mice. WT C57Bl/6 mice were infected i.n. with 5 x 10^4 PFU γHV-68 or mock-infected with saline, and AECs were purified from the lungs of both groups 14 d.p.i. AECs were then cultured for 24h, and the culture supernatants were assayed for total TGF-β1 by ELISA. TGF-β1 from AECs of latently-infected mice is represented by the black bar, and TGF-β1 from AECs of mock-infected mice is represented by the white bar (n=4-6 per group). B. Lung epithelial cells infected with γHV-68 *in vitro* produce more active TGF-β1 than uninfected lung epithelial cells. The bar graph shown reflects the results of a luciferase assay used to measure active TGF- β1 production from epithelial cells in culture for 3d or 7d. The cells were either uninfected or infected at a MOI of 0.005 or 0.05. Active TGF-β1 produced is presented relative to amount of active TGF-β1 produced by uninfected cells after 3d in culture, which was set at 1 (n=6-8 mice per group; ***=p<0.001 compared with uninfected cells from the same harvest).

Latent γHV-68 infection induces cysLT production in AECs

We next tested latently-infected AECs for alterations in cysLT production. We infected mice i.n. with 5 x 10^4 PFU of γHV-68 or mock-infected mice with saline, and AECs were purified from the lungs of the infected mice 21 d.p.i. AECs were then cultured for 24h in the presence of the calcium ionophore, A23187, a stimulus for maximal LT production and the culture supernatants were assayed for cysLTs by ELISA. The level of cysLTs was significantly higher in supernatants from AECs isolated from latently-infected mice compared to supernatants from AECs isolated from mock-infected mice (Fig. 20). We conclude that latent γHV-68 infection alters AECs to produce more cysLTs.

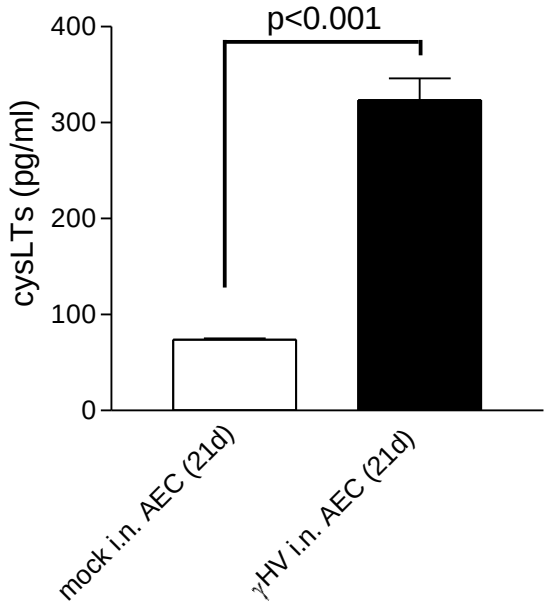


Figure 20: Latent γHV-68 infection induces cysLT production in AECs
WT C57Bl/6 mice were infected i.n. with 5 x 10^4 PFU γHV-68 or mock-infected with saline, and AECs were purified from the lungs of both groups 21 d.p.i. AECs were then cultured for 24h with A23187 (5 μM), and the culture supernatants were assayed for cysLTs by ELISA. CysLTs from AECs of latently-infected mice are represented by the black bar, and cysLTs from AECs of mock-infected mice are represented by the white bar (n=3 per group).

Direct infection of fibrocytes and fibroblasts does not cause early proliferation or pro-fibrotic differentiation

γHV-68 does reactivate and replicate persistently at a low level throughout long-term latency in the lung [230]. We hypothesized that infection-induced alteration of mesenchymal cells could account for some of the increased fibrosis we observed during chronic infection. Thus, we investigated whether direct infection with γHV-68 differentiates fibroblasts and fibrocytes to a more pro-fibrotic phenotype. Expression of α-SMA is an indicator of mesenchymal cell differentiation to a myofibroblast phenotype [244]. Fibrocytes and fibroblasts were isolated from wild-type mice and infected *in vitro* at a MOI of 0.01 or 0.1. After 48h, RNA was isolated from some cells, and cell lysates were made from others, followed by Western blots using antibodies for collagen 1 and α-SMA. Using real-time RT-PCR, we found that γHV-68 does infect both fibrocytes and fibroblasts *in vitro* (data not shown). Prolonged infection (greater than 6 days) leads to lytic destruction of the cells *in vitro*, and viral infection has little influence on proliferation of fibrocytes and fibroblasts during the first 24h after infection (Fig. 21A). 48h hours after infection, collagen 1 expression in fibrocytes is unchanged compared to uninfected fibrocytes, and expression of α-SMA is diminished (Fig. 21B). γHV-68 infection also does not increase collagen 1 or α-SMA expression in fibroblasts (Fig. 21C). Thus, we conclude that infection of fibrocytes and fibroblasts in the lung does not directly lead to enhanced proliferation or differentiation of fibrocytes of fibroblasts.

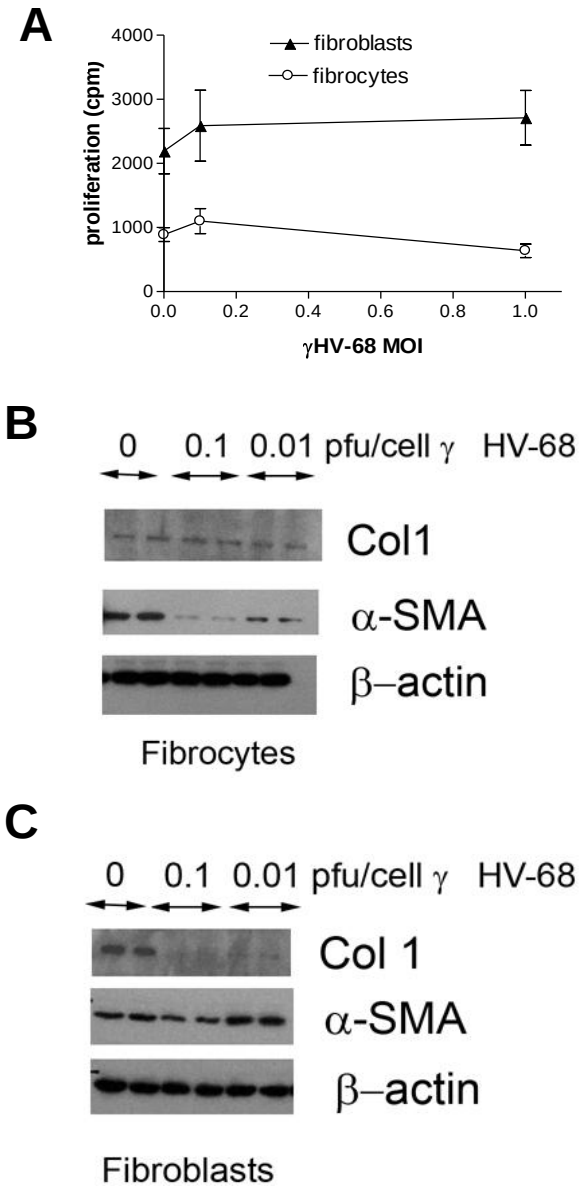


Figure 21: Direct infection of fibrocytes and fibroblasts does not cause early proliferation or pro-fibrotic differentiation

A. Fibrocytes or fibroblasts were purified from lung mince cultures from WT C57Bl/6 mice on day 14. 5 x 10^3 cells/well were left untreated or infected *in vitro* at a MOI of 0.1 or 1 in complete media for 24h, and proliferation was measured over the incubation period via ^{3}H-thymidine incorporation. There was no significant difference between the proliferation rates of uninfected and infected cells of either type, n=6. B. and C. Murine fibrocytes (B) or fibroblasts (C) were purified from WT C57Bl/6 mice, and 4 x 10^5 cells per well were infected *in vitro* at a MOI of 0.01 or 0.1. After 48h, cell lysates were made. 10μg of protein from each lysate were run and analyzed by Western blotting with anti-mouse collagen 1 antibody or anti-mouse α-SMA antibodies. Blots were stripped and probed for β-actin as a housekeeping control.

The influence of cysLTs on fibrocytes

FITC treatment stimulates cysLT production

Our data in Figure 13 indicate that latent γHV-68 infection prior to FITC challenge results in increased accumulation of fibrocytes in the lung compared to FITC treatment alone despite the fact that the production of the chemokines CCL2 and CCL12 are similar in mock- or γHV-68- infected mice following FITC administration (Fig. 15). These results could reflect the fact that the accumulation of fibrocytes in the latently-infected fibrotic mice is the result of the additive recruitment of cells in response to γHV-68 and FITC alone. Additionally, it is possible that the fibrocytes in the γHV-68 + FITC-treated mice are responding to proliferative signals. In this regard, we were intrigued by the finding that latently infected AECs produce cys LTs (Fig. 20). CysLTs have been shown to confer pro-fibrotic effects; mice deficient in 5-LO are protected from bleomycin-induced fibrosis and LTs exert direct effects on migration [116], proliferation [110], and matrix protein synthesis [118] by fibroblasts. The effects of LTs on fibrocytes remain unknown. Whether FITC deposition would result in further increases in cys LT levels in the lungs was also unknown. Thus, we undertook the next series of studies to determine whether FITC-induced fibrosis is associated with cysLT production, whether cysLTs regulate the development of fibrosis, and if so, what effect cysLTs have on fibrocytes.

