γ-Herpes virus-68, but not Pseudomonas aeruginosa or influenza A (H1N1), exacerbates established murine lung fibrosis

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Am J Physiol Cell Physiol 307: L219–L230, 2014. First published May 30, 2014; doi:10.1152/ajpcell.00300.2013.—Patients with idiopathic pulmonary fibrosis (IPF) often do worse following infection, but the cause of the decline is not fully understood. We previously demonstrated that infection with a murine gamma herpes virus (γHV-68) could exacerbate established lung fibrosis following administration of fluorescein isothiocyanate (McMillan et al. Am J Respir Crit Care Med 177: 771–780, 2008). In the present study, we anesthetized mice and injected saline or bleomycin intratracheally on day 0. On day 14, mice were anesthetized again and infected with either a Gram-negative bacteria (Pseudomonas aeruginosa), or with H1N1 or γHV-68 viruses. Measurements were then made on days 15, 21, or 35. We demonstrate that infection with P. aeruginosa does not exacerbate extracellular matrix deposition post-bleomycin. Furthermore, fibrotic mice are effectively able to clear P. aeruginosa infection. In contrast, bleomycin-treated mice develop worse lung fibrosis when infected with γHV-68, but not when infected with H1N1. The differential ability of γHV-68 to cause increased collagen deposition could not be explained by differences in inflammatory cell recruitment or whole lung chemokine and cytokine responses. Alveolar epithelial cells from γHV-68-infected mice displayed increased expression of TGFβ receptor 1, increased SMAD3 phosphorylation, and evidence of apoptosis measured by cleaved poly-ADP ribose polymerase (PARP). The ability of γHV-68 to augment fibrosis required the ability of the virus to reivate from latency. This property appears unique to γHV-68, as the β-herpes viruses, cytomegalovirus, did not have the same effect.

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However, *P. aeruginosa* is responsible for life-threatening infections in immunocompromised patients, the elderly, and following prolonged hospitalization (42). Clearance of *P. aeruginosa* from the lungs requires a functional innate immune system with the involvement of macrophages and polymorphonuclear leukocytes (PMNs) (20). Influenza A is a RNA virus that replicates in the respiratory epithelium leading to the infiltration of inflammatory cells, mainly mononuclear leukocytes and small numbers of PMNs. Innate defense against influenza A infection involves the production of high levels of type I interferons by infected epithelial cells, alveolar macrophages (AMs), recruited conventional dendritic cells (cDCs), PMNs, and NK cells (9, 16, 32). DCs lining the airways play key roles in activating effector CD8 T cells mediating viral clearance and protection (3, 22). In contrast, γHV-68 can infect a variety of cells within the lung including epithelial cells, fibroblasts, macrophages, and B cells (36, 37). There is low level induction of type I interferon, and plasmacytoid DCs are necessary to activate cDCs in vitro (49). Production of both IFNγ and perforin are important for viral control (34, 40). In this study we used bleomycin to establish fibrosis in mice, and then compared the ability of *P. aeruginosa*, H1N1, and γHV-68 to exacerbate the fibrotic response.

**MATERIALS AND METHODS**

**Animals.** Male wild-type C57BL/6 (B6) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed under pathogen-free conditions and provided food and water ad libitum. All animal experiments complied with university and federal guidelines for humane use and care. The University of Michigan Committee on Use and Care of Animals approved these experiments.

*P. aeruginosa* PA01 infection. As previously described, *P. aeruginosa* PA01 inoculum was prepared and mice were injected intratracheally with a sublethal dose of 5 x 10^5 colony forming units (CFU) (2, 21). For exacerbation studies, mice were infected with *P. aeruginosa* on day 14 post-bleomycin.

Quantification of bacterial burden in the lung and blood. Mice were euthanized 24 h following intratracheal infection with *P. aeruginosa*. Blood and whole lung samples were collected. Bacterial burden in whole lung and blood samples was analyzed by CFU assay as previously described (2).

Total lung leukocyte preparation. Whole lung samples were harvested from mice and collagenase-digested as previously described (14). For each sample total viable cells were counted on a hemocytometer by trypan blue exclusion. Differential analysis was done using the total lung cells isolated from the collagenase-digested whole lung samples to determine the percentage of neutrophils, eosinophils, and monocytes/macrophages as previously described (14).

Flow cytometry. Whole lungs were enzymatically digested using collagenase and DNase (24) and leukocytes were isolated. Leukocytes were incubated with Fc block (1:100) clone 24G2 (BD Pharmingen, San Diego, CA) for 15 min, then stained with combinations of anti-mouse CD45, CD4, CD8, T cell receptor β, NK1.1, and CD19 directly conjugated antibodies available from BD Pharmingen. Cells were analyzed on a FACScan (BD Biosciences, Mountain View, CA). Gene specific primers and probes were purchased from Sigma-Aldrich (St Louis, MO). Relative expression was calculated using the comparative CT method with beta actin (β-actin) as an internal standard gene control. Fold change in mRNA was quantified using the ΔΔCT method.

Western blot analysis. AECs were lysed in radioimmunoprecipitation assay buffer with protease inhibitor cocktail (Sigma) for 15 min at 4°C and centrifuged. Total protein concentrations in the supernatants were determined by the Bicinchoninic acid assay (Pierce). Equal amounts of protein from each sample were separated on a 4–20% gradient SDS-polyacrylamide gel and transferred to a PVDF membrane (Amersham/GE Healthcare, Pittsburgh, PA). PVDF membrane was then probed with rabbit polyclonal PARP, SMAD3 (Cell Signaling, Beverly, MA) and β-actin (Sigma).

**Statistical analysis.** Statistical analysis was performed by analysis of variance (3 or more comparisons) with a Bonferroni post hoc test or Student t-test (2 comparisons) using GraphPad Prism 6 software (San Diego, CA). Data shown represent means ± SE. *P* < 0.05 was considered significant.

**RESULTS**

*P. aeruginosa* infection has no effect on bleomycin-induced pulmonary fibrosis. To determine the effects of *P. aeruginosa* infection on established pulmonary fibrosis, mice were given intratracheal saline or bleomycin on day 0. On day 14, mice were then given 5 x 10^5 CFU *P. aeruginosa* intratracheally or were mock infected. All lungs were harvested on day 21 and lung collagen content was measured by hydroxyproline assay. As expected, bleomycin-treated mice showed significant increases in collagen content compared with saline controls. However, subsequent infection of bleomycin-treated mice with *P. aeruginosa* showed no significant increase in collagen content compared with bleomycin-treated mice that were mock infected (Fig. 1). Thus *P. aeruginosa* infection in wild-type mice does not exacerbate bleomycin-induced fibrotic response in the lungs. Furthermore, at this dose of infection, there was no difference in the survival of bleomycin-treated mice that were mock infected or infected with *P. aeruginosa* (data not shown).

Fibrotic mice do not show increased susceptibility to *P. aeruginosa*. To determine whether the bleomycin-treated mice were more susceptible to infection with *P. aeruginosa*, mice were treated with saline or bleomycin as previously described. On day 14, both groups of mice were infected with 5 x 10^5
Exacerbation of established pulmonary fibrosis could occur on day 21, a time point noted for maximal bacterial clearance, we could not detect virus by plaque assay in the lungs at either day 3 or day 7 postinfection in saline or bleomycin-pretreated mice (data not shown). In contrast, the γHV-68 DNA pol gene was significantly elevated 7 dpi in bleomycin-treated compared with saline-treated mice confirming earlier observations (23). In addition, we were able to demonstrate virus by plaque assay following γ-HV-68 infection (Fig. 5C).

**Exacerbation of lung fibrosis by γHV-68 requires the ability to reactivate from latency and is not a property shared by cytomegalovirus (CMV).** A v-cyclin mutant γHV-68 (ΔORF72) is 100-fold decreased in its ability to reactivate from latency (43). In our hands, mice infected with ΔORF72 display decreased replication within the lung compared with wild-type marker rescue virus by day 3 postinfection (data not shown). To see if on-going viral replication was required to promote fibrosis, we infected bleomycin-treated mice with mock control, marker-rescue virus, or ΔORF72 mutant virus and analyzed collagen deposition at day 21 (Fig. 6A). Wild-type (marker rescue) virus significantly enhanced fibrosis whereas ΔORF72 did not. Surprisingly, infection with a β herpes virus, CMV, did not exacerbate bleomycin-induced fibrosis either (Fig. 6B). We were unable to plaque infectious CMV from the lungs of these