In order to verify that FITC deposition resulted in cysLT release, we treated WT C57Bl/6 mice with FITC on day 0 and homogenized lungs on days 0, 3, and 7. Lipids were extracted from lung homogenates using C_{18} Sep Pak cartridges. Levels of cysLTs increased on days 3 and 7 post-FITC (Fig. 22A). We next measured the production of

cysLTs from alveolar cells purified by BAL on days 0 and 7 post-FITC. Plastic-adherent cells from the BAL which likely include AMs, fibrocytes and neutrophils were cultured for 1 h in the presence or absence of the calcium ionophore A23187 (Fig. 22B) to provide a maximal stimulus for arachidonic acid release and LT synthesis. Synthesis of cysLTs was significantly elevated in cells purified from FITC-treated mice ($p=0.0002$). Similar results were seen in FITC-treated 129SvEv mice (data not shown). These results indicate that inflammatory cells likely contribute to increased lung cysLTs post-FITC.

5-LO-/- mice are protected from FITC-induced fibrosis

Previous studies have demonstrated that 5-LO-/- mice are protected from bleomycin-induced lung fibrosis [119]. In order to verify that 5-LO-/- mice were protected from FITC-induced fibrosis, we injected WT (129SvEv) or 5-LO-/- mice with FITC on day 0 and measured collagen accumulation in the lungs by hydroxyproline assay on day 21 post-FITC. Figure 22C demonstrates that 5-LO-/- mice are significantly protected from FITC-induced fibrosis.

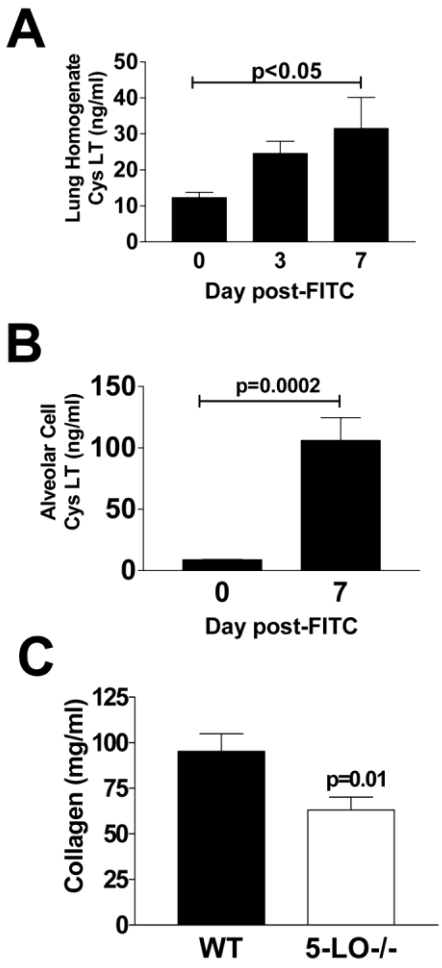


Figure 22: FITC deposition results in release of cysLTs

A. WT (C57Bl/6) mice were injected with FITC on day 0. Lung homogenates were collected on days 0, 3 and 7 post-FITC. Lipids were extracted and levels of cysLTs were determined by specific EIA, n=4-6 per group, $p<0.05$ by ANOVA at day 7. B. Mice were injected with FITC on day 0 and plastic-adherent BAL cells were harvested on days 0 or 7. These cells (which consist mostly of AMs but may contain some fibrocytes and neutrophils post-FITC) were cultured at 5×10^5/ml for 1 h in the presence of 5 μM A23187 and supernatants were analyzed for cysLTs via specific EIA, n=4, p=0.0002 by Student's t-test. C. WT (129SvEv) or 5-LO-/- mice were injected with FITC intratracheally on day 0. On day 21, mice were euthanized, lungs were removed and collagen content was determined via hydroxyproline assay, n=10, p=0.01 by Student's t-test.

Fibrocytes express both the cysLT1 and cysLT2 receptors

We next investigated the expression of LT receptors on fibrocytes. Fibrocytes were purified from C57Bl/6 mice and total mRNA was prepared. The mRNA was analyzed for expression of the two cysLT receptors, cysLT1 and cysLT2, by real-time RT-PCR using mRNA levels in AMs as a positive control. To directly compare the expression of cysLT1 and cysLT2 in fibrocytes, the expression of cysLT1 in AMs was set to 1, and then the expression of cysLT1 and cysLT2 on fibrocytes was compared to this value. As noted in Figure 23A, the levels of cysLT1 mRNA were approximately 12 fold higher in fibrocytes compared to AMs. The levels of cysLT2 mRNA in fibrocytes were approximately half the level of cysLT1 mRNA. We next analyzed the expression of cysLT1 and cysLT2 protein in isolated murine AMs, fibrocytes and fibroblasts by Western blot analysis (Fig. 23B). Despite the differences in mRNA levels noted above, the levels of cysLT1 protein appear similar in both fibrocytes and AMs. In contrast, cysLT1 is expressed at low levels in fibroblasts. CysLT2 protein levels in AMs and fibrocytes appear similar. Interestingly, the level of cysLT2 noted in fibroblasts is greater than the levels on either fibrocytes or AMs. CysLT1 and cysLT2 protein levels in fibrocytes isolated from human peripheral blood were detected at similar ratios to those found in cells from mice (Fig. 23C). The receptors for LTB_4, BLT1 and BLT2, were analyzed by conventional RT-PCR followed by Southern blotting using internal probes, but were found to be undetectable in murine fibrocytes (data not shown). Thus, LT effects on fibrocytes are likely mediated via the cysLT1 and/or cysLT2 receptors.

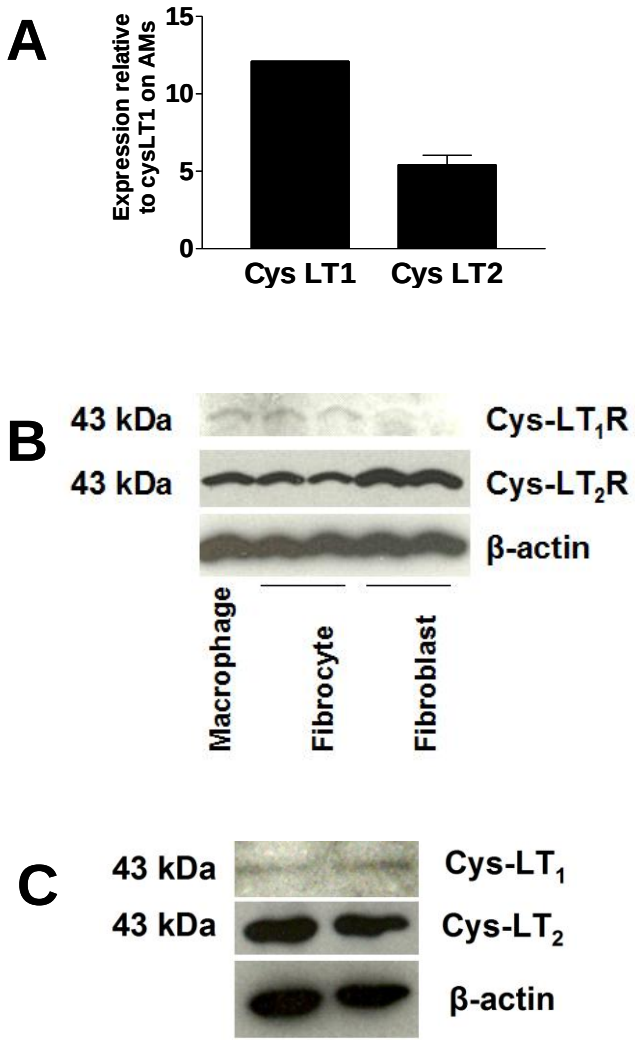


Figure 23: Murine and human fibrocytes express CysLT1 and CysLT2

A. Murine fibrocytes were purified from WT C57Bl/6 lung digest cultures, and total RNA was prepared and analyzed for the expression of CysLT1 and CysLT2 by real-time RT-PCR. The expression level of each gene was first normalized to β-actin and next compared to the expression level of cysLT1 in alveolar macrophages. These data are representative of two independent experiments. B. Murine fibrocytes and fibroblasts were purified from lung digest cultures. AMs were isolated from BAL. 4μg of protein from each lysate was run and analyzed by Western blotting with anti-human CysLT1 or anti-murine CysLT2 receptor antibodies. Each lane represents cells from a single animal. Blots were stripped and probed for β-actin as a housekeeping control. C) Human fibrocytes were isolated from buffy coats of two normal volunteers. Western blotting was performed on 4μg of cell lysates as above.

Fibrocytes produce cysLTs

While it is known that leukocytes produce cysLTs, the ability of fibrocytes, which do maintain some leukocyte markers, to make LTs is unknown. Fibrocytes were cultured overnight in SFM and supernatants were collected for analysis of total cysLTs and LTB_4 by specific EIA. Levels of cysLTs in overnight unstimulated cultures of fibrocytes were 57.2 ± 10.8 pg/ml for cysLTs (Fig. 24). However, when cells were treated with the Ca^{2+} ionophore, A23187, for 1 h which provides a maximal stimulus for arachidonic acid release and LT biosynthesis, levels of cysLTs measured in the fibrocyte cultures were 470 ± 63 pg/ml. For comparison, the levels of cysLTs produced by ionphore-treated fibroblast cultures were only 24.6 ± 15 pg/ml. Levels of LTB_4 in the fibrocyte cultures were undetectable in the overnight unstimulated conditions and reached 25 ± 6 pg/ml in the ionophore-stimulated conditions (not shown). Thus, fibrocytes retain the ability to produce LTs and thus may be responsive to cysLT signaling in either an autocrine or paracrine manner.