**Fig. 1. Pseudomonas aeruginosa infection does not exacerbate bleomycin-induced fibrosis.** Wild-type mice were given bleomycin or saline intratracheally on day 0. On day 14, half of the mice in each group were given *P. aeruginosa* (PA) intratracheally or saline as a vehicle control. Lungs were harvested on day 21 for hydroxyproline assay. Data shown represent *n* = 6–10 mice per group pooled from 3 independent experiments. *P* < 0.05. ns, not significant.

**Fig. 2. Bleomycin-treated mice show no defect in the clearance of *P. aeruginosa* infection.** Mice were first treated with saline or bleomycin on day 0 followed by infection with *P. aeruginosa* on day 14. On day 15 lungs (A) and blood (B) were collected for colony-forming units (CFU) analysis. Data represent *n* = 5–8 mice per group from 2 independent experiments. NS, not significant.
Although histologic evidence in Fig. 4 suggests that between these groups (Fig. 8). Thus differential accumulation of NK-T, CD19, monocyte, PMN, or eosinophil cell types be discernable differences in the percentages of CD4, CD8, NK, leukocyte subsets between these two groups. There were no whereas HV-68 infected mice did not exacerbate fibrosis infections occurred at day 14, and lungs were harvested at day 21 or day 35; n = 3–6 mice per group. *P < 0.05.

C57Bl/6 mice, but we did confirm that expression of viral 1E1 and envelope gB proteins were detectable by real-time RT-PCR (data not shown).

The profibrotic effects of HV-68 compared with H1N1 and P. aeruginosa infection are not explained by inflammatory cell recruitment. To determine whether the inflammatory cell composition was different following the various infections, mice were treated with bleomycin on day 0. Next, bleomycin-treated mice were infected with P. aeruginosa, H1N1, or HV-68 on day 14. Lungs were harvested on days 15 or 21, which represent 1 and 7 dpi, respectively. Single-cell suspensions were isolated, counted, and analyzed by flow cytometry to assess leukocyte populations. As demonstrated in Fig. 7A, total cells were not different between groups at day 15, but there was a noticeable increase in the percentage of CD45+ leukocytes in the P. aeruginosa infected mice at this time point (Fig. 7B). This increase was attributable to an influx of PMNs in these mice, as would be expected for this bacterial infection (data not shown). This increased percentage of leukocytes in the P. aeruginosa group was not maintained at day 21, consistent with rapid clearance of the organism from the lungs (Fig. 7C). In contrast, the viral infected mice showed increased percentages of CD45+ leukocytes on day 21, consistent with the recruitment of leukocytes in response to viral infection. Both H1N1 and HV-68 infected mice showed similar increases in leukocyte accumulation in the lung (Fig. 7D), although histologic evidence in Fig. 4 suggests that HV-68-infected mice show both diffuse and focal inflammatory infiltrates. Because H1N1 infected mice did not exacerbate fibrosis whereas HV-68 infected mice did, we analyzed the various leukocyte subsets between these two groups. There were no discernable differences in the percentages of CD4, CD8, NK, NK-T, CD19, monocyte, PMN, or eosinophil cell types between these groups (Fig. 8). Thus differential accumulation of leukocyte subsets could not explain why HV-68 infection augments fibrotic responses in the lung whereas H1N1 infection did not.

Differences in profibrotic mediators do not explain the ability of HV-68, but not H1N1, to exacerbate fibrosis. We next examined production of several pro- and antifibrotic mediators by ELISA. Expression of CCL12 (Fig. 9A) was elevated in both viral infections, but only reached significance in the H1N1-infected mice. Expression of CCL2 (Fig. 9B) is decreased post-HV-68 infection, but is unchanged post-H1N1. Expression of active and total TGFβ (Fig. 9C) is similar in all groups.