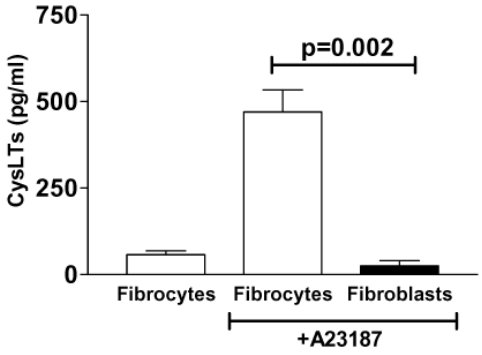


Figure 24: Fibrocytes produce LTs
Fibrocytes and fibroblasts were purified from WT (C57Bl/6) mice and cultured at 10^5/ml overnight in SFM. The next morning, A23187, a calcium ionophore was added for 1 h at 5 μM. Supernatants were collected and analyzed for the production of CysLTs by specific EIA, n=4. Ionophore-stimulated fibrocytes produce significantly more cysLTs than do fibroblasts, p=0.002 by Student's t-test.

LTs enhance fibrocyte proliferation

We examined the effect of endogenous LTs on fibrocyte proliferation by comparing basal proliferative rates of fibrocytes from WT (C57Bl/6) and 5-LO-/- mice over 48 h in complete media. Fibrocytes from 5-LO-/- mice exhibited a diminished proliferative capacity (Fig. 25A). Thus, endogenous LT production can influence fibrocyte proliferation *in vitro*.

We next tested the influence of exogenous LTC_4 and LTD_4 on the proliferation of fibrocytes from WT (129SvEv, Fig. 25B) and 5-LO-/- mice (Fig. 25C). Exogenous addition of LTD_4 increased fibrocyte proliferation of both WT and 5-LO-/- fibrocytes in a dose-dependent manner within 24 h. In contrast, the addition of LTC_4 had little effect in

either cell type. LTD_4 is the most potent agonist of the cysLT1 receptor [135]. The efficacy of LTD_4 compared to LTC_4 suggested that that these effects were being mediated via CysLT1 rather than CysLT2.

To verify receptor utilization, exogenous LTD_4 was added to fibrocytes from WT (C57Bl/6) mice in the presence or absence of cysLT1-specific receptor antagonists, MK571 (Fig. 26A) and Ly171883 (Fig. 26B). Fibrocytes treated with 10 nM LTD_4 proliferated significantly more than fibrocytes did in SFM. Treatment with the cysLT1 antagonists MK571 or Ly171883 abrogated this LTD_4 enhancement of fibrocyte proliferation. The significant effects of the cysLT1 receptor antagonists to block all effects of exogenous LTD_4 confirm that LTD_4 signals proliferation of fibroblasts via the cysLT1 receptor.

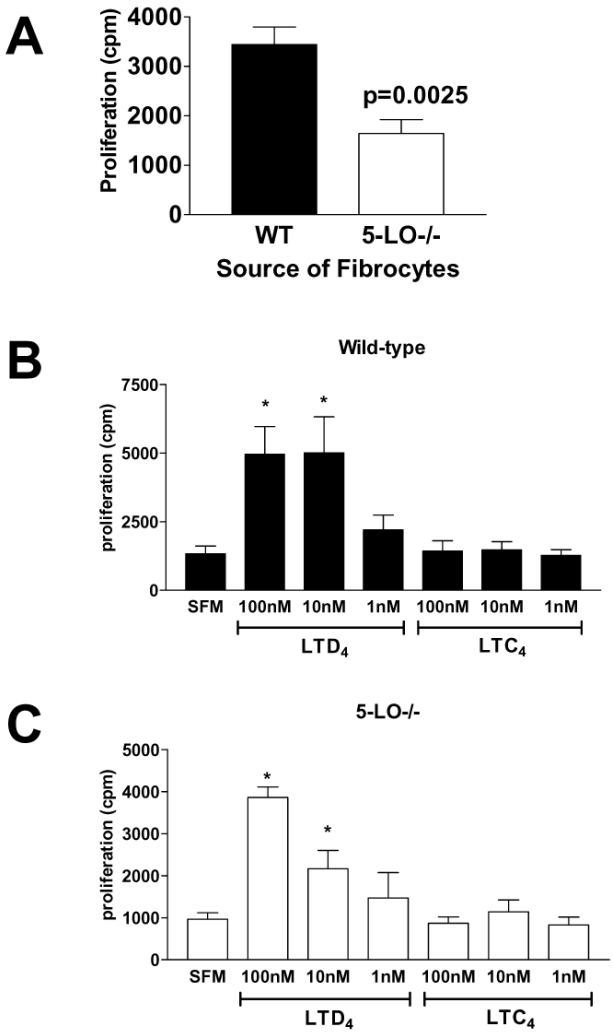


Figure 25: LTs regulate fibrocyte proliferation

A. Fibrocytes were purified from lung mince cultures at day 14 from either WT (C57Bl/6) or 5-LO-/- mice. Fibrocytes were cultured at 2 x 10^5/ml in complete media for 48 h and proliferation was measured via ^{3}H-thymidine incorporation over the final 16 h, n=6, p=0.0025 by Student's t-test. B and C. Fibrocytes were purified from WT (129SvEv) mice (B) or strain-matched 5-LO-/- mice (C) and cultured at 2 x 10^5/ml in SFM with or without 1-100 nM LTD_4 or LTC_4 for 24 h. Proliferation was measured over the final 16 h as previously described, n=6, *p<0.05 by ANOVA.

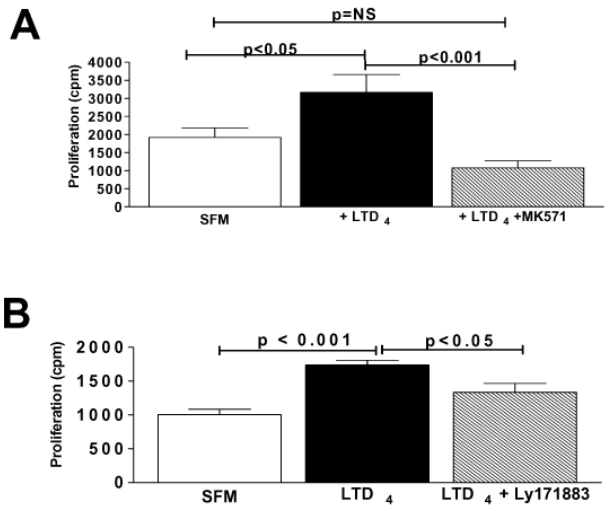


Figure 26: Exogenous LTD_4 can augment fibrocyte proliferation in a Cys LT1-dependent manner

Fibrocytes were purified from lung mince cultures of WT (C57Bl/6) mice and cultured at 2 x 10^5/ml in SFM in the presence or absence of 10 nM LTD_4 and either MK571 at 10 nM (A) or Ly171883 at 1 μM (B) for 48 h, n=6. LTD_4 was able to significantly stimulate fibrocyte proliferation in both experiments. MK571 and Ly171883 both significantly reduced LTD_4-stimulated proliferation ($p<0.05$ for both by ANOVA).

CysLTs do not enhance the differentiation of fibrocytes to fibroblasts *in vitro*

To determine whether cysLTs affect the rate of fibrocyte differentiation to fibroblasts, purified fibrocytes (>98% $CD45^+$ on day 0) from WT (C57Bl/6 mice) were cultured in the presence or absence of 10nM LTD_4 for 4 days. The percentage of $CD45^+$ cells remaining in each culture was then determined by flow cytometry. The percentages of $CD45^+$ cells remaining in the culture were similar for both treatments at day 4 (47.2% untreated versus 43.8% + LTD_4) indicating that LTD_4 did not enhance the differentiation of $CD45^+$ fibrocytes to $CD45^-$ fibroblasts (data not shown).

Fewer fibrocytes are cultured from the lungs of FITC-treated 5-LO -/- mice

We next analyzed the impact of LTs on fibrocyte accumulation by comparing fibrocyte numbers that could be cultured from the lungs in WT (129SvEv) and 5-LO-/- mice. We challenged both groups of mice with FITC, and 5 days later performed a BAL. Cell pellets from the BAL were cultured for 14 days to allow leukocyte populations to die off and mesenchymal populations (fibrocytes) to expand. We found that the number of fibrocytes that can be cultured from 5-LO-/- mice is less than the number of fibrocytes that can be cultured from wild-type mice post-FITC (Fig. 27). These results were verified in WT and 5-LO-/- mice on the C57Bl/6 background. $17 \pm 0.5 \times 10^4$ fibrocytes were cultured from FITC-treated C57Bl/6 mice whereas only $8.6 \pm 0.3 \times 10^4$ fibrocytes were cultured from 5-LO-/- mice post-FITC, $p=0.0006$. Because these experiments were conducted by culturing the cells isolated from the WT or 5-LO-/- mice treated with FITC, it was not clear whether these findings reflected a difference in the initial recruitment of fibrocytes to the lung or differences in the proliferative capacity of the fibrocytes during the two-week culture period.

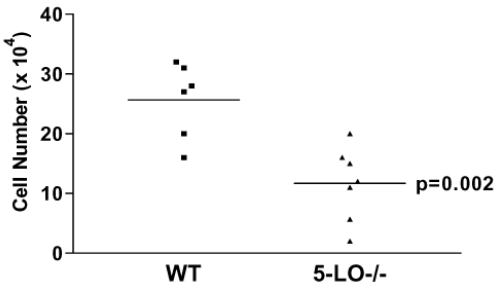


Figure 27: Fewer fibrocytes are cultured from the lungs of FITC-treated 5-LO-/- mice
WT (129SvEv) or 5-LO-/- mice were challenged with FITC on day 0. On day 5 post-FITC, BAL was performed and cell pellets were cultured for 14 days to enumerate fibrocytes. Fewer fibrocytes were cultured from the BAL of 5-LO-/- mice compared to WT mice, n=6-7 per group, p=0.002 by Student's t-test.

LTs are not essential for fibrocyte recruitment *in vivo*

To analyze the initial recruitment of fibrocytes to the lung in response to FITC, we used flow cytometry to identify $CD45^+$, collagen 1^+ cells in either the BAL fluid post-FITC or in lung-digests post-FITC. We compared the recruitment of fibrocytes in WT (C57Bl/6) versus 5-LO-/- mice (Fig. 28), and also the recruitment of fibrocytes in wild-type mice treated with FITC in the presence or absence of the cysLT1 receptor antagonist, MK571 (not shown). We have previously demonstrated that day 5 represents the peak time point for fibrocyte accumulation post-FITC [38]. In the present studies, we found that the number of fibrocytes recruited to the lung on day 5 post-FITC was not affected either by 5-LO genetic ablation or by MK571 treatment. Taken together, these

results suggest endogenous LTs influence proliferation rather than recruitment of fibrocytes.

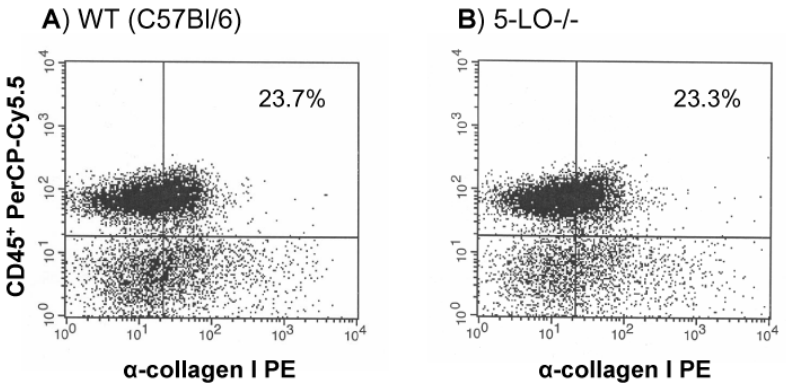


Figure 28: LTs are not necessary for fibrocyte recruitment in response to FITC *in vivo*
WT (C57Bl/6) or 5-LO-/- mice were treated with FITC on day 0. On day 5 post-FITC, lungs were subjected to collagenase/DNAse digest and cells were stained for expression of CD45 and collagen 1. Similar percentages of $CD45^+$, col 1^+ fibrocytes were noted in both groups. Similarly, absolute numbers were not different either. Similar results were obtained when we analyzed the recruitment of fibrocytes to WT mice treated with FITC in the presence or absence of MK571, analyzed BAL cells rather than lung digests, or analyzed WT and 5-LO-/- mice on the 129SvEv background. Thus, LTs do not appear to be essential for fibrocyte recruitment *in vivo*. Graphs are representative of n=3 mice per group. Note that these analyses were not gated in the same manner as those in Figure 7.

Human fibrocytes proliferate in response to LTD_4

Our previous results indicated that human fibrocytes express both cysLT1 and cysLT2 (Fig. 23C). In an effort to determine whether circulating human fibrocytes proliferate in response to LTD_4, peripheral blood was collected from 5 normal volunteers and fibrocytes were isolated. Fibrocytes were then plated in quadruplicate at 20,000

cells/well in SFM or SFM containing 10 nM LTD_4. LTD_4 significantly stimulated the proliferation of the fibrocytes from each subject (p=0.01 compared to SFM only for each). The means of the unstimulated and LTD_4-stimulated proliferation rates for each subject are shown in Fig 29. Thus, both murine and human fibrocytes proliferate in response to LTD_4.

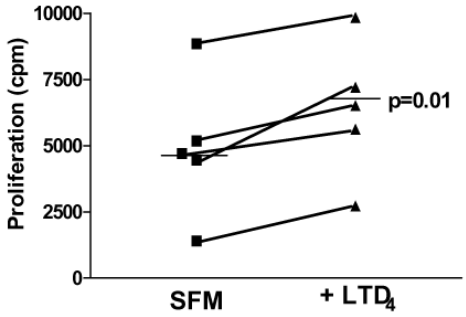


Figure 29: LTD_4 stimulates human fibrocyte proliferation
Fibrocytes were isolated from buffy coats of n=5 normal volunteers grown in the presence of complete media with 20% FCS for 14 days before being trypsinized and plated at 2 x 10^5cells/ml in 96 well flat-bottomed plates in the presence of SFM or SFM + 10 nM LTD_4 for 48 h. Proliferation was measured by incorporation of ^{3}H-thymidine over the final 16 h. Addition of LTD_4 significantly stimulated the proliferation of fibrocytes from each subject (p=0.01 by paired t-test). The mean of each group is shown as the black horizontal line.

CHAPTER FOUR:

DISCUSSION

A role for latent virus in the development of fibrosis

There is accumulating evidence that associates chronic viral infection with the pathogenesis of IPF. Serology, immunohistochemistry, and PCR have correlated the presence of adenovirus, hepatitis C, and particularly herpesvirus infection with the presence of IPF in human patients [159, 160, 163, 165, 173-175]. Tang *et al*. reported the most striking results when they detected 1 or more of 4 herpesviruses in the lungs of 97% of patients with IPF but only 36% of controls [170]. Mouse models have been used to examine whether gammaherpesvirus, γHV-68, contributes to the development of fibrotic lung disease because γHV-68 is genetically and biologically similar to the human herpesvirus, EBV. Lok *et al*. demonstrated that lytic γHV-68 promotes the development of pulmonary fibrosis in the presence of a subsequent exogenous injury [217]. Mora *et al*. have shown that lytic γHV-68 induces pulmonary fibrosis in immunocompromised Th2-biased mice with a phenotype favorable for tissue-remodeling [182]. In response to these findings, we hypothesized that latent γHV-68 could augment pulmonary fibrosis induced by a subsequent fibrotic stimulus in wild-type C57Bl/6 mice. The results of our experiments designed to test this hypothesis demonstrate for the first time that either short-term or long-term gammaherpesvirus latency in the lung significantly augments the

response to a subsequent fibrotic stimulus. Identifying latent γHV-68 as a fibrogenic cofactor is particularly relevant because gammaherpesviruses persist chronically in humans for entire lifetimes in a latent form. Our observations extend prior reports about a role for gammaherpesviruses in lung fibrosis in several other important ways. First, we demonstrate that latent γHV-68 can augment a subsequent fibrotic stimulus and that this augmentation is not associated with substantial reactivation to its lytic phase. Mora *et al*. found reactivation of γHV-68 to be essential for the induction of pulmonary fibrosis in their model using Th2-biased mice [231]. Second, we demonstrate that latent γHV-68 can augment pulmonary fibrosis in immunocompetent wild-type mice. Mora *et al*. had demonstrated the fibrogenic effects of latent γHV-68 that chronically reactivates in IFN-γR-/- mice [182, 231]. We believe our model is more relevant than the Th2-skewed model because no human lungs have complete knockouts of IFN-γ receptors, and although humans do not normally breathe in FITC, they are exposed to numerous other types of occupational and environmental particulate matter. Third, we demonstrate that latent γHV-68 can serve as a fibrotic cofactor by having additive effects with a subsequent fibrotic stimulus whether that stimulus is FITC or bleomycin. The model presented by Mora *et al*. demonstrates that γHV-68 induces fibrosis in Th2-skewed mice through persistent "hits" by chronically reactivating. Lok *et al*. describe lytic γHV-68 participating in a "double-hit model" of fibrogenesis, but this double-hit occurred when the virus was in a lytic state. Our model is the first to describe a latent gammaherpesvirus participating in a "double-hit model" of fibrogenesis. Fourth, we found that the presence of latent γHV-68 at the time of the fibrotic stimulus causes pulmonary fibrosis to occur even in cases when the fibrotic stimulus is inadequate to cause pulmonary fibrosis on its

own. Previously, Lok *et al*. demonstrated only that lytic γHV-68 virus could do this. Moreover, our results are the first to identify potential mechanisms whereby latent gammaherpesvirus infection can augment subsequent fibrotic responses; these will be discussed later.

To test our hypothesis about latent γHV-68, we had to first determine when lytic γHV-68 was cleared from the lungs of infected mice. Our finding that lytic virus was cleared from the lung by 14 d.p.i. leaving only latent virus agrees with previous characterizations of γHV-68 that determined titres of replicating γHV-68 decline to undetectable levels in the lungs between 10 and 15 d.p.i. after i.n. infection [245, 246]. Conversely, we detected latent viral RNA through the latest time-point we tested 30 d.p.i. We are confident that latent virus was present in the lungs even when we measured hydroxyproline after the longest period of latency 91 d.p.i. because Stewart *et al*. have shown that latent γHV-68 persists in the lungs of normal mice for at least 12 months post-infection [214].