When evaluating Th1, Th2, and Th17 cytokines, IFNγ was elevated in HV-68-infected mice compared with bleomycin plus mock infection (Fig. 10A), confirming earlier results in FITC and HV-68-infected mice (23). Interestingly, levels of IFNγ were reduced in H1N1-infected mice, perhaps consistent with the observation that H1N1 replication is resolving at this time point. Levels of IL-13 (Fig. 10B) and IL-17 (Fig. 10C) were reduced in both groups of virally infected mice compared with the bleomycin control group, but only reached significance in HV-68-infected animals. This is also consistent with the earlier observation that HV-68 can augment fibrosis in the absence of Th2 cytokines (23). Previous studies have shown that aged mice, which are susceptible to HV-68-induced fibrosis, have elevated levels of TGF-β receptors on lung fibroblasts (29). However, in the present studies, levels of TGFβRI (Fig. 10D) were not different, and levels of TGFβRII (Fig. 10E) were elevated only in H1N1-infected mice when measured in the whole lung.

When we compared the induction of IFNγ or TNFα in mice treated with either virus alone compared with the amount made in response to viral infection post-bleomycin, we noted that.

Fig. 3. H1N1 infection does not exacerbate bleomycin-induced pulmonary fibrosis. A: mice were given bleomycin (Bleo) or saline intratracheally on day 0. On day 14, bleomycin- or saline-treated mice received γ-herpes virus-68 (γHV-68), H1N1, or saline intranasally. Lungs were harvested for collagen determination on day 21. B: on day 18, bleomycin-treated mice received H1N1 or saline intranasally. Lungs were harvested 3 days postinfection to measure collagen content and were compared with mice treated with saline alone. Data represents n = 5–8 mice per group collected in 2 independent experiments. C and D: in a separate experiment, mice were treated with saline or bleomycin on day 0, viral or mock infections occurred at day 14, and lungs were harvested at day 21 or day 35; n = 3–6 mice per group. *P < 0.05.
pretreatment with bleomycin did not alter levels of either cytokine in response to H1N1 infection significantly. However, the ability of bleomycin-treated mice to produce TNF-alpha was significantly inhibited post-
HV-68 infection, and production of IFN-gamma tended to be lower (Fig. 11). These results may indicate a suboptimal antiviral response to HV-68 in bleomycin-pretreated mice.

Alveolar epithelial cells are more sensitive to TGF-beta signaling and show evidence of apoptosis post-
HV-68 infection. Human studies that have associated exacerbation of lung fibrosis with herpes virus have shown presence of virus in alveolar epithelial cells (AECs), and because latent infection of AECs with HV-68 have demonstrated elevated production of cysteinyl leukotrienes (44), we isolated AECs from bleomycin-infected mice on day 21 and analyzed them for expression of leukotriene synthetic enzymes and expression of TGF-beta receptors and analyzed their protein lysates for evidence of SMAD3 phosphorylation and apoptosis via cleaved PARP (Fig. 12). Expression of leukotriene synthetic enzymes [5-lipoxygenase (5-LO) and...

![Fig. 4. Histological analyses. Immunohistochemistry showing representative lungs of mice treated with bleomycin + vehicle control (saline), bleomycin + 
HV-68 infection, or bleomycin + H1N1 infection. Shown are hematoxylin and eosin (H&E) or Masson’s trichrome staining. Magnification is 200×. Left panels: mice treated with bleomycin were harvested on day 21 postinjection and show diffuse mononuclear infiltrates and collagen deposition noted as blue coloration in the bottom panel. Middle panels: mice were treated with bleomycin on day 0 and infected with 
HV-68 on day 14. Lungs were harvested on day 21 and show both focal areas of dense mononuclear inflammatory cells as well as diffuse inflammation. Collagen deposition is noted within the interstitium. Right panels: mice were injected with bleomycin on day 0 and H1N1 on day 14. Lungs were harvested on day 21 and show diffuse mononuclear infiltration. While collagen deposition is seen within the interstitium, it is similar to that noted in mice treated with bleomycin alone. Overall, the mice treated with bleomycin + 
HV-68 show the greatest degree of lung involvement. Representative of n = 4 lungs in each group.

![Fig. 5. 
HV-68 replicates to a greater extent than does H1N1 post-bleomycin. Mice were injected on day 0 with saline or bleomycin. On day 14 mice were infected with 
HV-68 or H1N1. On day 21, lungs were collected and levels of H1N1 viral M1 (A) or 
HV-68 DNA polymerase (DNA pol) (B) gene expression were measured by real-time RT-PCR. C: similarly, lungs were collected from mice infected with 
HV-68 on days 17 and 21, and virus titers in the lungs were measured by plaque assay. Data shown are from n = 3–5 lungs/group representative of 2 experiments.