We present data to demonstrate augmentation of statistically-significant FITC-induced fibrosis in Figure 2, but the experimental variability of FITC also provided an opportunity to test the effect of the virus on sub-threshold fibrosis induced by FITC. These were situations where mice that had been administered FITC did not have statistically different amounts of hydroxyproline in the lungs 21 days later compared to the lungs of mice that had been administered saline. We present data in Figure 4 to demonstrate that latent γHV-68 still augmented subsequent sub-threshold fibrosis. We think this may be a particularly relevant finding as the causes of human IPF are idiopathic. It is possible that a latent viral infection could predispose patients to develop

fibrotic responses to subsequent injuries that are not themselves sufficient to induce fibrosis.

Since injury to AECs has emerged as a hypothesis for the underlying cause of IPF, and since lytic virus causes injury to AECs, we originally hypothesized that reactivation of latent γHV-68 was necessary for the augmentation we observed. It has proved difficult to demonstrate reactivation of γHV-68 *in vivo*, but in one study using a highly-sensitive plaque assay, Flano *et al*. demonstrated that persistent replication does occur at a low level during long-term latency and is detectable in the lungs of immunocompetent mice 90 d.p.i [230]. We investigated whether the fibrotic stimulus induced further reactivation in our model, and we found that FITC reactivated transcription of lytic viral RNA to a level two orders of magnitude below the levels at the peak of viral gene expression 3-5 days after the primary infection (Figure 5 and data not shown). Given this low level of induction, we were skeptical about the requirement for reactivation in our model. To further investigate this question, we infected mice with a mutant strain of gammaherpesvirus with a 1000-fold reduced ability to reactivate [240]. Despite a severely reduced ability to reactivate, latent ΔORF72 γHV-68 still caused augmentation of subsequent pulmonary fibrosis. Mora *et al*. found lytic viral replication to be critical for γHV-68 fibrogenesis in Th2-skewed mice, but in our model where latent virus is combined with a separate exogenous injury, persistent replication is not necessary for γHV-68 to have pro-fibrotic effects. In other words, persistent lytic virus-induced injury leads to fibrosis in Th2-skewed mice, but in our model latent infection coupled with a secondary injury is also sufficient to elicit fibrosis. Our interpretation of these results is that the development of fibrosis likely requires at least "two hits." In the Mora

model, the two hits are constituted by multiple rounds of lytic injury in the presence of the Th2-skewed environment. Another recent murine model demonstrates that the reactivation of latent murine CMV with a "second hit," in this case cecal ligation and puncture, leads to evidence of pulmonary fibrosis in immunocompetent mice [247]. In our model, we believe the first hit likely involves the enhanced inflammatory response (and perhaps the alteration of cellular phenotypes) caused by the virus such that the subsequent instillation of FITC can drive the augmentation of fibrosis, perhaps by providing a Th2 stimulus as well as inducing pro-fibrotic cytokines. It is possible that the FITC injury substitutes for the "reactivation" injury seen in Mora's studies.

Our results also provide new mechanistic insight into the contribution of γHV-68 to fibrogenesis. We found that several pathogenic elements that are thought to be dysregulated during fibrosis are altered in the lung environment in the weeks following i.n. γHV-68 infection. We were particularly interested in whether these changes persist during latency. Previously, a number of studies have characterized the host immune response to γHV-68 and shown that inflammation persists during latency [182, 245, 248]. McMillan *et al*. from our laboratory showed that fibrocytes are part of the inflammatory response to γHV-68 when enumerated in the lungs 5 d.p.i. [241]. We demonstrate for the first time that the accumulation of fibrocytes persists in the lung beyond the clearance of active γHV-68 infection. Fibrocyte numbers remain significantly elevated in the lung for at least 45 d.p.i. but decrease back to basal levels by 70 d.p.i. The increased number of fibrocytes at the time that the fibrotic stimulus is administered may help explain the augmented fibrosis we observe in the lungs of mice infected for 45 or fewer days because adoptive transfer of fibrocytes has previously been shown to augment FITC-induced

fibrotic responses [80]. Interestingly, we observed that the presence of latent γHV-68 augmented fibrosis triggered 70 d.p.i. despite the fact that we did not note elevated fibrocyte numbers at this time point.. As a result, there is reason to believe that the augmentation induced in the setting of long-term latency proceeds via different mechanisms than the augmentation induced in the context of short-term latency. We can speculate that the augmentation observed with long-term latency is due to residual tissue-remodeling that took place while the fibrocytes were in the lung, but it seems likely that long-term latency may change the lung by altering a great number of other factors that boost an ongoing fibrotic response.

We were not surprised to find that levels of secreted CCL2 and CCL12, chemokines responsible for fibrocyte recruitment, are increased in the lungs beyond the clearance of lytic γHV-68 infection. Our data agree with Weinberg *et al*. who have previously shown that CCL2 transcript levels are increased for at least 44 days in the lungs following γHV-68 infection [243], and also with our previous findings that CCL12 gene expression is upregulated during lytic γHV-68 infection [241]. CCL2 is also known to up-regulate the production of collagen by fibrocytes [38]. CCL2 expression is increased in the lungs of patients with IPF, and CCL12, a murine homologue of human CCL2, is the CCR2 ligand most responsible for fibrocyte recruitment and fibroproliferation in mice [80, 106]. These data suggest that the upregulation of chemokines is one mechanism by which infection with γHV-68 leads to enhanced recruitment of fibrocytes and increased extra-cellular matrix deposition by fibrocytes. Although the viral infection alone is able to recruit fibrocytes, it is likely that maximal

differentiation of those fibrocytes to myofibroblasts may require factors present only in the setting of the "second hit" (e.g. the further induction of TGF-β1 by FITC).

Consistent with the idea that γHV-68 does not cause fibrosis on its own, we did not find a significant change in the number of fibroblasts in the lungs after γHV-68 infection. However, it is also possible that our digestion protocol which utilizes collagenase is not sufficient to extract fibroblasts that are firmly embedded in extracellular matrix, particularly if that matrix is not collagen-based. We think this may be particularly relevant in the setting of infection + FITC (see below).

Our observation of persistent inflammation through 70 d.p.i. is consistent with the initial characterization of γHV-68 in mice by Sunil-Chandra *et al*. who found localized areas of lymphoid infiltrate persisted for at least 170 d.p.i [245]. This is significant because pulmonary fibrosis has been described as the end-point of a chronic inflammatory process consisting of various cell-types including AMs, T cells, neutrophils, eosinophils, and natural killer cells, and an increase in the number of mononuclear phagocytes in lung biopsies from patients with idiopathic pulmonary fibrosis (IPF) worsens prognosis [81, 249, 250]. It is possible that at 70 d.p.i., latent γHV-68 alone recruits macrophages that support a pro-fibrotic environment via the secretion of pro-fibrotic factors [250]. It is also possible that direct infection of AMs causes a pro-fibrotic alteration in the cytokines AMs produce. For instance, *in vitro* infection of primary AMs results in production of both CCL2 and CCL12 (J.B. Weinberg and Moore laboratories, unpublished results). A persistent increase in neutrophils could lead to increased oxidants in the lung and result in more AEC injury in light of the fact that neutrophils from patients with IPF display a more activated phenotype than

neutrophils from control subjects [82]. Clearly these alterations do not cause fibrosis in the setting of viral infection alone, but they may explain the ability of long-term latency to augment FITC-induced fibrosis. Such mechanisms could contribute to the fibrotic augmentation we observed during long-term latency after FITC despite the fact that fibrocyte numbers had declined to baseline in the lungs. In the setting of latency + FITC, the impact of those factors induced during long-term latency may be directed more to the accumulated fibroblasts and myofibroblasts as opposed to fibrocytes at this time-point.

Considering the chronic inflammation during latency, we were surprised that we did not observe a significant change in TNF-α levels in the whole-lung following infection alone. TNF-α over-expression correlates with worse fibrosis in FITC-induced murine fibrosis [104], and McMillan *et al*. showed that lytic γHV-68 augments TNF-α levels when the virus infection occurs after the establishment of FITC-induced fibrosis [241]. TNF-α does not appear to be a significant factor in the augmentation in our model of latent infection prior to FITC challenge, however. In our model, we found that the presence of latent γHV-68 did not change the level of TNF-α in the lungs after FITC administration (data not shown).

Our results do demonstrate that the level of total TGF-β1 is increased in the lungs after γHV-68 infection and beyond the clearance of active infection. Considering TGF-β1 is the most powerful known promoter of extracellular matrix secretion and a number of other fibrogenic processes[26, 58], we expect that γHV-68-induced TGF-β1 production is a mechanism that contributes to the augmentation of fibrosis in our model. It is important to note that measuring levels of active TGF-β1 is difficult in the whole-lung. Our studies were done on acid-treated lung homogenates to allow the measurement

of total TGF- β1 and it is unknown what percentage of the total TGF-β1 we measured was actually active. We assume that since the total levels of TGF- β1 increased, that it is likely that this may represent an increase in active TGF-β1 as well.

Despite many documented changes in the lung environment, γHV-68 infection does not cause pulmonary fibrosis alone in wild-type mice, and additional insults to the lung are necessary for γHV-68 to be a fibrotic cofactor. As a result, we investigated how the combined stimuli of latent γHV-68 plus a subsequent fibrotic insult affected cellular accumulation and the production of pro-fibrotic factors. Our results demonstrate that latent γHV-68 significantly augments the total number of cells and fibrocytes as well as the total amount of TGF-β1 in the lungs after the instillation of the second insult. We believe that these additive effects are responsible in part for the γHV-68-induced augmentation of subsequent pulmonary fibrosis that we observed.

7d after FITC administration and 21 d.p.i., we observed more total TGF-β1 in the lungs of latent γHV-68 + FITC mice than we observed in the lungs of mice that were administered only FITC. The increase is not substantial, but we speculate that any difference in TGF-β1 during the development of fibrosis has a big impact biologically because local concentrations of TGF-β1 may be significantly elevated to elicit an array of pro-fibrotic effects. It is important to note that our model results in focal areas of fibrosis that do not encompass more than about 30% of the lung, thus pro-fibrotic factors found in the focal areas may be diluted significantly when whole lung levels are measured. We did not observe a change in total TGF-β1 levels in the lungs of latent γHV-68 + FITC mice 21d after FITC administration compared to the lungs of mice that had only received FITC. This suggests that latent γHV-68 does not further increase the level of total TGF-

β1 in the lungs at a time-point when fibrosis has been established. These data are not surprising considering that FITC-induced fibrosis is not progressive once fibrosis is established.

In the same mice, 7d and 21d after FITC administration, there were approximately twice the number of fibrocytes in the lungs of latent γHV-68 + FITC mice as there were in the lungs of mice that were administered only FITC. We speculate that at these two time-points, the increase could be a result of the fibrocytes present as a result of γHV-68 infection at 24 or 38 d.p.i. adding to the normal number of fibrocytes that accumulate in response to FITC alone. The number of fibrocytes may also be further increased by cysLT-induced fibrocyte proliferation after FITC administration. It is unclear whether the latent virus would have an additive effect on fibrocyte numbers in the setting of subsequent FITC injury at 70 d.p.i. In the setting of viral infection alone, fibrocyte numbers are decreasing by 70 d.p.i., but we have not yet analyzed fibrocyte accumulation in the setting of subsequent FITC injury at this time-point. This is a focus for future experiments. What we do know is that FITC-induced fibrosis is augmented when FITC is administered 70 d.p.i. Thus, latent viral infection continues to augment fibrosis at the latest time point we tested, and it remains to be determined whether the mechanism involves fibrocyte accumulation at this time-point or whether the mechanism may involve direct pro-fibrotic effects on the accumulated fibroblasts, myofibroblasts, and other inflammatory cells.

We found no significant additive increase in fibroblast numbers in the lungs of latent γHV-68 + FITC mice, but as mentioned above, this could reflect a technical inability to digest fibroblasts out of fibrotic lung tissue. We are surprised we did not find

more fibroblasts in the lungs of γHV-68 + FITC mice given the presence of more fibrocytes, more collagen and more TGF-β1 in these lungs. A focus of future experiments could be to explore histological methods or alternative digestion methods to enumerate fibroblasts and myofibroblasts in these lungs.

The total number of cells in the lungs was augmented in latent γHV-68 + FITC mice 7d and 21d following FITC administration. Histological staining indicates that the presence of latent γHV-68 also changed the nature of the inflammatory response after FITC administration. In lungs of γHV-68 + FITC mice, inflammatory cells formed focal clusters in lungs that were not seen in mice that were administered FITC alone. This suggests that local cytokine and chemokine milieus are organized differently in the lungs of the two groups as well. However, we did not observe an augmentation in the level of CCL2 and CCL12 in the lungs of γHV-68 + FITC mice. The levels of chemokines post-FITC and post-virus alone are similar so it is likely that either stimulus is a maximal stimulus on the cells making the chemokines. This could be the reason there was no additive effect on CCL2 and CCL12 levels after the stimuli were combined. It is not yet known whether the focal distribution of the chemokines was altered or whether other chemokine levels are altered in this setting.

AECs are latently-infected *in vivo* in our model for at least 21 d.p.i. The level of M3 gene expression declines in AECs isolated during the third week after infection when compared to AECs isolated one week post-infection. The decline could represent death of infected AECs *in vivo* or possible differentiation to fibroblasts via EMT. It could also be indicative of a gradual silencing of viral gene expression at later time-points during latency. Our data agree with another study that demonstrates AECs to be a reservoir of

latent γHV-68 for at least 54 d.p.i. *in vivo* [214]. EBV has previously been localized to AECs of IPF patients and has been associated with endoplasmic reticulum stress, the unfolded protein response, and a worse-prognosis [251, 252]. Specifically, the expression of EBV latent membrane protein 1 in AECs is associated with poor prognosis [177].

As a result, we hypothesized that latent γHV-68 alters the AEC phenotype to contribute to the augmentation of fibrocyte numbers, inflammation, and TGF-β1 we had observed in the whole lungs. Our results demonstrate that latent infection of AECs induces transcription of CCL2 and CCL12 mRNA, production of more TGF-β1, and production of more cysLTs. Data collected by Antoniades *et al*. and Mercer *et al*. corroborate our findings by showing AECs are a prominent source of CCL2 during pulmonary fibrosis [106, 253]. We observed a delay in the induction of CCL2 gene expression until 21 d.p.i. in AECs that does not correlate directly with total CCL2 mRNA levels in the lungs of latently infected mice measured by our group and others. Much of the CCL2 gene expression in the lung during this early period after infection may be contributed by inflammatory cells. We also found that AECs harvested from latently-infected mice produce significantly more total TGF-β1 than AECs from mock-infected mice do. In addition, we were able to use mink lung epithelial cells stably transfected with a reporter construct encoding a luciferase gene with a PAI-1 promoter to demonstrate that active TGF-β1 was being produced directly by AECs after γHV-68 infection. Since epithelial cells infected *in vitro* do not live for more than 2 weeks in culture, we only measured the effect of lytic γHV-68 and found that it significantly increases active TGF-β1 production for 7 d.p.i. These data provide further support for

the idea that concentrations of TGF-β1 are elevated locally in areas of latently-infected AECs whether whole-lung levels of TGF-β1 are markedly increased or not. Our findings are consistant with the studies of Malizia *et al*. who found that AEC injury with EBV upregulates TGF-β1 expression in a human cell line [252]. We also demonstrated that AECs harvested from the lungs of latently-infected mice produce significantly more cysLTs than AECs harvested from the lungs of mock-infected mice despite our observation that γHV-68 increases cysLT levels significantly in the whole-lung during lytic infection but not during latency. These data further suggest that latent viral infection of AECs changes the local milieu around AECs during viral latency even if changes in the amount of a particular factor are not obvious when measuring the lungs as a whole. We think these results may help to explain the focal nature of the fibrotic process that occurs in our model. Interestingly, fibrosis is heterogenous in IPF patients as well and it may relate to local alterations in AEC functions.

We found that direct infection of mesenchymal cells does not appear to contribute to the increased fibrosis we observed. Direct infection of fibrocytes and fibroblasts *in vitro* does not induce pro-fibrotic differentiation, and it also does not induce proliferation in the first 24h post-infection. Indirectly, however, we speculate that the increased TGF-β1 and CCL2 we have observed in the infected lungs contribute to pro-fibrotic differentiation of fibrocytes or fibroblasts *in vivo* at later time-points.

The influence of cysLTs on fibrocytes

Our results have also demonstrated a new mechanism through which fibrocytes can proliferate in our model and other models of fibrotic disease. We found that cysLT

production, whether induced by latent γHV-68, FITC or another stimulus, can cause fibrocytes to proliferate. Our studies about the relationship between fibrocytes and cysLTs are important because fibrocytes have convincingly been shown to be recruited to murine lungs in response to fibrotic injury [38, 39], and now we have shown that fibrocytes accumulate in the lungs during γHV-68 latency. As such, a better understanding of the factors which recruit and activate fibrocytes is needed.

Fibrocytes may be able to augment fibrosis through a variety of mechanisms. First, they contribute to collagen production directly. Second, they secrete pro-fibrotic and pro-inflammatory factors which likely augment fibrotic responses. Third, they may differentiate into myofibroblasts to participate in lung contraction and extracellular matrix deposition. Increases in the total number of fibrocytes within the lung, either by direct chemotactic mechanisms or *in situ* proliferation would likely augment fibrotic responses. Our studies are the first to demonstrate the ability of fibrocytes to make and respond to LTs.

We wished to test the hypothesis that LTs regulate fibrocytes in a manner that contributes to the development of pulmonary fibrosis. These mechanistic studies were carried out in the absence of viral infection. We first demonstrate that LT production was specifically stimulated by the instillation of FITC as it is by bleomycin [119] and that LTs play a causal role in FITC-induced fibrosis. We show that fibrocytes produce cysLTs and respond to them in an autocrine or a paracrine fashion by proliferating and migrating *in vitro*. We also demonstrate that bFGF is a fibrocyte mitogen (data not shown). These are the first studies to provide evidence for any factor that regulates murine fibrocyte proliferation. One previous report demonstrated that addition of

endothelin-1 or TGF-β1 to human circulating fibrocytes can stimulate BrdU incorporation over a period of 4 days [75]. It is interesting to note that in other systems, endothelin-1 and TGF-β1 have been noted to upregulate cysLT production [254, 255]. Thus, it is possible that cysLTs are common effector molecules for fibrocyte proliferation, but they do not enhance fibrocyte differentiation into fibroblasts *in vitro*. Our *in vivo* studies show that the initial recruitment of fibrocytes to fibrotic injury is not inhibited in the absence of LTs. Because fibrocytes can be recruited to sites of FITC-induced injury by CCR2, CXCR4, and CCR7-mediated signals [38, 39], we speculate that factors such as CCL12 or SDF-1 (CXCL12) may be responsible for fibrocyte recruitment in the absence of LTs. As a result, the fact that fewer fibrocytes can be cultured from BAL of 5-LO-/- mice post-FITC-induced injury likely reflects reduced proliferation rates. Given these findings, we propose that protection from fibrosis exhibited by 5-LO-/- mice [[119] and the current studies] or mice treated with cysLT1 antagonists [256, 257] likely results, at least in part, from diminished *in situ* proliferation of fibrocytes in the challenged lungs.