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5-LO activating protein (FLAP)] were increased in response to both infections. However, only infection with γHV-68 resulted in increased expression of TGFβR1, increased evidence of SMAD3 phosphorylation, and increased evidence of apoptosis as noted by cleaved PARP.

**DISCUSSION**

The cause of IPF is unknown, yet several lines of evidence have suggested that viral infections may play a role either as initiating or exacerbating agents. Mounting clinical evidence suggests that patients with IPF have T cells with low expression of CD28 (10, 13). This suggests chronic activation of T cells in IPF patients, potentially due to underlying and undiagnosed infections. Thus the goal of this study was to determine whether both bacterial and viral infections could exacerbate bleomycin-induced fibrosis.

Even though *P. aeruginosa* has the capability of infecting epithelial cells (33) and IPF patients tend to do poorly when...
they develop bacterial pneumonia (31), *P. aeruginosa* infection was effectively cleared from the lungs and did not exacerbate fibrosis. While our results suggest that this bacteria would not worsen fibrosis due to enhanced ECM deposition, there is caution in extrapolating these results to humans. The progressive nature of IPF is not modeled by bleomycin; thus, it is likely that in humans with chronic disease and more diminished lung capacity, a bacterial infection could be far more devastating, and certainly an influx of inflammatory cells might worsen dyspnea even if it does not alter ECM deposition.

Our results using bleomycin verified earlier results using FITC as a fibrotic stimulus (23) and demonstrated that γHV-68 could exacerbate ECM deposition post-bleomycin stimulus. The ability of γHV-68 to do this likely involves ability of the virus to reactivate from latency as the ΔORF72 mutant was not able to do this. Our unpublished observations suggest that the first 3 days of replication by ΔORF72 and γHV-68 are similar, but viral gene expression is significantly diminished by 7 days postinfection in ΔORF72-infected mice. Thus exacerbation of fibrosis likely requires on-going viral replication or spread within the AECs. These data are consistent with earlier studies showing that ongoing viral replication is necessary for fibrosis in Th2-biased mice as well (26). It may also be a unique feature of γHV-68 or perhaps of β-herpes viruses in general since CMV (a β-herpes virus) did not enhance fibrosis. It should be noted that our experiments used the same dose of γHV-68 and CMV; however, C57Bl/6 mice are relatively resistant to CMV infection and we could not plaque infectious CMV from the lungs on day 7 postinfection (data not shown) whereas we could demonstrate infectious virus in γHV-68-infected mice.
We could demonstrate by RT-PCR that CMV viral RNA was expressed (data not shown), albeit at low levels. However, our earlier finding that murine adenovirus type 1 (MAV1) was also unable to exacerbate fibrosis following FITC challenge (23) and our current results with H1N1 suggest that this is not a feature of all viral infections that can infect AECs. Caution should be used when interpreting these data, however, since it is clear that the rates of infection are different for all these viruses.