Fibrocytes are cells which express both mesenchymal and leukocyte markers. Our studies demonstrate that fibrocytes, like leukocytes, retain the ability to produce LTs. This is the first description of eicosanoid production by fibrocytes. The ability of fibrocytes to produce LTs would be expected to augment the chemotaxis of both leukocytes and resident fibroblasts. Our studies confirm that resident lung fibroblasts express both cysLT1 and cysLT2 receptors. In addition, previous reports have demonstrated that cysLTs can augment proliferation of resident lung fibroblasts [33, 107, 108]. The secretion of LTB_4 may serve as a potent neutrophil chemotactic molecule

[258]. The role that neutrophils play in fibrotic responses is unclear, but certainly the release of reactive oxygen species and neutrophil elastase may contribute to local tissue injury [259]. The fact that cysLTs are elevated in fibrotic lungs is well-documented in both murine and human studies [33, 119], and in the mouse, cysLTs are the predominant LT generated in the lung [119]. Thus, it is likely that the recruitment of fibrocytes to the lung via any chemotactic stimulus would result in the local expansion of these cells via cysLT-mediated proliferation. As fibrocytes have also been shown to be recruited to airways in the setting of asthma [75], the presence of cysLTs in this disease would also be expected to enhance the local proliferation of fibrocytes and this may be an important aspect of the airway remodeling that occurs in this disease setting. In this regard, it is important to note that human fibrocytes also proliferate in response to LTD_4.

Our results demonstrate that LTD_4 is a more potent mitogen for fibrocytes than is LTC_4. Additionally, cysLT1 receptor antagonists block the effects of LTD_4 on fibrocyte proliferation. These results suggest that LTD_4 mediates its effects on fibrocytes solely via the cysLT1 receptor. We speculate that the proliferative effects of LTD_4 on fibrocytes are important to the pathogenesis of lung fibrosis. We utilized two different cysLT1 receptor antagonists in these studies, MK571 and Ly171883. MK571 has previously been shown to be a competitive inhibitor of the multidrug resistance protein adenosine triphosphate (MRP ATP)-binding cassette transporter, which can regulate cysLT secretion, but only at doses above 1 μM [260]. Similarly, Ly171883 has been shown to be both a peroxisome proliferator-activated receptor (PPAR)-α [261] and PPAR-γ [262] ligand, but again at doses 10-10,000-fold higher than used in our studies. Thus, we believe the findings that both of these inhibitors blocked LTD_4-induced

proliferation when used at the doses employed strongly support a role for cysLT1-mediated proliferative effects.

5-LO-/- mice are protected from lung fibrosis induced by both bleomycin [119] and FITC at day 21 post-challenge. Our studies provide mechanistic insight into these observations and suggest that 5-LO-/- mice are protected at least in part because LTD_4-induced mitogenic effects on fibrocytes mediated via cysLT1 are lacking in the 5-LO-/- mice. Our results are consistent with previous studies which demonstrated that dosing of mice with either of the cysLT1 receptor antagonists MK571 [256] or montelukast [257] limited bleomycin-induced lung injury and fibrosis in mice. Additionally, our findings are consistent with the observation that cPLA2-/- mice have reduced levels of LTs in their lungs in response to bleomycin challenge and are protected from fibrosis [263]. LTC_4 synthase-deficient mice are similarly protected from bleomycin-induced fibrosis [264]. However, our findings are not consistent with those of Beller *et al*. who demonstrated that cysLT1-/- mice showed evidence of septal thickening and deposition of reticular fibers that was enhanced compared to WT mice at day 12 post-bleomycin [264]. Our observations are also not consistent with the findings from the same group that cysLT2-/- mice were protected from bleomycin-induced fibrosis on day 12 [265]. Based on our findings of cysLT2 on resident fibroblasts, we would anticipate that cysLT2-/- mice might show some protection from fibrosis. However, it is not clear why the reported results in the cysLT1-/- mice are different from the results using cysLT1 antagonists *in vivo* [256, 257] or our results in the 5-LO-/- mice, but this may reflect differences in the time-points of assessment (d12 vs. d21), model systems utilized, or methodology (Ashcroft scores vs. hyrdoxyproline assays) .

Given our findings that viral infection can result in both fibrocyte accumulation and cysLT production, it is interesting to speculate that latent γHV-68 infection augments fibrosis via a cysLT-dependent mechanism. To address this, we hope to perform studies of viral augmentation of FITC-induced fibrosis in 5-LO-/- mice. We feel it is important to do these studies in the C57Bl/6 background, however, and these mice have been on backorder from Jackson Laboratories for over a year. We are hopeful that we can perform these experiments for future studies.

Final thoughts, future directions, and implications

In sum, we constructed a mouse model to further our understanding about pulmonary fibrosis etiology and pathogenesis. Our model proved that a latent gammaherpesvirus infection can act as a cofactor in the development of subsequent pulmonary fibrosis. Our results demonstrate for the first time that short-term or long-term latency significantly augment a subsequent fibrotic stimulus in immunocompetent mice even if the stimulus is sub-threshold.

Moreover, our results are the first to identify potential mechanisms whereby latent gammaherpesvirus viral infection can augment subsequent fibrotic responses. We interpret our findings to suggest that the development of fibrosis likely requires at least "two hits." The first hit likely involves the enhanced inflammatory response (and perhaps the alteration of cellular phenotypes such as AECs) caused by a virus or another exogenous factor. Our results demonstrate that CCL2, CCL12, TGF-β1, and the number of fibrocytes are each increased after the first "hit" in our model. Total inflammation, fibrocyte number, and TGF-β1 (at least 7d after FITC) are augmented along with

hydroxyproline after a combination of two hits. It is likely that factors produced in the context of latent viral infection elicit pro-fibrotic effects on the elements dysregulated by FITC-induced fibrosis and vice-versa. For instance, we also demonstrated that AECs are infected during latency and that latently-infected AECs produce more chemokines, TGF-β1, and cysLTs. The altered mediator milieu generated by these AECs can promote pro-fibrotic differentiation and proliferation of fibrocytes or fibroblasts accumulating in response to viral infection or FITC. (The effects of latently-infected AECs are illustrated in Fig. 30). We demonstrate for the first time that cysLTs regulate fibrocyte proliferation. We also showed for the first time that production of cysLTs is upregulated in the lungs of mice during FITC-induced fibrosis. We already know that multiple other pro-fibrotic factors are up-regulated after FITC administration so a variety of factors including cysLTs could cause proliferation or activation of fibrocytes or fibroblasts accumulating in response to FITC and/or virus infection.

Using our animal model of latent viral augmentation of subsequent fibrosis is a unique way to study the pathophysiology of pulmonary fibrosis with the ultimate goal of defining future treatments for IPF. First, it should be noted that there is still research to be done so that we have a better understanding about how results from our model can be translated to human pathology. Despite similarities between γHV-68, EBV, and HHV-8, more research is necessary to completely understand the differences between the consequences of infection in humans and mice. In addition, differences between FITC- and bleomycin-induced fibrosis and fibrosis in humans have been documented [184]. In the meantime, the model can be used to determine in more detail the molecular

mechanisms involved in the dysregulated repair of injured lungs with the specific goal of identifying particular cell-types or pro-fibrotic factors critical to virus-induced augmentation of fibrosis. We are interested in investigating whether latent γHV-68