When examining a variety of pro-and antifibrotic mediators and the composition of the inflammatory cell influx that followed both infections, no notable differences could explain the discrepancy between the ability of γHV-68, but not H1N1 to exacerbate fibrosis. This prompted us to look at changes that...
might be specific to AECs, the initial site of infection, and viral replication within the lung. We have observed that γHV-68 can replicate in AECs in culture without lytic destruction of all the cells. In contrast, H1N1 may be more likely to ultimately destroy all infected epithelial cells. Our results in vivo suggest that γHV-68-infected mice have AECs that are more responsive to TGFβ signaling and show signs of apoptosis. This is consistent with earlier work showing that γHV-68 infection in aged mice is associated with AEC apoptosis and ER stress (39). One caveat was that our analyses of AECs were done at day 7 postinfection, a time point when H1N1 replication was diminished, but γHV-68 replication was on-going. It is possible that the prolonged replication of γHV-68 at 7 dpi may cause more epithelial stress. We have previously demonstrated that AECs isolated from mice with latent γHV-68 infection overproduce profibrotic factors such as TGFβ and cysteinyI leukotrienes (37, 44). Our current data confirm that infection with γHV-68 and H1N1 both significantly upregulate FLAP in AECs, enzymatic machinery necessary for cysteinyI leukotriene synthesis; however, induction of FLAP was highest with γHV-68. Because cysteinyI leukotrienes can promote fibrocyte proliferation (45) and activation of resident lung fibroblasts (5, 30), this could promote ECM deposition post-viral infection. When we looked for evidence of TGFβ signaling in AECs, we observed elevated TGFβRI in AECs from γHV-68-infected mice. This resulted in increased evidence of TGFβ activation of these cells at this time point as demonstrated by increased phospho-SMAD3 expression. Ultimately, we believe these AECs may be undergoing apoptosis in vivo as there was evidence of cleaved PARP, a marker of apoptosis. Thus we speculate that the ability of γHV-68 to undergo persistent rounds of reactivation and an enhanced sensitivity of the infected AECs to respond to TGFβ leads to ongoing apoptosis in AECs along with induction of profibrotic factors such as cysteinyI leukotrienes, which ultimately promote ECM deposition in these mice. Because H1N1 has also been shown to
induce apoptotic machinery in AECs as a way to promote viral replication (46), it is not clear why our results differ with these two viral infections. These results may merely reflect the doses of virus used, the extent of ultimate damage to the AECs, alterations in the ability to repair damaged epithelium following each infection or additional signaling cascades induced by the distinct viruses that we have not yet identified.

While our results in AECs highlight cell-specific increases in susceptibility to TGFβ signaling post-γHV-68 infection, we did not observe differences in total or active TGFβ in the lungs of bleomycin-treated mice infected with γHV-68 or H1N1. As this cytokine is often activated locally on the cell surface, it is likely that measurements in lung homogenates do not accurately reflect levels available during cell-cell communication. However, we were surprised that levels of TGFβRII were actually elevated in H1N1-infected mice within the whole lung. One caveat of these interpretations however is that receptor expression levels were measured in whole lungs, not in isolated fibroblasts. As TGFβ receptors can be expressed on numerous cell types, it is not clear what cells may be overexpressing TGFβRII in H1N1-infected mice. Because TGFβRII can interact with various other cellular proteins such as cyclin B2 (18) endoglin (CD105) (4) or TGFβRIII (12), this may result in differential cell activation of some cell types in the H1N1-infected mice that may further explain the discrepancies between outcomes with H1N1 vs. γHV-68.

Finally, the differences in cell types that are readily infected by each virus may play an additional role. We have demonstrated that γHV-68 is readily found as both lytic and latent infection in lung AECs, fibroblasts, macrophages, and B cells (37). However, H1N1 tends to restrict replication predominantly to the epithelial cells within the lung (48) and in our hands does not replicate well in macrophages. It is interesting that one study has suggested that H1N1 can replicate more
effectively in type II AECs from IPF patients (7). Additionally, a recent case report noted acute exacerbation of IPF following H1N1 vaccination (41). Thus, as mentioned before, bleomycin may not be effectively modeling all the epithelial changes noted in patients with IPF. It should also be noted that while H1N1 infection at the doses used in this study do not appear to worsen ECM deposition, that is not to say that H1N1 infection is not detrimental to fibrotic lungs. At higher doses of H1N1 (500 PFU), bleomycin-treated mice were highly susceptible to rapid death, most likely from acute lung injury (data not shown). However, this dose was also lethal in some control mice.

In summary, γHV-68 is able to exacerbate bleomycin-induced fibrosis or FITC-induced fibrosis (23) and stimulate collagen deposition. Infection with P. aeruginosa, H1N1, and CMV did not exacerbate bleomycin-induced fibrosis at the doses tested in our studies. The difference in the ability of γHV-68, but not the other pathogens tested, to exacerbate collagen deposition requires the ability of γHV-68 to undergo reactivation from latency as demonstrated by our experiments with the ΔORF72 mutant virus. Additionally, we have demonstrated evidence of enhanced sensitivity to TGFβ signaling in AECs from γHV-68-infected mice, likely leading to enhanced profibrotic release of cysteiny1 leukotrienes, AEC stress, and apoptosis.

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