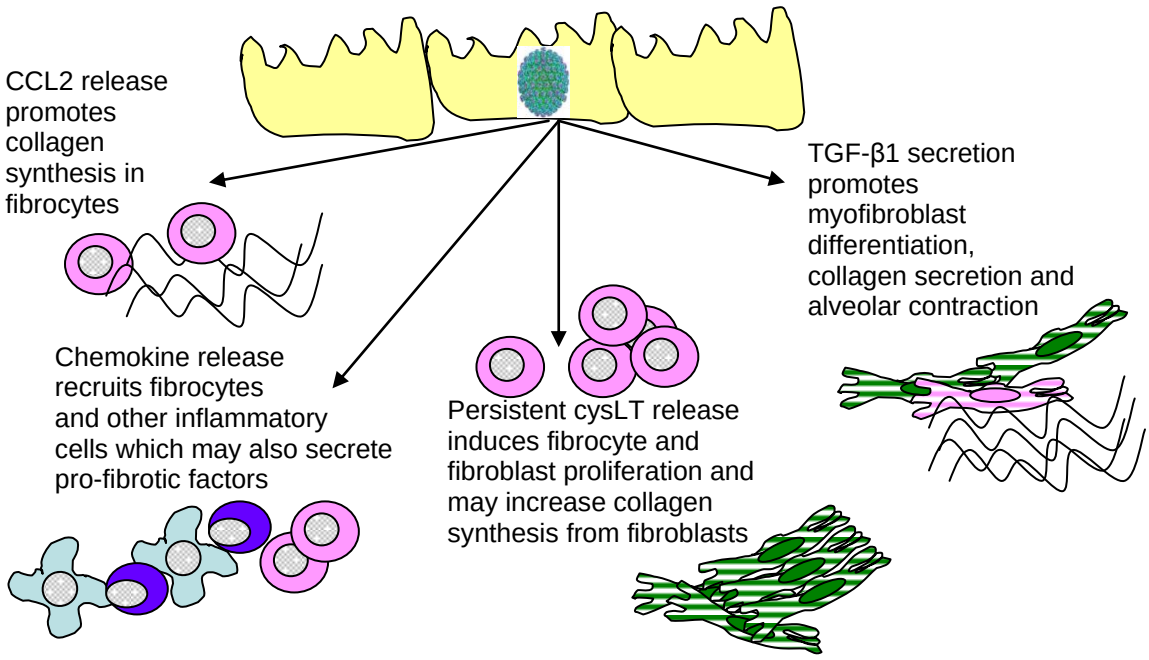
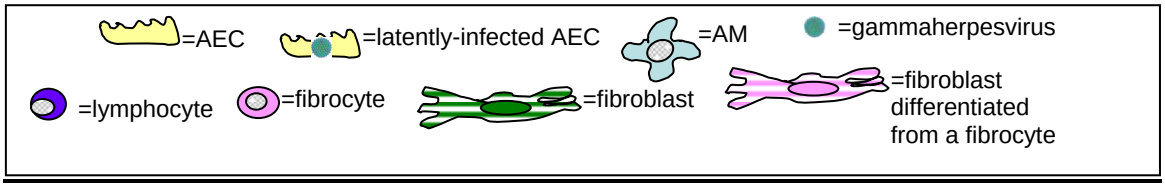


Figure 30: Focal changes induced by latently-infected AECs that promote fibrosis
Schematic representation depicting a microenvironment in the lung around a latently-infected AEC.



infection augments fibrosis via a cysLT-dependent mechanism using cysLT1-/- mice. If these mice are unavailable, 5-LO-/- mice could be used to investigate whether latent γHV-68 infection augments fibrosis via a LT-dependent mechanism. We speculate that fibrocytes are critical to the viral-induced increases in fibrosis, but we have not yet

explored the results of virus + FITC in mice with fibrocyte recruitment defects. To address this, we hope to perform studies of viral augmentation of FITC-induced fibrosis in CCR2-/- mice. Experiments with CCR2-/- have been difficult to complete thus far because of the lack of availability of CCR2-/- mice and a high rate of mortality among mice treated with virus + FITC. It is possible that CCR2 regulates fibrotic or injury processes that are independent of fibrocyte accumulation. While our data suggest that fibrocytes are contributing to the augmentation we observe, it also indicates that fibrocytes do not account for the entire viral impact on fibrosis because we still observed an augmentation of fibrosis induced by virus that had been latent for 70 days despite the fact that virus alone does not keep fibrocytes elevated for that long.

The studies by Mora *et al*. suggested that anti-viral therapies may improve fibrotic outcomes. Since our studies suggest that viral reactivation is not critical to the augmentation we observed, anti-viral drugs are unlikely to be a useful therapy in our model. A more fruitful area for therapeutic investigation may be to try to find mechanistic pathways that are similar in both models. Thus, it will be important to further understand how latent virus infection alters the phenotypes of AECs, macrophages, fibrocytes, and fibroblasts to understand what factors they produce. Our findings about latency-induced changes in the phenotype of AECs indicate that local changes in the lung environment and between cell-types are likely playing a significant role in the virus-induced augmentation we observed. For instance, herpesviruses have been shown to induce the production of the coagulation protein, thrombin, to enhance viral infection, and it has also been demonstrated that thrombin can activate AECs to produce CCL2 during IPF [253, 266]. Focal changes could be studied further with

immunohistochemical techniques or *in situ* hybridization. It will be interesting to know more about the balance between Th1 and Th2 cytokines in these micro-environments as well as whether γHV-68 expresses cytokines itself. Since fibroblasts are known to be the primary producers of extracellular matrix during fibrotic disease, it will be important to better understand their place in our model. We plan to explore different histological methods or alternative digestion methods to enumerate fibroblasts and myofibroblasts in fibrotic lungs.

Our description of the role of cysLTs as mitogens for fibrocytes provides a new rationale for employing LT inhibitors or cysLT1 receptor antagonists in anti-fibrotic therapy. One potential advantage of targeting long-term therapy to blockade of the cysLT1 pathway rather than blockade of all LT production may be the ability to deliver anti-fibrotic therapy without diminishing the ability of LTB_4 to participate in innate immune actions on AMs and polymorphonuclear leukocytes (PMNs). It will be interesting to test whether cysLTs can activate fibrocytes or fibroblasts to increase collagen expression, and it will also be interesting to investigate if and how cysLTs can regulate TGF-β1 production by AECs. LTD_4 has been shown to induce TGF-β1 production in human bronchial epithelial cells [267]. Conversely, it will also be interesting to test the effects of TGF-β1 induced by viral infection on the relationship between AECs and PGs and PG receptors because γHV-68 induces PGE_2 production to enhance its own replication.

Lastly, we recognize that IPF is likely multifactorial, and that it is likely that individual patients each suffer from unique patterns of disease. Our model is one example of how this devastating disease may occur. Our hope is that the mechanisms

that we have elucidated as being involved may represent common final pathways in many forms of fibrosis and that our studies may inform future therapeutic investigations.

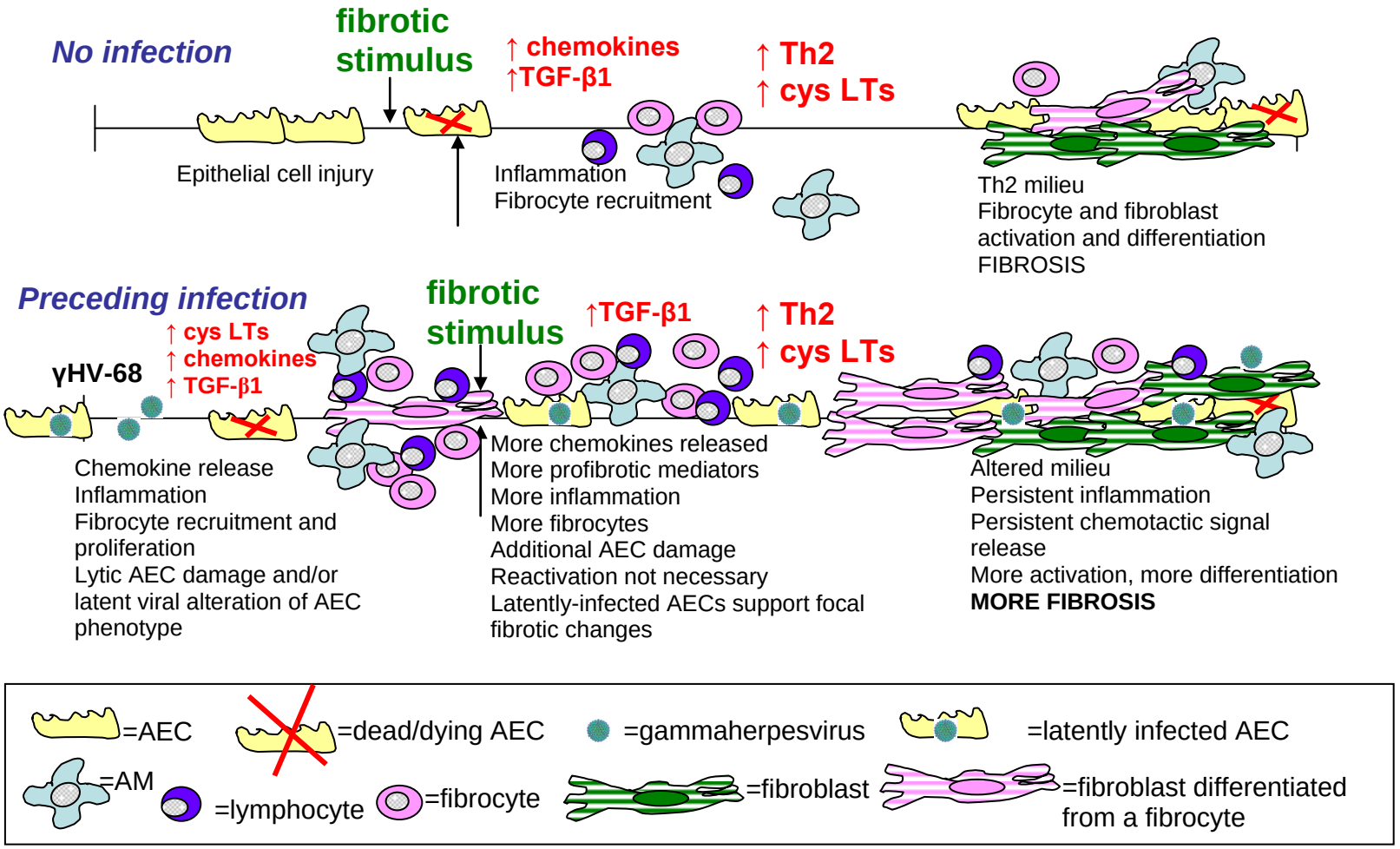


Figure 31: Potential mechanisms involved in the gammaherpesvirus-induced augmentation of fibrosis

Latent herpesvirus infection augments a subsequent fibrotic stimulus in the lung via the recruitment of fibrocytes and the induction of pro-fibrotic factors.

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